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Review

# Polyphenol oxidases in plants and fungi: Going places? A review

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**Abstract**

The more recent reports on polyphenol oxidase in plants and fungi are reviewed. The main aspects considered are the structure, distribution, location and properties of polyphenol oxidase (PPO) as well as newly discovered inhibitors of the enzyme. Particular stress is given to the possible function of the enzyme. The cloning and characterization of a large number of PPOs is surveyed. Although the active site of the enzyme is conserved, the amino acid sequence shows very considerable variability among species. Most plants and fungi PPO have multiple forms of PPO. Expression of the genes coding for the enzyme is tissue specific and also developmentally controlled. Many inhibitors of PPO have been described, which belong to very diverse chemical structures; however, their usefulness for controlling PPO activity remains in doubt. The function of PPO still remains enigmatic. In plants the positive correlation between levels of PPO and the resistance to pathogens and herbivores is frequently observed, but convincing proof of a causal relationship, in most cases, still has not been published. Evidence for the induction of PPO in plants, particularly under conditions of stress and pathogen attack is considered, including the role of jasmonate in the induction process. A clear role of PPO in a least two biosynthetic processes has been clearly demonstrated. In both cases a very high degree of substrate specificity has been found. In fungi, the function of PPO is probably different from that in plants, but there is some evidence indicating that here too PPO has a role in defense against pathogens. PPO also may be a pathogenic factor during the attack of fungi on other organisms. Although many details about structure and probably function of PPO have been revealed in the period reviewed, some of the basic questions raised over the years remain to be answered.


*Keywords:* Polyphenol oxidase; Structure; Genes coding; Multiplicity; Distribution; Induction; Pathogens; Herbivores; Inhibitors; Function of enzyme

**Contents**



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## 1. Introduction

Polyphenol oxidases or tyrosinases (PPO) are enzymes with a dinuclear copper centre, which are able to insert oxygen in a position *ortho*- to an existing hydroxyl group in an aromatic ring, followed by the oxidation of the diphenol to the corresponding quinone. Molecular oxygen is used in the reaction. The structure of the active site of the enzyme, in which copper is bound by six or seven histidine residues and a single cysteine residue is highly conserved. The enzyme seems to be of almost universal distribution in animals, plants, fungi and bacteria. Much is still unknown about its biological function, especially in plants, but also in fungi. Enzyme nomenclature differentiates between monophenol oxidase (tyrosinase, EC 1.14.18.1) and catechol oxidase or *o*-diphenol:oxygen oxidoreductase (EC 1.10.3.2), but in this review the general term polyphenol oxidase (PPO) will be used.

The topic of PPO has been reviewed frequently, and among the more recent general reviews is that of Steffens et al. (1994). In addition reviews of specific aspects of the biochemistry of PPO have appeared. PPO in plants has been reviewed by Yoruk and Marshall (2003), but much of their review covers ground also stressed in other surveys. The mechanism of reaction of tyrosinase has been discussed in great detail by Lerch (1995) and Sanchez-Ferrer et al. (1995), who emphasise the importance of the enzyme in melanogenesis. A survey of mushroom tyrosinase, including lists of inhibitors, the characteristics of the enzyme and its potential uses for clinical purposes has appeared (Seo et al., 2003). The browning of mushrooms, *Agaricus bisporus* is of major economic importance and the underlying mechanisms have been reviewed by Jolivet et al. (1998), with particular stress on the involvement of tyrosinase in the process. The most recent review of fungal tyrosinases and their applications in bioengineering and biotechnology is by Halalouili et al. (2006), who cover most aspects of this PPO in depth. The potential use of PPO in organic synthesis is reviewed by Burton (2003), although the emphasis in the review is on laccases rather than on PPOs. A comparative analysis of polyphenol oxidase from plants and fungal species, with particular emphasis on secondary protein structure and similarities to hemocyanin was published very recently (Marusek et al., 2006), amplifying an earlier review (van Gelder et al., 1997). Their later review emphasizes the amino acid sequence of the enzyme from different sources and especially the N- and C-terminal domains of the enzyme. The review by Marusek et al. (2006) is especially important because it deals with aspects of PPO structure not previously discussed in detail elsewhere.

Lastly it should be mentioned that the importance of PPO in browning reactions continues to occupy many researchers as indicated by an ACS Symposium (Lee and Whitaker, 1995), and very many subsequent publications describe browning reactions in a variety of species and their tissues.

Since the 1994 review hundreds of papers dealing with plant and fungal PPO have been published. The reason for this plethora of papers is probably the relative ease with which the enzyme activity can be assessed, despite the fact that there are many potential pitfalls in its assay. Many of the published papers report on correlations between levels of PPO activity and environmental factors, attacks by pathogens or changes during food processing or storage. Although useful contributions to the store of information they do not advance the basic understanding of the function of the enzyme and proof of causal relationships between observed phenomena and levels of PPO are mostly missing.

It is clear from the perusal of the literature that PPOs are quite diverse in many of their properties, distribution and cellular location. It could therefore be asked whether it is justified to review such a very diverse group. Jaenicke and Decker (2003) write "Probably there is no common tyrosinase: the enzymes found in animals, plants and fungi are different with respect to their sequences, size, glycosylation and activation". Discussing the phylogenetic tree of PPO, Wichers et al. (2003), conclude that tyrosinases (PPOs) cluster in groups for higher plants, vertebrate animals, fungi and bacteria. "Homologies within such clusters are considerably higher than between them". However, the PPOs have at least one thing in common, they all have at their active site a dinuclear copper centre, in which type 3 copper is bound to histidine residues, and this structure is highly conserved. Despite the huge variability of PPO it still seems justified to try and provide an overview of what is happening. The intention of this review is to attempt to provide such an overview for the period from 1994 until

to today, so that the reader can see where the biochemistry of this group of enzymes is going.

## 2. Structure and molecular weight of PPO

The crystal structure of one PPO in its active form, from *Ipomoea batatas* has been solved (Klabunde et al., 1998). No comparable data are available for the latent forms of PPO. The crystal structure of a tyrosinase from *Streptomyces*, bound to a "caddie protein" has been resolved. This tyrosinase (Fig. 1) shows several features which differ from the plant catechol oxidase (Matoba et al., 2006). These authors ascribe the ability of this tyrosinase to act as a monophenolase as due to some of the observed structural differences. However, it must be remembered that many plant PPOs are able to both hydroxylate monophenols and oxidize dihydroxy phenols, so that monophenolase activity is not a unique characteristic of the *Streptomyces* enzyme. Indeed, the study of another bacterial tyrosinase, from *Ralstonia* has shown that possibly the unusually high ratio of hydroxylase/dopa oxidase activity of this particular PPO was linked to the presence of a seventh histidine unit, binding Cu (Hernandez-Romero et al., 2006). The importance of the histidine residues of a fungal PPO, the tyrosinase from *Aspergillus oryzyae*, expressed in *Escherichia coli*, has revealed the importance of a previously unrecorded histidine residue (Nakamura et al., 2000). These authors used site directed mutagenesis in their study of the enzyme. They propose that while CuA is linked to three histidine units and one cysteine, CuB is liganded by four histidines, including the newly described one. Thus new information about the detailed structure of PPOs is still being uncovered. No fungal PPO has yet been crystallized, either in its active or its latent form. Perhaps the procedures for crystallization described by Matoba et al. (2006) will give an impetus to further attempts in this direction. From the structural studies it is also apparent that PPOs do have distinct features and that not only the amino acid sequences of PPOs differ, but that there are also some differences even at the highly conserved active site.

The amino acid sequence of a considerable number of PPOs, on plants, fungi and other organisms derived from cloning of the enzyme, has now been published and many of the reports and reviews give such comparative information, e.g. van Gelder et al. (1997), Wichers et al. (2003), Cho et al. (2003), Marusek et al. (2006), Halaouili et al. (2006), Hernandez-Romero et al. (2006), Nakamura et al. (2000) and Matoba et al. (2006). As already stated, except for the active site, amino acid sequences show considerable variability and it seems to this reviewer that the salvation for understanding the role of PPO in plants and fungi will not come from the description of yet more amino acid sequences.

Reports on the molecular weight of plant PPO are very diverse and variable. It must be assumed that part of this variability is due to partial proteolysis of the enzyme during its isolation. Furthermore, since there obviously is a family of genes coding for plant PPO, some multiplicity must be a result of genetic variability. This problem is addressed by Sommer et al. (1994) and van Gelder et al. (1997).

## 3. Distribution and expression

### 3.1. Plant PPO

While the list of species in which PPO have been described and at least partly characterized is growing steadily, the majority of the reports fill out details and do not add any new dimension to the subject. For this reason we will mention only a few of the newer reports, particularly those which also identify the genes coding for the enzyme.

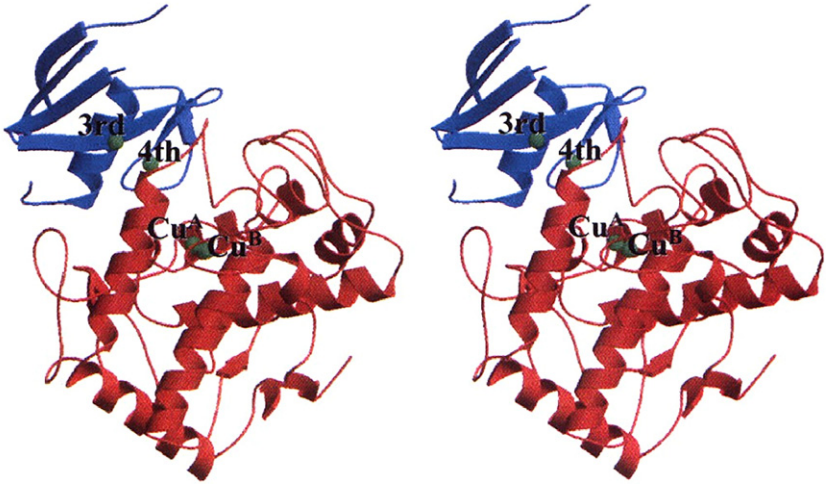



Fig. 1. Overall structure of the tyrosinase from *Streptomyces castaneoglobisporus*, complexed with a "caddie" protein, ORF378. The tyrosinase is shown in red and the ORF 378 in blue. Copper atoms are shown as green spheres (after Matoba et al., 2006, with permission).

The gene coding for PPO in the moss *Physcomitrella patens*, the properties of the enzyme, and changes in the expression of the gene during growth of the protonema of the moss in liquid culture has been reported (Richter et al., 2005) and this is probably the first full report on a PPO in bryophyte. There appears to be only a single gene coding for PPO in this moss, which has one unusual feature, the presence of an intron, absent in most plant PPO genes reported so far. However, in banana an intron is also thought to be present (Gooding et al., 2001), and banana tissues contain at least four distinct genes coding for PPO.

The major progress in the description of PPO in plant tissues has been the research on the multiplicity of genes coding for PPO, their description and the characterization of the expression pattern of some of these genes. Some of this ground breaking work was by Steffens and his collaborators (Newman et al., 1993), as partially described in the review by Steffens et al. (1994). Differential, tissue specific, expression of six genes coding for PPO in potatoes has been reported by Thygesen et al. (1995), and for seven genes in different tissues of tomatoes (Thipyapong et al., 1997). Other early contributions to this aspect are the observations that apple PPO is encoded by a multiple gene family, whose expression is up-regulated by wounding of the tissue (Boss et al., 1994; Kim et al., 2001). The DNA coding for one of the PPOs from apple fruit was cloned and expressed in *E. coli*. The PPO contained a transit protein and was processed to a mature PPO, $M_r$ 56 kDa. Although the protein expressed in *E. coli*, $M_r$ 56 kDa, was detected using antibodies, the gene product was enzymically inactive (Haruta et al., 1998). Two different genes are expressed at different stages of apple flower development, one gene coding for PPO being expressed only at the post-anthesis stage, but the two genes had 55% identity in their amino acid sequence (Kim et al., 2001). This multiplicity of genes, their differential expression in different parts of the plant and at different stages of development is one of the most important features of recent work on plant PPO. The sequences of PPO in any one species are highly conserved, but there is a lot of divergence in the sequences among different species (Thygesen et al., 1995). However, this divergence may not be greater than has been reported for other genes coding for enzymes, and a comparison is difficult. Surprisingly, early work by Robinson and his co-workers indicate the presence of only a single PPO gene in grape vine (Dry and Robinson, 1994).

### 3.2. Methyl jasmonate and PPO

The response of expression of PPO to wounding has been shown in poplar (Constabel et al., 2000), who also showed that methyl jasmonate induced expression of PPO genes, a fairly general phenomenon (Constabel and Ryan, 1998). However, not all species respond to methyl jasmonate by induction of PPO, so that although common, induction of PPO activity by methyl jasmonate, is by no means a universal response. It is also by now well established that methyl jasmonate induces formation of other proteins involved in the defense response of plants (Constabel et al., 1995; Howe, 2004). The presence of PPO in glandular trichomes of tomato and potato has been described by Steffens et al. (1994). The trapping of insects, mediated by PPO, was a groundbreaking result on the function of the enzyme in a clearly defined system. More and more examples of PPO induction by jasmonate or methyl jasmonate are appearing. In the leaves of *Datura wrightii*, PPO is induced in the trichomes, irrespective of whether the trichomes are glandular or non-glandular (Hare and Walling, 2006). Other enzymes were not induced, e.g. peroxidase, nor was alkaloid production enhanced. However, acyl sugars were preferentially synthesised in one form of the trichomes, the glandular ones. Clearly the inductive effect of jasmonate is very complex. Such methyl jasmonate induced expression has also been demonstrated by Koussevitzky et al. (2004), who showed that import and processing of the PPO into chloroplasts from tomato was enhanced by pre-treatment with methyl jasmonate. This observation adds a new and important facet to the mechanisms which control PPO activity in plant tissues. The step most affected by methyl jasmonate seemed to be transport to the thylakoids.

A localized effect of methyl jasmonate has been shown to exist in tomato seeds (Maki and Morohashi, 2006). At the stage of radical protrusion, the level of PPO increased about fourfold at the micropylar end of the endosperm, but not in other parts of the endosperm. Wounding had a similar effect on the level of PPO activity. The wound induced PPO was distinct from the PPO in other parts of the endosperm. Treatment of half seeds of tomato with methyl jamonate, either ruptured or non-ruptured showed that methyl jasmonate only induced PPO activity in the micropylar part of the endosperm. The $M_r$ of PPO induced in ruptured and un-ruptured micropylar endosperm was different, but this could be the result of different processing. The formation during germination can probably be ascribed to wounding, which occurs during radicle protrusion, but its role is by no means clear. It must be assumed that such a localized formation of PPO in a well defined and controlled developmental process is of biological significance, but its function requires further study.

The ability of jasmonic acid, applied to the leaves of *Physalis*, to induce PPO activity was dependent on the time of year when it was applied, maximum induction being obtained in young plants during the summer (Doan et al., 2004). No data seem to be available whether different enantiomers of methyl jasmonate show differences in their ability to induce PPO activity.

Jasmonate can have clear effects even under field conditions. Tobacco plants grown in the vicinity of sagebrush (*Artemisia tridentata*) responded to clipping of the sagebrush by an increased formation of PPO, the response being mediated by methyl jasmonate (Karban et al., 2000). Moreover, the tobacco plants near the clipped sagebrush experienced reduced damage by grasshoppers and

cutworms. Obviously communication between plants plays a role in controlling PPO levels. Expression of the iso-forms of PPO is differential (Haruta et al., 2001; Wang and Constabel, 2003).

### 3.3. PPO in diverse genera

The presence of PPO has been described in a variety of plants, some unusual or exotic. In most cases the descriptions cover molecular weight and often multiplicity. The characteristics of the PPOs mostly show no special features, but a few instances will be mentioned. The PPO from the aerial roots of an orchid *Aranda* was found to be present in four iso-forms, which were partially characterized, including the N-terminal sequences of the iso-forms (Ho, 1999). Since aerial roots contain chloroplasts, it is probable that these PPOs were located in the plastids.

Two distinct PPOs are present in leaves and seeds of coffee (Mazzafera and Robinson, 2000), in the parasitic plant *Cuscuta* (dodder) (Bar Nun and Mayer, 1999), and in Chinese cabbage (Nagai and Suzuki, 2001). The latter has been partially purified and appears to be an example of a PPO in the Cruciferae. *Annona muricata* (Bora et al., 2004), oregano (Dogan et al., 2005a), persimmon (Ozen et al., 2004), artichoke (Dogan et al., 2005b), marula (*Sclerocarya birrea*) (Mduli, 2005), loquat (*Eriobotrya japonica*) (Sellés-Marchart et al., 2006) and *Uapaca kikiana* fruit, a plant belonging to the Euphoriaceae (Muchuweti et al., 2006), all contain PPO. The PPO level in apricot fruits remains high even at a stage when it's mRNA can no longer be detected, indicating that the protein is stable for long periods (Chevalier et al., 1999). This raises the question, is the presence of mRNA a good indicator of function or importance of the enzyme in a given tissue. Most work characterizing PPO and its DNA and mRNA implicitly assume that levels of mRNA are directly related to function, but this may not be true in all cases.

The PPO present in red clover, which has an important role during ensiling of leaves, has been cloned and characterized. At least three PPO genes were detected, which had a high degree of identity, and which were differentially expressed in different parts of the plant (Sullivan et al., 2004). One of these genes was successfully expressed in *E. coli*. The proteins encoded by these three genes all had sequences which predict that they would localize in chloroplast thylakoids. An unsuspected significance of this particular PPO is that silages prepared from clover forage with high PPO activity are of better quality than those with lower PPO activity (Lee et al., 2004). An important aspect the work by Sullivan et al. (2004) is that it is apparently the only report of recombinant expression of PPO with partial activity of the expressed protein. Obviously this opens up many possibilities for further study of PPO. Although it is generally agreed that PPO is plastid located, the site at which it is present in potato tubers is not entirely clear. PPO in undamaged tissue was located to starch grains and the cytoplasm, but upon several hours after mechanical bruising the PPO becomes more generally distributed including in the vacuolar region (Partington et al., 1999). This is presumably due to breakdown of membrane integrity and thus direct evidence of "leakage" from defined sites was demonstrated, using immuno-gold localisation integrity appearing in the cytoplasm.

### 3.4. Chromosomal location of PPO

The observation that hexaploid wheat kernels have six genes coding for PPO, of which at least three are expressed during development of the kernel (Jukanti et al., 2004) is worth noting. The deduced amino acid sequence and similarity with other PPOs has been recorded for at least one PPO from wheat (Demeke and Morris, 2002). This is significant in view of the browning phenomena in cereal products. Some variability in reports on purified wheat PPO is apparent. Kihara et al. (2005) report a $M_r$ of 35 kDa or 40 kDa, depending on assay, for a homogeneous preparation of PPO from *Triticum aestivum* and say that its amino acid sequence resembles that of other PPOs, but Anderson and Morris (2003) report a $M_r$ of 67 kDa and state that it resembles other PPOs as much as other wheat PPO. The purified PPO from wheat bran appeared to be the mature form and lacked the transit peptide locating it to the plastids (Anderson and Morris, 2003). Perhaps these results are not surprising in view of the fact that six genes coding for PPO in wheat are known and perhaps forms differing in maturity were isolated. Genetic analysis of the location of PPO in wheat points to a complex situation. One gene from of *T. turgidum* coding for PPO was mapped to chromosome 2D (Jimenez and Dubcosky, 1999). Reports on QTLs for PPO in *T. aestivum* indicate that a number are present on different chromosomes (Demeke et al., 2001). Genetic mapping of PPO in wheat seems to be the most detailed reported for any PPO, and has implications for selection for wheat with low PPO activity.

Vickers et al. (2005) manipulated the levels of PPO in transgenic sugar cane using constructs of sense and antisense to the native PPO gene and found that as a result the degree of browning of the juice could be changed. Over-expressing PPO led to enhance browning, the PPO content of the juice being elevated.

### 3.5. Fungal PPO

Since fungal tyrosinase has been reviewed in great detail recently (Halaouili et al., 2006; Seo et al., 2003), no attempt will be made here to report on its distribution, which in any case appears to be universal in fungi. Perhaps the isolation of the latent form of PPO in the ascocarp of *Terfezia claveryl* (a truffle), should be recalled (Perez-Gilabert et al., 2001), the general behaviour of this PPO falling in line with that of other fungal PPOs.

## 4. Location and properties of PPO in plants and fungi

### 4.1. Plant PPO

An early report by Rathjen and Robinson (1992) suggested that PPO in grape berries could accumulate in what appeared to be an aberrant form, with a molecular weight of 60 kDa, and not the expected one of 40 kDa. They suggested that PPO in the variegated grapevine was synthesized as a precursor protein which was then processed to a lower molecular weight form. It was also shown that the PPO of broad bean, which is latent, can be activated by SDS, and can undergo proteolytic cleavage with out loss of activity (Robinson and Dry, 1992). Sommer et al. (1994) investigated the pathway by which plant PPO reaches the chloroplast. They studied in detail the synthesis, targeting and processing of PPO. Using an in vitro system and pea chloroplasts they showed that tomato PPO, coded by cDNA, was processed in pea chloroplasts in two steps during its import. The precursor PPO with $M_r$ 67 kDa was imported into the stroma of the chloroplasts by an ATP-dependent step. It was then processed into a 62 kDa form by a stroma peptidase. Subsequent transport into the lumen was light dependent and resulted in the mature 59 kDa form. Apparently such processing is a feature of all chloroplast-located PPOs. The precursor protein contains a transit peptide, which must be removed in order that the PPO reaches its site in the chloroplast. The processing is carried out by a stromal peptidase, which was purified and characterized (Koussevitzky et al., 1998). The import and processing did not require $Cu^{2+}$, but import was inhibited by micromolar concentrations of $Cu^{2+}$. Further studies revealed that this inhibition was probably due to inhibition of the stromal peptidase involved in the processing of the precursor protein (Sommer et al., 1995).

It is clear therefore, that the synthesis of PPO and its transport to its site in chloroplasts, where plant PPOs are thought to be located, is a complex process, but which has the general features of import of nuclear coded proteins into sub-cellular organelles.

A curious finding on the possible ability of PPO to act as a protease has been reported (Sokolenko et al., 1995; Kuwabara, 1995; Kuwabara et al., 1997; Kuwabara and Katoh, 1990). According to these reports a protein said to be identical with PPO in structure and properties can under certain conditions oxidatively degrade a low molecular weight protein located in chloroplasts. The evidence for the identity of this PPO-like protein is still not totally convincing and it has not been cloned or fully characterized using the techniques of molecular biology. Whether this activity is of any physiological significance remains to be demonstrated.

### 4.2. Fungal PPO

The location of fungal PPO is not entirely clear. Generally it appears to be a cytoplasmic enzyme. However, a PPO from *Pycnoporus* over-produced in *Aspergillus niger*, could be targeted to the extracellular growth medium (Halaouili et al., 2006). An additional PPO is present in the mycelium of *Pycnoporus saguineus*, which has a very high tyrosinase activity and is able to convert coumaric acid to caffeic acid in vitro (Halaouili et al., 2005). This enzyme differs from other PPOs from *Pycnoporus* and existed in four iso-forms, with $M_r$ of 45 kDa. The enzyme was not *N*-glycosylated.

At least in some cases fungal PPO is associated with the cell wall, occurring apparently in the extra cellular matrix (Rast et al., 2003). The molecular weights of fungal PPO show considerable diversity. Part of this diversity is genetic as is indicated by the clustering in the phylogenetic tree of the enzyme (Halaouili et al., 2006), and part can be ascribed to artifacts arising during isolation of the enzyme. This question is discussed by Halaouili et al. (2006). Fungal PPO, as plant PPO, can be present in latent form which is activated by SDS or proteolysis or acid shock. SDS activation in beet root PPO is said to be reversible (Perez-Gilabert et al., 2004), while trypsin activation is not. Gandia-Herrero et al. (2005b) suggest that a common peptide is involved in activation both by SDS and trypsin. The evidence is not totally compelling and it should be remembered that very old work on sugar beet chloroplast PPO already showed that even peptides with $M_r$ of around 10 kDa still retain considerable PPO activity. This shows that by no means the entire protein is required for activity (Mayer, 1966). Further insight into the activation of a latent PPO comes from the work of Kanade et al. (2006) on PPO from *Dolichos lablab*, which shows that both acid and SDS change the environment of a single glutamic residue, close to the di-copper active site. As a result the active site is unblocked or opened and enzyme activity enhanced. An intriguing suggestion by the authors, as yet unproven, is that wounding or methyl jasmonate could cause localized acidification which results in conversion of a latent to an active enzyme. An interesting feature of two tyrosinases from *Agaricus* is that the proteins contain putative glycosylation and phosphorylation sites, although no glycosylation or phosphorylation has so far been reported for fungal PPO (Wichers et al., 2003).

It is fairly clear that fungal PPOs also undergo some processing, by proteolysis, and that the synthesized form of the enzyme is trimmed. What is less clear at the present is what exactly happens during the conversion of latent to active forms of the fungal PPOs?

Tyrosinases from crustaceans have been shown to occur in vivo as a hexamer, made up from a single subunit of molecular weight 71 kDa (Jaenicke and Decker, 2003). This of course recalls the many older reports of associations of plant and fungal PPO into aggregates, but in the case of the crustacean PPO, the hexamer has been shown to exist in vivo and its structure demonstrated by electron microscopy. The similarities of this structure with haemocyanin are apparent (Gerdemann et al., 2002). Nevertheless aggregation into a hexameric form, existing in vivo, has not been shown for any plant or fungal PPO.

## 5. Inhibitors of PPO

Because of the importance of browning caused by PPO in the food industry (Vamos-Vigyazo, 1995) and its great significance in melanogenesis (Seo et al., 2003), research on potential inhibitory compounds continues. An inhibitor, often used by later authors as a reference compound is kojic acid (5-hydroxy-2(hydroxyl-methyl)-4*H*-pyran-4-one) (Kahn, 1995), which is effective at 10–50 μM. One of the more promising compounds, whose use is permitted in foods (Vamos-Vigyazo, 1995), is hexylresorcinol, which is active at around 100 μM, and this compound has proven to be useful in differentiating between laccase and PPO (Dawley and Flurkey, 2003).

### 5.1. Inhibitors related to phenolic compounds

The search for naturally occurring inhibitors has led to the discovery of a number of active compounds. Among the more interesting ones discovered were chalcones and related compounds (Nerya et al., 2004). The most active compounds were glabridin, effective at around 1 μM and isoliquiritigenin, active at 8 μM (Nerya et al., 2003). A study of chalcone derivatives, related to these compounds (Nerya et al., 2004; Khatib et al., 2005), showed that the number and position of hydroxyl groups in the A and B rings of chalcones was important in determining their inhibitory activity. The precise mechanism of action of these chalcone derivatives is not entirely clear. The possibility of actually using such compounds in preventing browning of intact mushrooms was examined and was found to be unsuccessful (Nerya et al., 2006), clearly indicating the pitfalls on the way between the laboratory and practical use. Not surprisingly other phenolics are also able to inhibit PPO.

Some flavanols active in the 50–100 μM, range have been found, acting perhaps as copper chelators (Kubo et al., 2000). More recently, flavanones isolated from *Garcinia subelliptica* were found to inhibit tyrosinase (Masuda et al., 2005). Among the most interesting recent reports is that procyanidins can inhibit PPO from apples (Le Bourvellec et al., 2004). The most active of these compounds, ProAV, with a degree of polymerisation of 80, was inhibitory at 0.02 g/l. Translated into molarity, assuming a molecular weight of about 300 for the monomer, this implies a very high inhibitory activity in the micromolar range. Other very active inhibitory compounds were the oxidation products of caffeoylquinic acid, also at very low concentrations.

### 5.2. New classes of inhibitors

New classes of inhibitors are tetraketones (Khan et al., 2006) active at 2.06 μM (50% inhibition), decanoate derivatives, which inhibited irreversibly at 96.5 μM (50% inhibition) (Qiu et al., 2005) and substituted 1,3,4-oxadiazole analogues (Khan et al., 2005). The most active compound among the oxadiazoles was inhibitory at 2.18 μM (50% inhibition), making it one of the most active inhibitors in the assay used.

This later paper described the use of microwave-assisted combinatorial synthetic approaches to analyse the inhibitory characteristics of the compounds examined. A novel approach for analysing inhibitory and non-inhibitory compounds was adopted by Casanola-Martin et al. (2006) in order to try and predict which structures would be inhibitory to tyrosinase.

*N*-Benzylbenzamides have been shown to be active as tyrosinase inhibitors, in which the hydroxylation pattern of the B ring was the most important in determining activity. The most active compound inhibited at 2.2. μM (Cho et al., 2006). Although not examined, inhibition presumably was competitive. Competitive inhibition by hydroxystilbenes has also been reported, although activity was not very great (Song et al., 2006). The *cis*-isomer of 3,5-dihydroxy stilbene form was about twice as effective as the *trans*-isomer and this has some implications with regards to the interaction between the inhibitor and the enzyme.

A word of warning is required about most of these new reports. In most of them a multi-well, spectrophotometric, assay of tyrosinase activity was adopted, often using a commercial preparation of mushroom tyrosinase. Oxygen uptake, which is the definitive assay for tyrosinase activity was not used. In many cases the nature of the inhibition was not determined, e.g. copper chelation, competitive inhibition, non-competitive or mixed inhibition. Furthermore, the newly discovered substances were not tested on other metal containing enzymes, and inhibition of tyrosinase was not compared with other copper containing enzymes such as laccase or ascorbic acid oxidase. Therefore any practical application of any of these compounds is a very long way off, regardless of whether they are to be used in the food industry or as agents to prevent melanogenesis. The structural diversity of the newly reported tyrosinase inhibitors is huge and this reviewer finds it difficult to find any common denominator in their activities. Some clearly act as competitive inhibitors at least for the substrate used in the assays, usually either tyrosine or dopa, while others are copper chelators.

Chitosans have been reported to extend post-harvest life of fruits and it has been claimed that they have a direct inhibitory effect on PPO. In the case of longan (*Dimocarpus longan*) fruit increases in PPO during storage were delayed when the fruit was treated with chitosan, but the effects were probably due to secondary changes, such as the protective coating by chitosan, and not a direct inhibition of PPO (Jiang and Li, 2001). Although chitosans can induce stress in plant tissues, treatment of suspension cultures of potato did not induce increased PPO activity (Dörnenburg and Knorr, 2001). Chitosans should not be considered as PPO inhibitors despite the fact that they are said to have antifungal properties (El Gaouth et al., 1992).

## 6. Function

The physiological and biochemical functions of PPO in both plants and fungi have continued to occupy researchers. Already one of the early reviews on this problem (Mayer and Harel, 1979) pointed to the lack of clarity in many of the considerations of PPO function, and to a degree this lack of clarity persists.

### 6.1. PPO in biosynthetic processes

The role of PPO in synthetic processes continues to be a focal point in the discussions of function. An alleged role has been ascribed to tyrosinase in the biosynthesis of betalains (Steiner et al., 1999; Strack et al., 2003). This suggestion is based on the observation that tyrosinases from *Portulaca grandiflora* and from *Beta vulgaris* were able to hydroxylate tyrosine to dopa (3,4-dihydroxyphenylalanine), which can then be oxidized to the corresponding quinone, to dopaquinone. The authors indicate that the constitutive tyrosinase activity is complemented by a dioxygenase activity (Mueller et al., 1997), and that initiation of this dioxygenase activity can lead to betalain formation. Although the results are very suggestive, the isolation of enzymes capable of carrying out a reaction, and correlation of enzyme activity with betalain accumulation does not necessarily prove the function of such a system in vivo. A further indication of the involvement of PPO in betalain biosynthesis comes from the work of Gandia-Herrero et al. (2005a). They suggest that PPO (tyrosinase) can hydroxylate tyramine to dopamine, which in the presence of betalamic acid could yield dopamine-betaxanthin, and this on subsequent further oxidation could yield 2-des-carboxy-betanidin. This interesting suggestion, since it is based on an in vitro system using mushroom tyrosinase, requires more convincing evidence. Further supporting evidence comes from the location of a PPO in the ripening fruits of *Phytolacca americana* (poke weed) (Joy IV et al., 1995). Two distinct cDNAs coding for PPO were cloned and the PPO characterized. At least one of them was a typical PPO, with a transit peptide targeting it to the chloroplasts. The two PPOs showed a high degree of sequence identity, and both were expressed only in the fruit. The fruit of *Phytolacca* accumulates betalains. Here again the correlations were convincing, but proof of actual involvement still is lacking.

A very significant discovery is that a homologue of PPO catalyses the oxidative formation of aureusidin. The enzyme, named aureusidin synthase, has been fully characterized, the amino acid sequence and substrate specificity determined, and the mechanism by which the chalcone is oxidized described (Nakayama et al., 2000; Nakayama et al., 2001). Its cDNA has also been cloned. The enzyme contains a dinuclear copper centre. Unusual about this enzyme is that it has a very low affinity for the traditional PPO substrates such as tyrosine and Dopa, a value less than 1% of that for its natural substrate, 2′,4,4′,6′-tetrahydroxychalcone. Despite its clear similarity to the conventional PPOs, the International Union of Biochemistry on the Nomenclature and Classification of Enzymes has given it a separate number EC 1.21.3.6. Nevertheless, the discovery of this enzyme is a major breakthrough in understanding the function of this group of enzymes. An exceptional feature of this enzyme is that it is localized in the vacuoles of *Anthirrhinum majus* flowers and that the mature enzyme, with $M_r$ of 39 kDa was formed after processing, which involved removal of a peptide to target it to the vacuole (Ono et al., 2006). The mature enzyme contained sugar moieties. Thus the question of whether it is a conventional PPO remains, but it seems likely that this work will lead to the uncovering of other plant PPOs, not located in the plastid.

A surprising finding is that the oxidative polymerization of flavanoids in the seed coat of *Arabidopsis* is catalysed not by PPO but by laccase (Pourcel et al., 2005). Indeed, contrary to expectation, PPO appears to be absent in *Arabidospsis* and no genes coding for it could be detected. Whether this is a special feature of *Arabidopsis*, or is common in the Crucifereae raises interesting questions about the evolution of PPO.

A remarkable report on an enantiomer specific PPO with a clear biosynthetic role is by Cho et al. (2003). These workers showed that a typical PPO from the creosote bush (*Larrea tridentata*) specifically hydroxylates only (+)-larreatricin to the corresponding (+)-3′-hydroxylarreatricin, while the enantiomer (−)-larrreatricin was not oxidized or hydroxylated. This is the first and apparently the only report that a PPO can show an exceptionally high degree of specificity and contrasts sharply with the general observations that PPO is not very substrate specific. Cho et al. (2003) do not report whether their enzyme, which is clearly a typical plastidic PPO with amino acid sequence similarity to a range of other PPOs, oxidizes any other substrate.

### 6.2. PPO in browning reactions

The role of PPO in browning phenomena is so well documented, that it need not be discussed further. A major difficulty is always to determine whether PPO is the direct reason for browning or whether the browning reaction is a secondary result of other metabolic events. This is illustrated by work on development of blackheart in pineapple, which occurs after chilling (Zhou et al., 2003). Although PPO was clearly involved in the browning reaction, its role in the development of blackheart was found to be secondary. The development of brown core in pears also involved PPO, but neither the level of PPO nor that of the of phenolics seemed to limit the development of the brown-core disorder (Veltman et al., 1999).

### 6.3. Role of PPO in resistance of plants to stress and pathogens

A major focus of research in PPO has been its potential role in defense mechanism in plants. While in the past this problem has been approached chiefly by correlative studies,

progress in understanding the molecular biology of PPO has led to a welcome change of emphasis. The common approach now is to examine the expression of specific genes coding for PPO during injury, herbivore or pathogen attack or during exposure to external stresses. In addition it has been possible to manipulate levels of PPO expression using a variety of modern techniques.

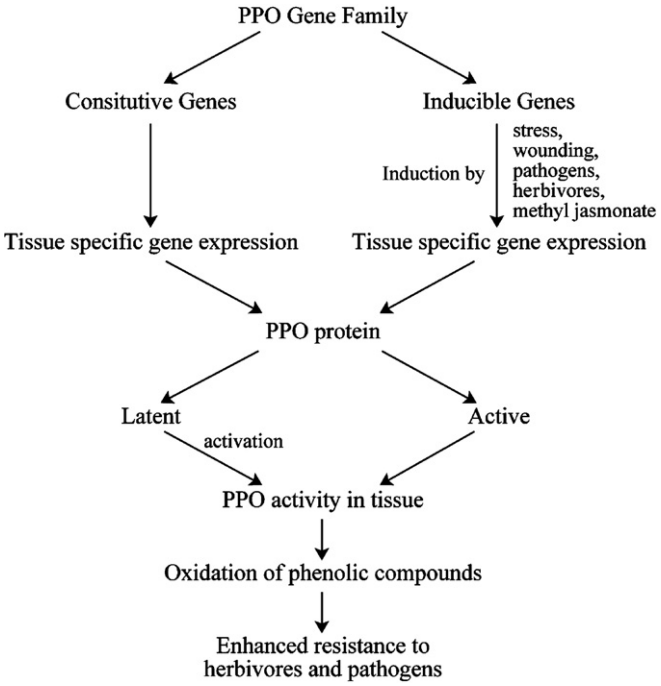
Thipyapong et al. (2004) introduced antisense PPO cDNA into tomato plants and examined the resistance of the plants to the pathogen *Pseudomonas syringae*. This resulted in the down regulation of all the members of the PPO gene family. PPO activity was reduced by a factor of about 40. Examination of the sensitivity of the plants to the pathogen revealed a dramatic increase in their susceptibility, although the overall growth and development of the tomato plants was not affected by the down regulation of PPO. In other experiments in which PPO was over-expressed in tomato plants (Li and Steffens, 2002) over expression was accompanied by enhanced resistance to the same pathogen. The levels of mRNA rose to a much greater extent than the levels of PPO protein. It is of course quite likely that the formation and accumulation of the enzyme protein is controlled not just by the level of mRNA and that other factors are also involved. These findings clearly implicate PPO in the defense of plants against the pathogen, but do not as yet provide an explanation of the underlying mechanism. This problem is discussed by Thipyapong et al. (2004), who offer a number of possible explanations. In addition to a reduced oxidation of phenolic compounds, which could result in reduced resistance to pathogens, the possibility is considered that PPO is also involved in the generation of reactive oxygen species (ROS). As a result the signaling pathways might also be affected by reduced expression of PPO coding genes. In this work on tomato, the oxidation of phenolic compounds was not examined directly. However, other research on the browning of potato clearly shows that reduced expression of PPO genes resulted in reduced PPO activity and reduced browning (Coetzer et al., 2001). In this work, surprisingly, insertion of tomato PPO cDNA in either the sense or the antisense orientation reduced PPO activity. Indeed the sense direction had the greatest effect on the level of PPO. The involvement of PPO in imparting resistance of pearl millet (*Penisetum glaucum*) to downy mildew (*Scerospora graminicola*) has been demonstrated by Raj et al. (2006). Resistant genotypes had localized, elevated levels of PPO, whose formation was rapidly induced following infection, while susceptible cultivars failed to accumulate PPO even after a considerable time.

Wounding and herbivore attack have also been shown to induce PPO activity.

An apparent antagonism between induction of PPO by methyl jasmonate in tomato plants and a compound mimicking the action of salicylic acid, a benzothiadiazole, has been reported (Thaler et al., 1999). The benzothiadiazole reduced induction of PPO activity and at the same time partially reduced resistance to the armyworm, *Spodoptera exigua*. Benzothiadiazole treatment improved protection against *P. syringae* in the tomato plants, but jasmonate seemed to antagonize this effect. At this stage it is difficult to understand this antagonism, since one might assume that PPO formation would have a general beneficial effect in defense against pathogens and predators, since resistance to herbivores and fungal pathogens have many common features (Mayer, 2004). However, information is lacking both about which specific PPOs are induced and the induction of which is prevented by the different treatments. Nevertheless, these results indicate the complexity of the response to methyl jasmonate and how careful one must be in drawing conclusions. An attempt to present schematically some of the events leading to PPO activity is shown in Scheme 1.

### 6.4. Role of PPO in defense against herbivores

In order to determine whether indeed these PPOs had a function in plant defense against herbivores, the expression of the genes was tested when plants were exposed to larvae of the forest tent-caterpillar (*Malacosoma disstria*) (Wang and Constabel, 2004a,b). Transgenic plants were constructed in which PPO genes were over-expressed. The transgenic plants had higher levels of mRNA and the PPO protein accumulated in them. Indeed in the transgenic plants, the forest tent caterpillar was adversely affected, although this depended on the time of hatching of the eggs. Therefore, PPO has at least a partial role as a defense protein in hybrid poplar. Probably various other defense mechanisms operate together with the PPO. It has been shown that a number of genes are expressed on systemic wounding of poplar (Christopher et al., 2004). Some unusual and perhaps unexplained observations relate to the




Scheme 1. Relationship between changing levels of PPO activity and some of its functions.

poplar system. The level of PPO in poplar plants transformed with anti-sense PPO constructs did not change (Wang and Constabel, 2004a,b), indicating that the control of PPO formation is perhaps more complex than might appear at first sight. The native substrate for poplar PPO is only released after some time from a glycosidic form (Haruta et al., 2001). Lastly, the PPO present in poplar is present in latent form (Wang and Constabel, 2003). Although it seems to be evident that PPO does play a role in defense against herbivores, the reaction sequence must be very complex indeed, involving gene expression, enzyme formation and activation as well as substrate formation. The response of potato and bean plants to the Colorado potato beetle has brought to light the fact that both wounding and regurgitant from the larvae can induce increases in PPO activity in the leaves of both *Solanum tuberosum* and *Phaseolus vulgaris* (Kruzmane et al., 2002). In the same system, formation of peroxidase was different in the two plants species, the bean leaves responding much more than the potato leaves. This seems to be the first report of a chemical factor produced by a herbivore which induces PPO formation, and this effect was thought to be mediated by ethylene, whose production was greatly enhanced by the regurgitant in both tissues.

A very unusual correlation between drought resistance and PPO expression has been reported by Thipayong et al. (2004). Tomato plants, either untreated or in which PPO expression was either suppressed or increased (over expression), were tested for their ability to withstand water stress. Plants in which PPO expression was reduced showed better stress tolerance than either the non-treated plants or those in which PPO was over expressed. Expression of PPO genes B and D was up-regulated in plants exposed to water stress especially in the abscission zone of leaves. The B gene was also up-regulated in leaf veins. It is not clear why PPO genes should be up-regulated when plants are exposed to water stress and what special function PPO activity fulfills under such conditions. It is also not quite clear why suppression of PPO activity should improve drought tolerance. The authors rule out involvement of the Mehler reaction in the improved drought tolerance, but suggest that the suppressed plants appear to show less oxidative stress. That PPO levels change when plants are exposed to drought or other forms of stress is well known, and has been discussed in the past (Mayer, 1987). Nevertheless, there is no satisfactory explanation at present for the underlying mechanism. It is not even clear whether the changed levels of PPO are beneficial or detrimental to the plant. A recent report shows that in the apical zone of maize roots exposed to salinity stress a putative laccase gene is expressed (Liang et al., 2006), and this expression was not simply due to stress, since osmotic stress did not lead to the same result. The increased expression could not simply be ascribed to reduced growth of the root. However, the authors suggest that the laccase may have a role in the formation of the Casparian strip in the roots, which contains suberin and hence phenolic compounds. It may be entirely fortuitous that an entirely different enzyme capable of oxidizing phenolic compounds is up regulated under certain stress conditions, but it does recall the increased expression of PPO under stress conditions. Constabel and his co-workers have used poplar as a model species to examine the response to wounding (Constabel et al., 2000; Haruta et al., 2001). Initially they reported only one or two PPO genes, but in later publications they showed that three genes coding for PPO were expressed differentially in different parts of a hybrid poplar (*Populus trichocarpa* × *P. deltoides*) (Wang and Constabel, 2004a,b). Two genes were cloned, their expression followed, and the biochemical characteristics of the PPOs studied (Wang and Constabel, 2003). These genes were expressed differentially upon wounding of the tissue or after application of methyl jasmonate.

The possible function of a tobacco flower-specific gene, which was cloned and characterized, coding for a polyphenol oxidase is discussed by Goldman et al. (1998). Again the usual suggestions were made that defense functions or control of the formation of phenolic compounds acting as signaling molecules are involved. Again no direct evidence was produced. It is nevertheless curious that there are a number of reports locating specific PPOs in flower parts, and this deserves more attention.

### 6.5. Role of PPO in fungal pathogenicity and fungal defense reactions

Induction of PPO in fungi has been researched much less than induction of plant PPO. Infection of the mushroom *A. bisporus* with *Pseudomonas tolaasii*, causes discolouration of the cap. This discolouration is accompanied by an induction of the fungal PPO (Soler-Rivas et al., 2000). The same effect could be induced by treatment of mushrooms with extracts containing the bacterial toxin tolaasin or with purified tolaasin. The induction of PPO activity could be ascribed to the conversion of a latent PPO, $M_r$ 67 kDa to an active form with $M_r$ of 43 kDa as a result of treatment with partially purified extracts, while addition of pure tolaasin induced the transcription of a gene coding for PPO. Thus treatment with the bacterial extract had a dual effect, post-translational modification of PPO and induction of mRNA formation. At this stage it is not clear whether the raised levels of PPO in the mushroom, following infection, is part of a defense reaction, or is simply the result of disruption of cellular metabolism in the host. The similarities with processes of induction of PPO in plants are clear, but further studies are badly needed.

In fungi, there is little doubt that PPO is active in the formation of melanin. The role of fungal melanin in pathogenesis has been reviewed by Jacobson (2000). In addition to the protective role of melanin, it apparently is needed in the cell walls of the appressorium in order to allow them to develop the osmotic potential needed to breach host cell walls. Support for this idea comes from the observation that mutants of *Magnaporthe grisea*, lacking melanin, were unable to develop high osmotic potentials. Although the review by Jacobson focuses on the importance of melanin

in fungal infections and in human disease, the implications for fungal plant interactions are obvious.

An interesting function of fungal PPO comes from an examination of fungal interactions (Score et al., 1997). In experiments in which different species of fungi were allowed to grow together either in pure or mixed cultures on Petri dishes. The formation of peroxidase, laccase and tyrosinase was followed in the medium. Although the experiments were qualitative, the results suggested that some of the fungi, including *Trichoderma* sp., released PPO when confronted with another fungal species, although PPO activity was not detected specifically in the interaction zone. However, the fact that tyrosinase was released into the medium is itself interesting. Although these results are suggestive of a function during fungal interactions, because the enzymes were not isolated or quantified, perhaps some reservations about their significance are in order.

## 7. Perspectives

Just 110 years ago the first report on polyphenol oxidase (tyrosinase) appeared (Bertrand, 1896). Forty-two years later the procedures for its isolation in large amounts were published by Keilin and Mann (1938) and Kubowitz (1938), making possible more detailed work on the properties of PPO. The presence of copper at the active site was clear and the similarities with haemocyanin apparent. By 1956, Mason discussed the structure and possible functions of PPO. In 1966 Mason stated that it is necessary to determine the number of copper atoms at the active site, the nature of the isozymes of PPO and the nature of the catecholase and cresolase activity and whether these are due to the same enzyme. These aims have largely been achieved but the question of catecholase vs. cresolase activity keeps on arising, although it is pretty clear that both activities are the attributes of a single enzyme. The function of PPO in plant and fungal metabolism was generally relegated to a second place in discussions. Probably advance since then can best be judged by comparing the discussions in the review by Mayer and Harel (1979) with those by Steffens et al. (1994). During the 12 years, since the latter review appeared, a huge amount of detailed information about the structure, multiplicity, induction, molecular properties of PPO has accumulated. Although new observations about the structure of PPO appear, it mechanism of action has been elucidated. However, some of the central problems remain unresolved. The function of the enzyme is in most cases still not unequivocally known or defined. Although molecular biology has been increasingly used to study it, this approach has not provided the hoped for answers, especially with regards to the mechanisms underlying its function. The reasons for the frequently observed latency of the enzyme, and how it is converted in vivo into the active form, are still not clear, nor do we know what regulates conversion from the latent to the active form. There exist both constitutive and induced PPOs, and the induction process and the role of jasmonate in induction are now well established, but molecular control remains unclear. More information is needed about the chromosomal location of the genes coding for PPO. The interaction between the sequestered enzyme and its substrates is still puzzling and how they interact under certain circumstances needs more study. The focus on future work on PPO should shift. More attention should be paid to its possible location in the cell, other than the chloroplast. Its natural substrates should be investigated to determine whether substrate specificity of the enzyme could be much greater than is thought at present. The role of plant PPO in resistance phenomena against different pathogens and herbivores requires further detailed investigations, using techniques of molecular biology, classical biochemistry and developmental physiology. Although correlations between PPO gene expression and defense reactions and stress are now well established, we still do not know just how PPO functions in increasing for example disease resistance. PPO activity and its level during the life cycle of plants and fungi must be addressed in the future and the function of PPO at each developmental stage determined. The major problems to be addressed by future research on PPO are the same as in the past, but a change of emphasis is needed. More attention should be given to mechanisms and function, and less to surveys of the presence of the enzyme. Despite the importance of PPO in food processing and browning reactions, there has been limited progress in this respect, and perhaps a new approach is needed. To the question in the title of this review: ''is PPO research going somewhere'' the answer seems to be it is meandering along, rather than reaching a defined target.

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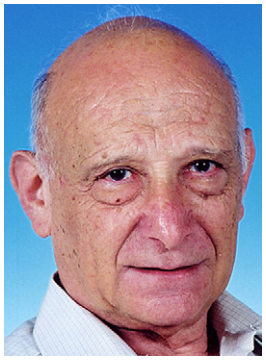

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