The di- and poly-amine oxidases of higher plants

TERENCE A. SMITH
Long Ashton Research Station, University of Bristol, Long Ashton, Bristol BS18 9AF, U.K.

Diamine oxidase

Enzymes which oxidize diamines occur sporadically throughout the plant kingdom (Smith, 1980, 1985a), though they are particularly active in the Leguminosae. The DAO found in pea seedlings (Pisum sativum) was the subject of early investigations by Werle in Germany (Werle et al., 1961) and by Mann and his co-workers in England (Hill & Mann, 1968). This DAO was purified to homogeneity and shown to contain copper which was readily removed from the enzyme by dialysis against chelating agents, with consequent inactivation. Activity could be restored on addition of small quantities of Cu²⁺. This property was exploited in the development of an extremely sensitive and highly specific method for the estimation of copper (Hill, 1973). All plant DAOs studied so far are dimers. Several have been shown to contain two copper atoms and one carbonyl residue per mol of enzyme (Nylen & Szybek, 1974; Kluetz et al., 1980a; Matsuda & Suzuki, 1981; Yanagisawa et al., 1981; Floris et al., 1983a; Rinaldi et al., 1983) thus attempts to demonstrate the presence of pyridoxal phosphate have so far failed, and the nature of the carbonyl residue is still unresolved. Recent work on the copper-containing enzyme, bovine serum amine oxidase, has shown the presence of pyrroloquinoline quinone (Lobenstein-Verbeck et al., 1984). It remains to be seen if the same is also the case for the pea seedling DAO. Estimates of the Mᵣ of the dimeric form of the pea seedling DAO are in the range 170000–185000 (McGowan & Muir, 1971; Macholán & Haubrová, 1976; Kluetz et al., 1980a; Yanagisawa et al., 1981).

Pea seedlings are the most active source of DAO, exceeding the classical hog kidney by 105 times in terms of crude material, and by 58 times in terms of purified enzyme (Smith, 1979). Moreover, the pea seedling enzyme is very stable during long-term storage and in assay. For these reasons the pea seedling DAO may be utilized for estimating amines. In one example of this, an arginine decarboxylase assay was developed in which the product of the decarboxylase (i.e. agmatine) was oxidized in the assay incubate by pea seedling DAO, and the peroxide produced was then estimated by means of the peroxidative oxidation of guaiacol (Smith, 1979). This assay is considerably more rapid and convenient than the conventional isotopic method more usually adopted for amino acid decarboxylase assay. The sensitivity could be greatly increased by utilizing peroxidase substrates which fluoresce on oxidation (Smith, 1983a). A method has also been developed for estimating lysine and arginine, by using membrane-bound DAO and the respective amino acid decarboxylases, in association with an oxygen electrode (Macholán, 1978). A mathematical model has been established for amine estimation by this electrode system (Toul & Macholán, 1975).

When investigating amine metabolism in plants, especially in the Leguminosae, it is important to be aware of the presence of DAO, since this may cause artefactual reactions catalysed by peroxidase (Suresh & Adiga, 1977). However, in vivo the peroxide formed may be utilized by the plant to affect natural metabolic pathways such as uric acid degradation (Tajima et al., 1983). Within the Leguminosae, DAOs with properties similar to those of the pea seedling enzyme have been found in

Abbreviations used: DAO, diamine oxidase; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; PAO, polyamine oxidase.

Arachis hypogea (Sindhu & Desai, 1980) Glycine max (Suzuki, 1973; Le Rudulier & Goas, 1977), Lathyrus sativus (M, of dimer 148000; Suresh et al., 1976; Suresh & Adiga, 1979), Lens esculenta (M, of dimer 154000; Floris et al., 1983a; Rinaldi et al., 1983), Lupinus luteus (Schütte et al., 1966) and Vicia faba (M, of dimer 126000; Matsuda & Suzuki, 1977, 1981). Certain non-leguminous plants also have the ability to oxidize putrescine (for a review of early work see Smith, 1980). Recently a DAO has been found in Euphorbia latex, with two Cu²⁺ ions and two carbonyl-like groups per dimer (M, 144000) (Rinaldi et al., 1982). A DAO has also been demonstrated in rice (Oryza sativa) seedlings which contains four molecules of FAD per dimer (M, 123000) (Chaudhuri & Ghosh, 1984). N-Methylputrescine oxidase, a copper-containing enzyme, is concerned in nicotine biosynthesis in tobacco roots (Mizusaki et al., 1972).

Substrate specificity. The following reactions are typical of those catalysed by DAO:

\[
\text{N}_2\text{H}_4\text{(CH}_3\text{)}_2\text{NH}_2 + \text{O}_2 \rightarrow \text{N}_2 + \text{H}_2\text{O} + \text{H}_2\text{O}_2
\]

Putrescine

\[
\text{N}_2\text{H}_4\text{(CH}_3\text{)}_2\text{NH(CH}_2\text{)}_2\text{NH}_2 + \text{O}_2 \rightarrow
\]

Spermidine

Aminopropylpyrroline

The pea seedling DAO is most active with putrescine and cadaverine [NH₂(CH₃)₂NH₂] as substrates (optimum pH7) though the Kₘ for spermidine is 5 × 10⁻⁷ M, smaller than the Kₘ for putrescine (4 × 10⁻⁵ M) (Smith, 1974). The pea seedling enzyme oxidizes a wide range of substrates including aromatic and aliphatic monoamines (Hill, 1971), and lysine and ornithine are also slowly oxidized. The optimum pH is substrate-dependent (Kenton & Mann, 1952). Even artefactual substrates are attacked, and this property has been utilized in the synthesis of new compounds. For example, monodansyldiamines are good substrates of DAO, and the product of oxidation (dansyl-5-aminovaleraldehyde) was used in the standardization and characterization of the product of exhaustive dansylation of lysine, with which it is identical (Smith, 1981a). Moreover, the oxidation of a series of amines capable of forming potential plant growth substances has been studied by using pea seedling DAO, and several of these amines were shown to have herbicidal activity in tomato and bean plants (Price & Wain, 1978).

Inhibitors. As might be expected from their dependency on copper, DAOs from dicotyledonal plants are subject to inhibition by a variety of chelating agents (Hill & Mann, 1962). They are also sensitive to many carbonyl reagents and especially to hydrazine (Yamasaki et al., 1982). This sensitivity was exploited in the development of the protective action endowed by hydroxyethylhydrazine on plants treated with 2,4-dichlorophenoxyethylamine, a precursor of the herbicide 2,4-D (Nash et al., 1968).

Purification. The pea seedling DAO has been purified to homogeneity by several groups of workers using a variety of approaches. In some studies (Werle et al., 1961; Hill, 1971; Nylen & Szybek, 1974; Macholán & Haubrová, 1976) the intact seedling was used, though, since the enzyme has a much higher specific activity in the epicotyl, some workers have chosen these as the starting material (McGowan &
Muir, 1971; Kluetz et al., 1980a; Yanagisawa et al., 1981; Hiramatsu et al., 1982. Indeed, the enzyme can be purified to homogeneity from this material (yield 35%) with only a 30-fold increase in specific activity (Yanagisawa et al., 1981). Recent results suggest that pre-soaking the pea seeds in water before germination tends to suppress the formation of DAO in cotyledons (Hirasawa, 1983). It appears that the proportion of DAO from the embryos to the cotyledons and embyros differ slightly, but most significantly in electrophoretic mobility. In 6-day-old seedlings the cotyledon enzyme migrated at twice the speed of the DAO from the embryos (Srivastava & Prakash, 1977). Up to four isoenzymes of the DAO from pea have been separated by electrophoresis (McGowan & Muir, 1971; Machołan & Haubrova, 1976; Hiramatsu et al., 1982).

Reaction mechanism and structure. Early work by Mann (1961) showed that the pea seedling DAO combines with the substrate in the absence of oxygen to form an intermediate, changing the normal pink colour ($\lambda_{max}$ 300nm) to yellow ($\lambda_{max}$ at 350, 435 and 465nm). The pink colour is restored on re-admission of oxygen. During the anaerobic reaction, ammonia and aldehyde are generated from the substrate, but peroxide formation is dependent on the presence of oxygen (Yamasaki et al., 1970; Kluetz et al., 1980b). Kluetz et al. (1980b) have shown by the use of low-temperature spectra that the copper is not reduced at any time during the catalytic cycle. The spectrum of the reduced form of the enzyme is independent of the substrate (Kluetz et al., 1980b; Rinaldi et al., 1984), indicating that the copper interacts directly with the organic cofactor of the enzyme. It is thought that the copper is essential for re-oxidation of the reduced enzyme after the addition of substrate, since removal of the copper in Lens DAO by dialysis against diethyldithiocarbamate prevents oxidation, even though O$_2$ is still bound to the metal-free enzyme (Rinaldi et al., 1984).

The pea DAO causes a stereospecific removal of protons from the substrate, particularly in the absence of oxygen, they can be rendered sensitive to dithiopyridine (Floris et al., 1983b). The specificity of the enzyme is inducible in both pea and oat cotyledons, suggesting that the enzyme is inducible (Srivastava et al., 1977). Phytochrome also stimulated activity (Prakash & Sindhu, 1979) but naturally occurring inhibitors have also been demonstrated (Joshi & Prakash, 1982; Srivastava et al., 1982).

The function of DAO in plants is still obscure. At first it was suggested that it was concerned with the conversion of tryptamine to indoleacetic acid, the principal auxin of higher plants (Clarke & Mann, 1957), though its large $K_m$ and small $V_{max}$ with tryptamine (McGowan & Muir, 1971) makes it unlikely that this is its major function. The low $K_m$ for spermine and especially for spermidine suggests that DAO may be concerned in controlling polyamine concentration (Smith, 1981b). However, the large concentration of DAO in the axis of pea seedlings (up to 3% of total protein) may have no significance relative to its activity (cf. urease of Jack bean). DAOs may be concerned in the biosynthesis of various alkaloids (e.g. nicotine) and model systems involving pea seedling DAO have been shown to effect the biosynthesis of certain alkaloids (e.g. Robins, 1982).

Polyamine oxidase

Unlike the DAO found typically in the Leguminosae, which has a broad specificity, an oxidase specific for polyanines which has no effect on diamines occurs throughout the Gramineae. This PAO has not as yet been found outside the Gramineae, though enzymes having a similar mechanism occur in several bacteria. The presence of PAO was first established in barley (Hordeum vulgare) seedlings. After feeding spermine to the cut shoots, these were shown to contain significant amounts of diaminopropionate, and a compound eventually identified as aminopropylpyrrole (Smith, 1970).

Mechanism and substrate specificity. This enzyme oxidizes spermidine to pyrrole, and spermine to aminopropylpyrrole, in each case with the additional production of diaminopropane and hydrogen peroxide:

$$NH_2(CH_2)_3NH(CH_2)_3NH_2 + O_2 \rightarrow$$

Spermidine

$$NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3N + H_2O_2$$

Aminopropylpyrrole

Presumed aminopropylpyrrole synthesized from spermine by the oxidative method of Wrede (1926), with metallic copper in alkali as a catalyst, was subsequently shown to have a bicyclic structure and to be 1,5-diazabicyclo[4.3.0]-nonane (Croker et al., 1983). Although this is identical in all respects with the product of the oxidation of spermine by PAO, and of spermidine by DAO, the form in which this compound exists in solution at physiological pH is still not known, though it is probable that the single-ring compound, aminopropylpyrrole, is predominant.

For the barley leaf PAO, the optimum pH for spermine oxidation was 4.8 and for spermidine the optimum pH was 7-8. The enzyme is unstable at pH values above 7, though in very brief incubations the optimum was near to 8 for spermidine (Smith, 1974). Diaminodipropylamine was also a substrate for the barley leaf enzyme, with a pH optimum of about 7 (Smith & Stevens, 1971). At their respective optima the PAO was more active by 14-fold with spermine as substrate than with spermidine. At their respective pH optima spermidine inhibits spermine oxidation ($K_i = 10^{-4}$m), and spermine inhibits spermidine oxidation ($K_i = 50$ by $3.5 \times 10^{-6}$m) (Smith, 1974). The oat (Avena sativa) leaf PAO was equally active with spermidine and spermine, and both substrates had the same optimal pH (6.5). Unlike the barley PAO, the oat enzyme would not oxidize diaminodipropylamine (Smith, 1977). The maize (Zea mays) seedling enzyme showed a greater similarity with the oat than with the barley enzyme. The optimum pH for spermidine and spermine oxidation was 6.3 and 5.5 respectively and the enzyme was slightly more active with spermidine than with spermine (Suzuki & Hirasawa, 1973).

The specificity of the cereal leaf PAO for spermidine and spermine has been exploited in a rapid method for the determination of spermidine synthase. In this technique decarboxylated S-adenosylmethionine is incubated with putrescine and the synthase, in the presence of the PAO, peroxidase and a fluorogenic peroxidase substrate. The spermidine is oxidized by the PAO as it is synthesized, and the H$_2$O$_2$ is utilized by the peroxidase to oxidize homovanillic acid to a fluorescent product (Suzuki et al., 1981; Sindhu & Cohen, 1983). This PAO has also been used in the
detection of spermine in forensic studies by spectrophotometry (Suzuki et al., 1982), and by immobilization on an oxygen electrode (Macholán & Jílková, 1983).

On purification to homogeneity, the maize seedling PA0 was yellow with absorption maxima at 278, 300 and 460 nm, and the enzyme was shown to contain 1 mol of FAD per mol (Suzuki & Yanagisawa, 1980). However, FAD could not be detected with certainty in the enzyme from oats, also published as apparent work by Suzuki & Yanagisawa. Only weak shoulders could be seen at 350 and 450 nm. Despite this, the specific activity of the oat enzyme was 13-fold greater than that of the maize PA0 (Smith, 1976, 1983).

The barley and oat leaf PAOs are characterized by a relatively large $K_m$ for $O_2$. For the barley PAO this is $3 \times 10^{-4} \text{M}$, which is approximately the concentration of $O_2$ in air-saturated water (Smith & Bickley, 1974; Smith, 1977).

The $M$, values estimated by gel filtration for the oat, barley and maize PAOs were 85000, 85000 and 65000 respectively (Smith, 1976; Suzuki & Yanagisawa, 1980) but density gradient centrifugation indicates a $M_r$ of 119000 for the oat enzyme (Smith, 1977). The $M_r$ of the maize enzyme determined by sodium dodecyl sulphate electrophoresis is 65000 and a subunit structure for the maize PA0 seems unlikely (Suzuki, 1978).

Physiology. The PAOs of barley, oats and maize are associated with a particulate fraction, and they may be eluted from this by washing in 0.5 M-sacH (Smith, 1972, 1976; Suzuki & Hirasawa, 1973). Since the enzyme could not be removed from the particulate fraction by treatment with nucleases, it was thought unlikely to be a nuclear enzyme (Smith, 1976). Only weak activity was still greatly reduced; nor was it due to product inhibition by diaminopropane or aminopropylpyrroline. Since these amines alone, or in combination, were effective was guazatine oxide-scavenger copper salicylate protected the enzyme. Protoplasts associated with a particulate fraction, and they may be inhibited by a spermidine analogue, amongst a homologous series of diguanidines $\left\{\left[NH_2\left(CH_2\right)_nNH_2\right]_{2}NH\right\}$, in which the methylene chain ranged from 2 to 12 carbon units in length.

The very rapid decline in activity during assay (Smith, 1977) was not due solely to oxygen depletion, since on re-oxygenation activity was still greatly reduced, nor was it due to product inhibition by diaminopropane or aminopropylpyrroline, since these amines alone, or in combination, were without any inhibitory effect. Omission of catalase from the assay greatly accelerated the loss of activity and provided presumptive evidence that peroxide is responsible for this inactivation. Neither superoxide dismutase nor the superoxide-scavenger copper salt-cathepsin protected the enzyme. Inactivation may therefore be due to superoxide formed at a site inaccessible to catalase (Smith, 1983).

Activity of oat leaf PA0 with spermine as substrate is stimulated up to 5-fold in the presence of salts of lithium, sodium, potassium and rubidium in order of increasing effectiveness, but salts of calcium and magnesium are inhibitory (Smith, 1977, 1985). The stimulation with alkaline metal salts is not so apparent when spermidine serves as a substrate.

The relation of amine oxidases with other pathways of amine catabolism

The erratic occurrence of the amine oxidases in the plant kingdom is difficult to reconcile with an essential function for these enzymes, although it is possible that they are one means adopted by certain plants for controlling or confining di- and poly-amines. This function may be fulfilled in other species by transamination (Kim, 1964; Hasse et al., 1967; Wink & Hartmann, 1979), or dehydrogenation (Tabor & Kellogg, 1970) as found in certain bacteria. In addition di- and poly-amines undergo conjugation, particularly with hydroxycinnamic acids, in many higher plants (Smith et al., 1983) and this process may be analogous to the polyamine acetylation which occurs in bacteria and mammals (Aigner-Held & Daves, 1980).
Polyamine oxidases

The enzyme has been purified to homogeneity (Tabor et al., 1954, 1955; Yasunobu & Smith, 1971; Turini et al., 1982; Mondovi et al., 1983), crystallized (Yamada & Yasonobu, 1962), and shown to act on spermine and spermidine as follows:

\[
\text{NH}_2(\text{CH}_2)_2\text{NH}_2(\text{CH}_2)_2\text{NH}_2(\text{CH}_2)_2\text{NH}_2 + 2\text{O}_2 + 2\text{H}_2\text{O} \rightarrow \text{Spermine}
\]

\[
\text{OCH}(\text{CH}_2)_2\text{NH}(\text{CH}_2)_2\text{NH}(\text{CH}_2)_2\text{CHO} + 2\text{NH}_3 + 2\text{H}_2\text{O}
\]

\[
N,N'\text{-Bis-(3-propionaldehyde)-1,4-diaminobutane}
\]

\[
\text{NH}_2(\text{CH}_2)_2\text{NH}_2(\text{CH}_2)_2\text{NH}_2 + O_2 + H_2O \rightarrow \text{Spermidine}
\]

\[
\text{NH}_2(\text{CH}_2)_2\text{NH}(\text{CH}_2)_2\text{CHO} + \text{NH}_2 + \text{H}_2\text{O}
\]

\[
N(4\text{-Aminobutyl})-3\text{-aminopropionialdehyde}
\]

The aldehydes produced were characterized by reduction with sodium borohydride and comparison with the corresponding synthetic aliphatic alcohols (Tabor et al., 1964). Three forms of the enzyme have been reported (Yasunobu et al., 1976) but the data on some of them is sparse. The enzyme has an \( M_r \) of about 180000 (Achee et al., 1968; Turini et al., 1982), made up of two subunits of \( M_r \), 90000, not covalently linked: association dissociation between monomer–dimer–trimer has been noted (Achee et al., 1968), providing an explanation for reported \( M_r \) values of the order of 263000 (Yamada et al., 1964; Nakano et al., 1974; Gaul & Pitot, 1982). Each subunit contains a disulphide bridge, and there are two copper atoms and one carbonyl group per molecule that are essential for activity. Early reports suggested pyridoxal phosphate as a cofactor (Yasunobu et al., 1968; Yasonobu & Smith, 1971) but its presence was never directly demonstrated and later work has tended to disprove this (Watanabe et al., 1972; Inamatsu et al., 1974; Yasunobu et al., 1976): indeed Suva & Abeles (1978) state categorically that pyridoxal phosphate is not a cofactor for this enzyme. Recently it has been shown that the prosthetic group is covalently bound pyrroloquinoline quinone.