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Polymorphism and function of some oxidizing enzymes of Scots pine (*Pinus sylvestris L.*)*

INTRODUCTION

Under investigation were enzymatic proteins from tissues of Scots pine with peroxidase (PO), polyphenoloxidase (PPO) and indole-3-acetic acid oxidase (IAA oxidase) activity. These are enzymes commonly found in plant tissues.

Peroxidases (donor H_2O_2 oxidoreductase E.C. 1.11.1.7.) are nonspecific enzymes with respect to substrates, which in the presence of H_2O_2 can oxidize phenolic compounds, cytochrome c, ascorbic acid, indole, amines and some inorganic ions. They are readily soluble but they occur also in forms bound with cell structures, namely with the cell wall (R i d g e and O s b o r n e 1970), ribosomes (P e n n o n et al. 1970, D a r i m o n t and B a x t e r 1973), mitochondria (D a r i m o n t and B a x t e r 1973), provacuolar membranes (P a r i s h 1975) and various unidentified membranes (van der M a s t 1970, D a r i m o n t et al. 1977). Activity of PO was also found in chromosomes (R a a 1973).

Polyphenoloxidases are a group of enzymes with so far an undefined function. They oxidize phenolic substrates using molecular oxygen. According to the recommendations of the International Biochemical Union from the year 1964 these enzymes have been divided into two groups: 1) catechol-crtho-diphenol oxidases: oxidoreductase (E.C. 1.10.3.1.) and 2) para-diphenol laccase: oxidoreductase (E.C. 1.10.3.2.). Later a revision of nomenclature was made combining these enzymes into E.C. 1.14.18.1 monophenol: monooxygenase. M a y e r and H a r e l (1979) believe that this revised nomenclature is inappropriate since catechol oxidase and laccase differ substancially in catalytic properties. Catechol oxidase referred to also as phenolase, polyphenoloxidase, catecholase, tyrosinase or crezolase catalyzes two reactions (M a y e r and H a r e l 1979): a) the

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transfer of oxygen from the ortho position to an existing hydroxyl group (crezolase activity), and b) oxidation to ortho-diphenol detaching hydrogen (catecholase activity). Laccase catalyses the reaction of dehydrogenating monophenols, triphenols, ortho-diphenols and as the only oxidase capable of doing it, para-diphenols. Catechol oxidase is easily detectable and thus it was one of the first of plant enzymes to be studied (Bertrand 1895 - 1896). This enzyme has been found in the soluble fraction of plant cells (Mayer 1961, Harel et al. 1964, Sanderson 1964, Coombs et al. 1974), in fractions bound with the cell wall (Palmer 1963, Delon 1974, Stom et al. 1975) and with membranes of organelles (Mayer and Friend 1960, Mayer 1961, Harel et al. 1964, Stafford 1969, Tolbert 1973, Kato et al. 1976).

Indole-3-acetic acid oxidase is an enzyme catalysing the destruction of IAA and it is known to be a ferroprotein. In the literature the majority of data indicates that generally various isoperoxidases are capable of oxidizing IAA though Sequeira and Mineo (1966) and van der Mast (1969) have isolated enzyme preparations capable of oxidizing IAA but demonstrating no PO activity.

The functions of PO, PPO and IAA oxidase have not been strictly determined yet. In vascular plants PO and PPO most probably participate in the production of wood through the oxidation of phenols which then spontaneously condense to lignin, though there is little evidence supporting this function. Freudenberg et al. (1958) have demonstrated the participation of laccase in the oxidative or dehydrogenative polymerisation in vitro of coniferyl alcohol molecules or else of these molecules with those of synapyl alcohol or p-coumaryl alcohol. Since PO in the presence of H_2O_2 can change p-coumaryl alcohol into polymers similar to lignin, as PPO in the presence of oxygen, Higuchi (1957) and Lyr (1957) have considered PO to be lignifying factor in plants. Harkin and Obst (1973) have shown that in the early stages of lignification PO is the only enzyme oxidizing phenols.

Laccase participates in the demethoxification of lignin during its degradation (Kirk et al. 1977) and in the case of pathogens it can detoxify fungicidal compounds produced by the host plant (Ward and Stoessl 1973, Rubin and Artsikovskaja 1964).

There is much data in the literature indicating the participation of oxidases in IAA metabolism. Thimann (1934) was the first to propose the theory that the phenol-phenoloxidase system acts inhibitively on auxin and Tomaszewski (1959) has found that the same enzymatic system may degrade IAA both in vivo and in vitro forming auxin-phenol complexes (Leopold and Plumer 1961).

The importance of PO in regulation of growth and development of plants has been frequently suggested in the literature. During the development of plants there occur changes in the pattern of electrophoretic

fractions for PO and in the enzymatic activity of specific fractions. There is relatively little information on the changes in isoenzyme patterns during development of forest trees (D u r z a n 1966, C o n k l e 1971, M a y b e r r y and F e r e t 1977). To determine the role of isoperoxidases in the development of plants the isoenzyme patterns have to be compared from extracts of developing tissues obtained from normal plants with those from an undifferentiated callus (B a j a j et al. 1973, H a-b a g u c h i 1977a, 1977b).

The pattern of electrophoretic fractions of oxidases also undergoes changes under the influence of growth substances however the mechanisms by which these compounds act are as yet unexplained. An increase in PO and PPO activity is observed under stress conditions (injury to tissue, action of pathogens, fumigation with industrial pollutants, low humidity, lack of mineral and hormonal substances) and during ageing. It is believed that an increase in activity is associated with a destruction of cellular structures and the liberation of oxidases from inactive forms (F a r k a s et al. 1964). Possibly also a change of the oxidases occurs from the bound condition with the cell structures into the free form (V o l k et al. 1977, 1978, H a r e l et al. 1966, K i n d-r o n et al. 1978).

There are various opinions concerning interrelationships between the activities of PO, PPO, and IAA oxidase: 1. The activity of PO, PPO and IAA oxidase are associated with the same protein molecule (P i l e t and S e v h o n k i a n 1969, J a n s s e n 1970, S r i v a s t a v a and v a n H u y s t e e 1973, K i e l i s z e w s k a - R o k i c k a 1980), 2. The activities of PO and IAA oxidase are strictly connected with each other while PPO is a clearly distinct enzyme (S h i n s h i and N o g u c h i 1975), 3. Some plant species do not have PPO (R a y 1960, B a s t i n 1964, C a m b i e and B o c k s 1966, D i n a n t and G a s p a r 1967).

In a previous investigation (K i e l i s z e w s k a - R o k i c k a 1980) the results of electrophoretic studies on PO and PPO in needles and roots of Scots pine have led to the suggestion that pine does not have a specific polyphenoloxidase. In the present study an analysis of the substrate specificity of electrophoretic forms from various organs of pine and from various cell fractions as well as from various chromatographic fractions was aimed at the confirmation or rejection of the view that pine lacks a PPO and the determination of the possible direction of oxidative action in this species.

MATERIAL AND METHODS

Plant material. Vegetative organs of several years old Scots pine (*Pinus sylvestris* L.) trees from an experimental forest and of young seedlings growing in a greenhouse at a 16 h photoperiod were investi-

gated. The seeds were sown in perlite acidified with 0.1 N phosphoric acid to pH 7 and then watered with a mineral nutrient solution according to L a i h o (1970). For some analyses commercial preparation of purified peroxidase of horse-radish (HRP, lyophilized, Calbiochem.) was used.

Enzyme preparations. 1. The crude extract was prepared on the basis of a method given by H a d a č o v a and S a h u l k a (1967). The tissue was homogenized in 0.2M phosphate buffer, pH 6, which contained 0.2% ascorbic acid, 0.1% L-cysteine hydrochloride monohydrate and 5% polyclar AT. The homogenate was centrifuged at $10\,000\times g$, the supernatant dialysed in 0.01 phosphate buffer, pH 6 and then used as an enzyme preparation. 2. Acetone powder. The tissue was infiltrated under vacuum with a solution containing 0.1M ascorbic acid and 0.1M potassium pyrosulphite and then homogenized in cold 80% acetone and washed with anhydrous acetone to obtain a white powder. From the acetone powder proteins were washed out with 0.1M borate buffer, pH 8 or 0.2M phosphate buffer pH 6. After centrifuging at $10\,000\times g$ the supernatant was dialysed in 0.01M borate buffer or 0.01M phosphate buffer respectively, and then used as an enzyme preparation.

Extraction of soluble bound enzymes. Pine tissues have been fractionated according to the method given by B i r e c k a and M i l l e r (1974) into: a) buffer soluble proteins, b) proteins soluble in 1% Triton X-100, c) proteins ionically bound with cell membranes, d) proteins covalently bound with cell membranes. All fractions were dialysed in 0.01M phosphate buffer pH 6.

Determination of protein content. The method given by P o t t y (1969) with albumine of bovine blood serum as standard was used.

Ion-exchange chromatography. The enzyme preparation in 0.1M borate buffer was fractionised in DEAE cellulose column (30×2 cm) according to the method of S h i n s h i and N o g u c h i (1975).

Enzyme assays. PO activity was determined according to the method of Q u o i r i n et al. (1974) with modifications. The reaction mixture contained 1 ml of 0.2M phosphate buffer, pH 6.1, 1 ml of 0.2M H_2O_2, 1 ml of 1% quaiacol and a suitable amount of enzyme. The activity was assayed at 430 nm. To determine the activity of IAA oxidase the decomposition of IAA by the enzyme preparations was investigated, using the method given by S h i n s h i and N o g u c h i (1975) with S a l k o w s k i reagent (G o r d o n and W e b e r 1951).

Electrophoresis. Polyacrylamide disc gel electrophoresis in anionic (D a v i s 1964) and cationic (R e i s f e l d et al. 1962) systems was carried out. Comparable amounts of protein were applied onto gel columns: 2 µg for peroxidase, 200 µg for IAA oxidase and polyphenoloxidase. Separation was conducted for 2.5 - 3 hours using 2 mA per one gel column 5 mm in diameter.

Staining of isozymes on polyacrylamide gels. The incubation mixture for PO contained 0.03M phenolic substrates as indicated in figures dissolved in an acetate buffer pH 5 and 0.02M H_2O_2 (modified method of S a h u l k a 1970). Benzidine was used in a saturated solution (S a f o n o v et al. 1969). The reaction was performed for 15 minutes at room temperature. PPO was stained with 0.03M phenolic substrates in 0.2M phosphate buffer, pH 6 (S a h u l k a 1970), or in the same buffer containing 60% ethanol. Some gel columns after 30 minutes of incubation in the buffer solution of substrate were incubated for another 30 minutes in 0.1% solution of p-phenylenediamine or m-phenylenediamine in order to obtain higher sensitivity of reaction (S h e e n and C a l v e r t 1969, v a n L o o n 1971). The oxidations of IAA and L-tryptophan were carried out in IAA and L-tryptophan respectively in the presence of $MnCl_2$ and 2.4-dichlorophenol with or without H_2O_2 (F r e n k e l 1972). After staining the gel columns were scanned in a Vitatron densitometer.

RESULTS

1. SUBSTRATE SPECIFICITY OF ENZYMATIC PREPARATIONS FROM PINE AND A COMMERCIAL PEROXIDASE PREPARATION FROM THE ROOT OF HORSE-RADDISH

1.1. Pine tissues

On acrylamide in an anionic and cationic systems the enzymatic preparations from various Scots pine tissues were electrophoretically separated. Enzymatic reactions was conducted in order to oxidize various phenolic and indolic substrates in the presence of H_2O_2 and without it. Figure 1 presents the patterns of electrophoretic fractions with an oxidase activity obtained from an enzyme preparation of vegetative buds of pine. The ability to oxidize IAA, L-tryptophan, p-cresol and benzidine was investigated. The oxidation of IAA occurred both in the presence of H_2O_2 and in atmospheric oxygen and the enzymatic fraction catalyzing these reactions has the same Rf value as the fractions oxidizing L-tryptophan, p-cresol and benzidine. L-tryptophan was oxidized exclusively in the presence of H_2O_2. The product of L-tryptophan oxidation had after a reaction with dimethylaminocinnamaldehyde a pink reaction similarly as the product of IAA oxidation. A similar result has been obtained earlier (K i e l i s z e w s k a - R o k i c k a 1980) when L-tryptophan was oxidized in the presence of H_2O_2 by electrophoretic fractions from poplar tissues.

In a solution of p-cresol without H_2O_2 on the gel two small darkly staining bands formed, however this reaction was not reproduced in the course of studies with other enzyme preparations. On the other hand, in the same solution of p-cresol in the presence of H_2O_2 the electrophoretic fractions have formed a stainless precipitate which suggests the dehydrogenation of a monophenol and its condensation into larger entities.
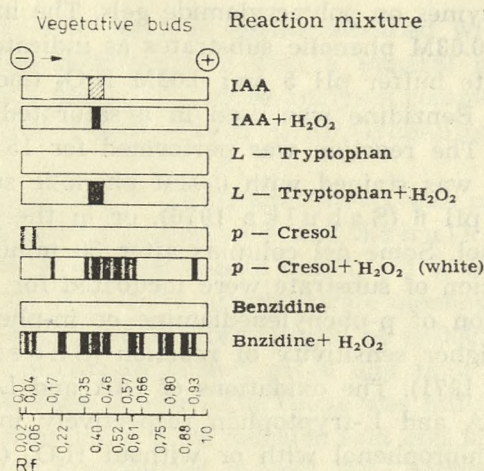
Fig. 1. Electrophoretic separation of an enzyme preparation from Scots pine vegetative buds on acrylamide gels at pH 8.3. The oxidation of IAA and L-tryptophan follows in the presence of $MnCl_2$ and 2.4-dichlorophenol in 0,2 M phosphate buffer pH 4.5. The staining mixtures for polyphenoloxidase contained 0.03 M p-cresol in 0.2 M phosphate buffer pH 6 and benzidine in the same buffer in a saturated solution. The staining mixtures for peroxidase contained 0.03 M p-cresol and benzidine in 0.2 M acetate buffer pH 5 and 0.02 M H_2O_2

In benzidine solution with H_2O_2 added there formed on the gel 12 brownish bands with PO activity. Without H_2O_2 no colour reaction was observed.

In further investigations the ability of the enzyme preparations from pine to oxidize various monphenols and polyphenols was studied in the presence of H_2O_2 and with atmospheric oxygen only. Enzymatic incubation in buffer solutions of phenols without H_2O_2 did not form any colour reactions. Thus for the enzymatic incubation we have used compounds which are known from the literature to couple with the products of dehydrogenation of phenols, namely m- and p-phenylenediamine and ethanol.

Figure 2 presents the electrophoretic patterns for the oxidases of cambium. In the presence of H_2O_2 the studied enzymatic preparation catalysed the dehydrogenation of polyphenols into staining compounds and monophenols into a colourless precipitate. The presence in the incubation mixture of 60% ethanol, 0.1% m-phenylenediamine or 0.1% p-phenylenediamine have resulted in the formation of staining products in spite of the absence of H_2O_2.

1.2. Peroxidase preparation from the root of horse-radish

For comparison the oxidation capacity of commercial peroxidase preparation from root of horse-radish (HRP) was analysed. Figure 3 shows the results of incubation of the HRP fractions separated on acry-

Reaction mixture

cambium — $(-)$ ... $(+)$

Guaiacol
Guaiacol+H_2O_2
Guaiacol+0.1% p-PDA
Guaiacol+60% ethanol

Catechol
Catechol+H_2O_2
Catechol+0.1% p-PDA
Catechol+60% ethanol

Caffeic acid
Caffeic acid+H_2O_2
Caffeic acid+0.1% p-PDA
Caffeic acid+60% ethanol

p-Coumaric acid
p-Coumaric acid+H_2O_2 (white)
p-Coumaric acid+0.1% p-PDA

Gentisic acid
Gentisic acid+H_2O_2
Gentisic acid+0.1% p-PDA
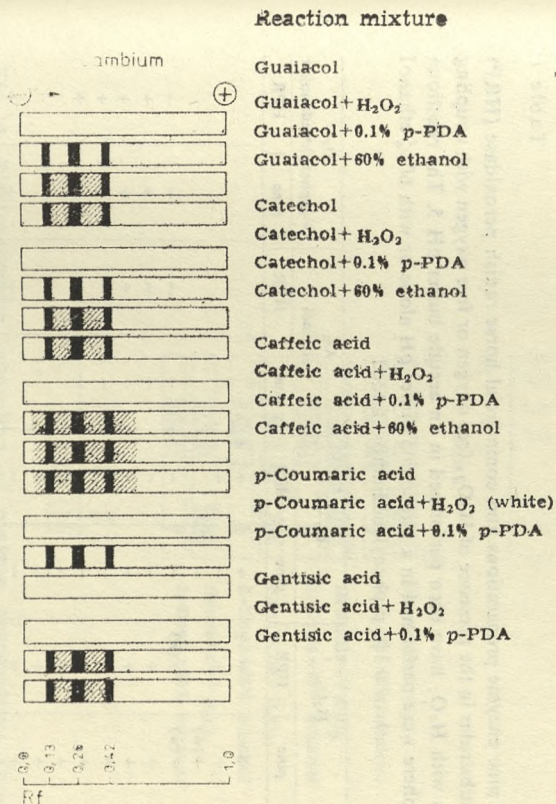
Rf 0.0 0.13 0.2? 0.42 ... 1.0

Fig. 2. Electrophoretic separation of an enzyme preparation from Scots pine cambium on acrylamide gels at pH 8.3. Staining mixtures for peroxidase contained phenolic substrates in 2M acetate buffer pH 5 and 0.02 M H_2O_2. Polyphenoloxidase activity was detected by incubating the gels in mixtures containing 0.03 M substrates in 0.2 M phosphate buffer pH 6. Some of reactions occur in the presence of 0.1% p-phenylenediamine (p-PDA) or 60% ethanol

Reaction mixture

HRP — $(-)$ ... $(+)$

IAA
IAA+H_2O_2

L-Tryptophan
L-Tryptophan+H_2O_2

p-Coumaric acid
p-Coumaric acid+H_2O_2 (white)
p-Coumaric acid+0.1% p-PDA
p-Coumaric acid+60% ethanol

Guaiacol
Guaiacol+H_2O_2
Guaiacol+0.1% p-PDA
Guaiacol+60% ethanol

Benzidine
Benzidine+H_2O_2
Benzidine+0.1% p-PDA
Benzidine+60% ethanol

Gentisic acid
Gentisic acid+H_2O_2
Gentisic acid+0.1% p-PDA
Gentisic acid+60% ethanol

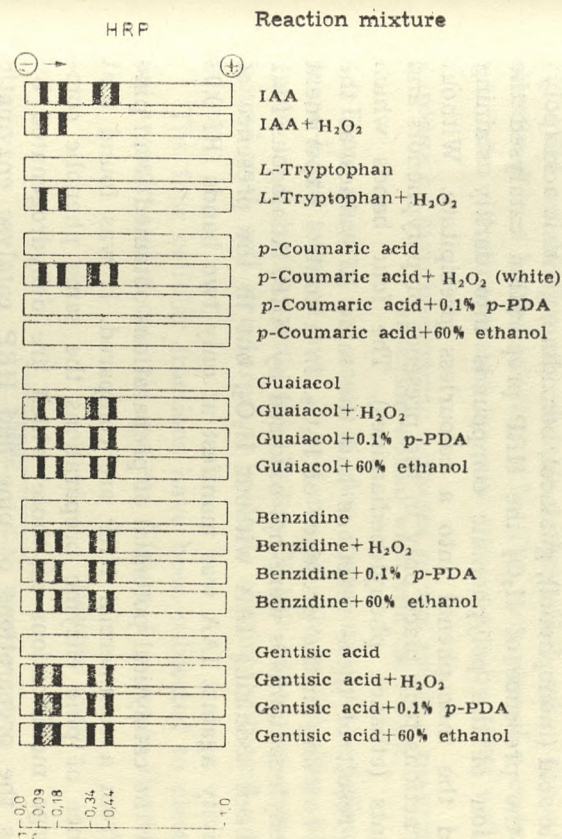Rf 0.0 0.09 0.18 0.34 0.44 ... 1.0

Fig. 3. Electrophoretic separation of horse-radish peroxidase (HRP) on acrylamide gels at pH 8.3. Capabilities of electrophoretic fractions to oxidize IAA and L-tryptopham have been detected as in Fig. 1. The staining mixtures for polyphenolooxidase were used as in Fig. 2

lamide in solutions of the following substrates: IAA, L-tryptophan indoles, p-coumaric acid (monophenol), guaiacol, benzidine, gentisic acid (polyphenols). In the presence of H_2O_2 the HRP preparation catalysed the dehydrogenation of the polyphenolic compounds into darkly staining products, and the monophenol into a colourless precipitate. Without H_2O_2 these reactions took place only in the presence of polyphenols and coupling agents (ethanol, phenylenediamines). The four bands which formed as a result of these reactions had the same Rf values as the bands of PO reacting in the presence of H_2O_2. In a control experiment m- and p-phenylenediamines were not oxidized by HRP. Analogous four bands have been oxidizing IAA without H_2O_2, but in the presence of H_2O_2 the activity against IAA was manifest at only two bands (Rf 0.08 and 0.18).

In Table 1 the catalytical potential of preparations obtained from pine tissues and from a commercial HRP are compared. It was found that in the presence of pine enzyme preparations the same phenolic compounds undergo modifications as those which are dehydrogenated by HRP. Besides the preparations of pine and HRP catalyse enzymatic reactions only in some, already identical conditions. The IAA oxidation by peroxidase in the presence of atmospheric oxygen has been known for some time (K e n t e n 1955, R a y and T h i m a n n 1956). The present results indicate the possibility of oxidation of other substrates also by PO of pine and of horse radish without H_2O_2 when in the reaction mixture ethanol or phenylenediamine are present. Pine and HRP prepara-

Table 1

Oxidative activity of pine enzyme preparations and commercial horse radish peroxidase (HRP) on various phenolic substrates in the presence of H_2O_2, free oxygen or free oxygen with coupling agents. The reactions with H_2O_2 have been performed in an acetate buffer pH 5. The reactions with O_2 from atmosphere were performed in a phosphate buffer pH alone or with 60% ethanol or 0.1% p-phenylenediamine added

Substrates	H_2O_2		O_2		O_2 + ethanol		O_2 + p-phenylenediamine	
	pine	HPR	pine	HPR	pine	HPR	pine	HPR
ndole-3-acetic acid	+	+	+	+				
L-tryptophan	+	+	−	−				
P-cresol	+(w)		−					
P-coumaric acid	+(w)	+(w)	−	−	−	−	−	−
Catechol	+	+	−	−	+	+	+	+
Guaiacol	+	+	−	−	+	+	+	+
Caffeic acid	+	+	−	−	+	+	+	+
L-dopa	+	+	−	−	+	+	+	+
Chlorogenic acid	+	+	−	−				
Hydroquinone	+	+	−	−				
Gentisic acid	+	+	−	−		+	+	+
Benzidine	+	+	−	−	+	+	+	+
Pyrogallol	+	+						

+ positive reaction,   − no reaction   w white precipitate

tions did not oxidize any phenols without H_2O_2 when the substrates were dissolved in a buffer solution only. The monophenol p-coumaric acid has not been dehydrogenated by pine tissues nor by HRP without H_2O_2.

2. FORMS OF ELECTROPHORETIC OXIDASES IN THE SOLUBLE FRACTION AND IN THE FRACTION BOUND WITH THE CELL WALL

From needles and roots of pine consecutively the following fractions were being washed out: a) proteins soluble in a buffer, b) proteins soluble in a buffer with 1% Triton X-100 added, c) proteins ionically bound with cell structures, d) proteins covalently bound with cell structures.

The four cellular fractions have been subjected to electrophoresis in an anionic and a cationic system. Figure 4 presents the electrophoretic
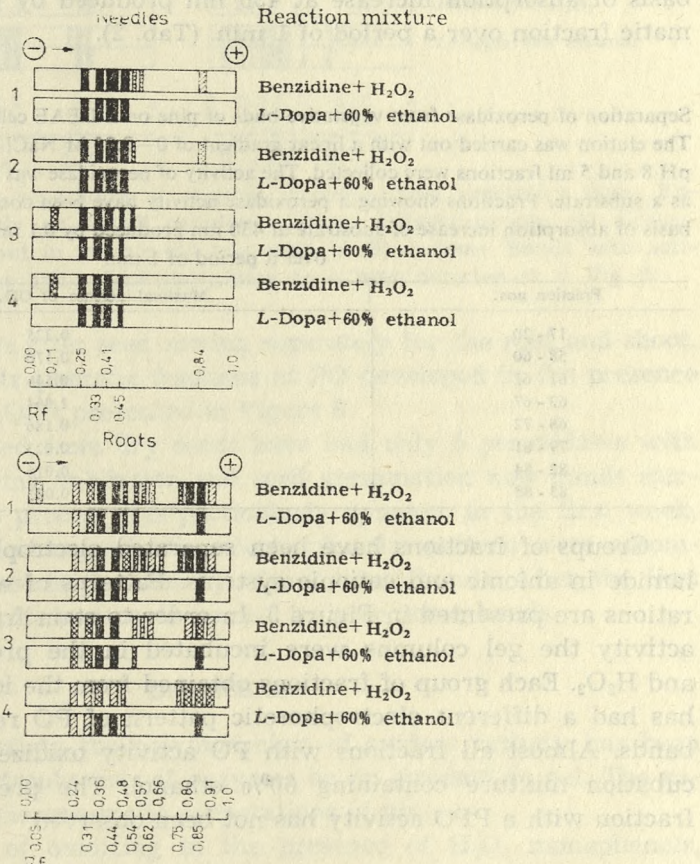

Fig. 4. Electrophoretic separation of enzyme preparations from needles (A) and roots (B) on acrylamide gels at pH 8.3. Pine tissues have been fractionated into: 1 — buffer soluble fraction, 2 — fraction soluble in 1% Triton X-100, 3 — ionically bound fraction, 4 — covalently bound fraction. The staining mixtures for peroxidase and polyphenoloxidase were used as in Fig. 2

bands of PO in individual cell fractions obtained as a result of incubation of gels in a solution of benzidine and H_2O_2 and of bands obtained through incubation of columns in a solution of an ortho-diphenol L-dopa in the presence of ethanol. The majority of PO bands have been oxidizing also L-dopa, but in none of the cellular fractions was the presence of a specific PPO demostrated.

3. ELECTROPHORETIC FORMS WITH PO AND PPO ACTIVITY IN ENZYMATIC PREPARATIONS FRACTIONATED ON AN ANION EXCHANGE COLUMN

The protein preparation from vegetative buds of pine has been separated in a column of ion-exchange cellulose Cellex-D in NaCl gradient. Fractions 5 ml in volume were collected and in the individual fractions the activity of PO was determined using guaiacol as a substrate. Fractions showing a PO activity have been combined into groups on the basis of absorption increase at 430 nm produced by 0.1 ml of the enzymatic fraction over a period of 1 min. (Tab. 2).

Table 2

Separation of peroxidase from vegetative buds of pine on a DEAE cellulose column (2×30 cm). The elution was carried out with a linear gradient of $0-0.25$ M NaCl in 0.01 M Tris-HCl buffer pH 8 and 5 ml fractions were collected. The activity of peroxidase was determined using guaiacol as a substrate. Fractions showing a peroxidase activity have been combined into groups on the basis of absorption increase of substrate at 430 nm produced by 0.1 ml of the enzymatic fraction over a period of 1 min

Fraction nos.	Maximal increase of OD_{430} /I'/ 0.1 ml
17 - 20	0.325
58 - 60	0.170
61 - 62	0.546
63 - 67	1.864
68 - 72	0.186
79 - 81	0.023
82 - 84	0.016
85 - 88	0.023

Groups of fractions have been separated electrophoretically on acrylamide in anionic and cationic systems. Patterns of electrophoretic separations are presented in Figure 5. In order to stain fractions having a PO activity the gel columns were incubated in the presence of benzidine and H_2O_2. Each group of fractions obtained from the ion-exchange column has had a different electrophoretic pattern of PO represented by 2 - 12 bands. Almost all fractions with PO activity oxidized L-dopa in an incubation mixture containing 60% ethanol. The presence of a specific fraction with a PPO activity has not been observed.

4. ISOPEROXIDASES IN THE EARLY STAGES OF PINE TREE DEVELOPMENT

Enzymatic preparations from germinating embryos, seeds and developing seedlings have been separated on acrylamide in an anionic system. Initially the enzymatic preparations have been made from the whole
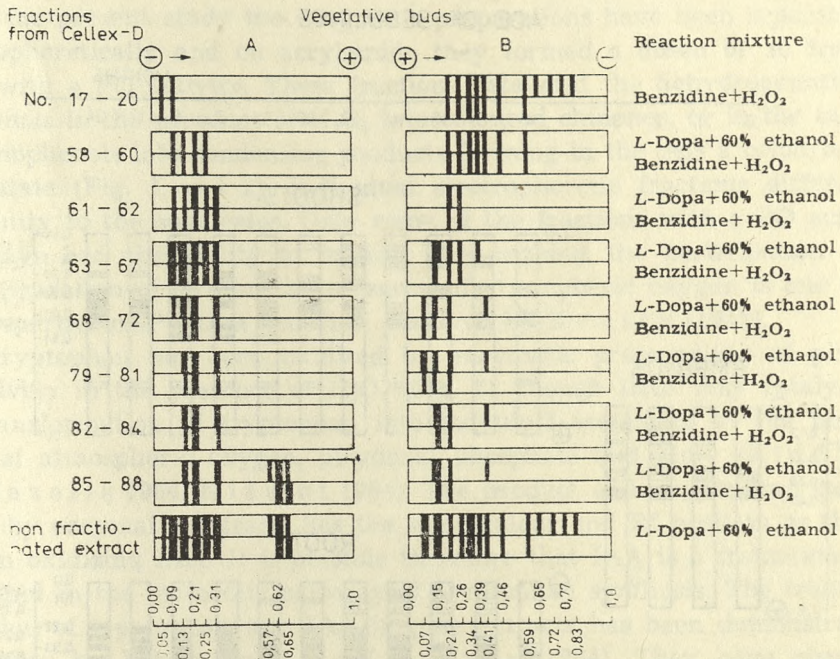
Fig. 5. Electrophoretic separation of enzyme fractions from vegetative buds. Extracts eluted previously on DEAE cellulose column. Polyacrylamide gel electrophoresis was carried out in anionic (A) and cationic (B) systems. Bands with activities of peroxidase and polyphenoloxidase have been detected as in Fig. 2

embryos, and 4 days after seed sowing separately for the root and shoot. The pattern of electrophoretic fractions of PO developed in the presence of benzidine and H_2O_2 is presented in Figure 6.

Embryos dissected from dry seeds have had only 5 peroxidases with a low activity. During imbibition and seed germination new bands started to appear. This process was particularly dynamic in the first week, and the qualitative changes in the electrophoretic pattern were accompanied by an increase in the activity of individual bands. After the first week of growth shoots and roots tended to loose some bands.

DISCUSSION

In the present study the polymorphism of oxidase activity has been analysed using electrophoresis of enzymes on an acrylamide gel. The results indicate that the enzymatic preparations of pine:

1. Are capable of oxidizing in the presence of H_2O_2 monophenols, o-polyphenols, p-polyphenols, IAA and L-tryptophan (peroxidase activity),

2. Are capable of oxidizing IAA in the presence of atmospheric oxygen (IAA oxidase activity),

AGE OF SEEDLINGS

Fig. 6. Patterns of eletrophoretic bands of peroxidase from embryos, shoots and roots of pine seedlings in early stages of development. Electrophoresis was performed on acrylamide at pH 8.3. The peroxidase bands have been developed with benzidine and H_2O_2 as in Fig. 2

3. Are unable to oxidize phenolic compounds and L-tryptophan without H_2O_2.

Results of investigations of some authors have shown that the activity of IAA oxidase cannot be separated from the activity of PO (P i l e t and L e v a n c h y 1969, G a s p a r et al. 1969, H o y l e 1972, S h i n s h i and N o g u c h i 1975). There are also reports where PO activity was not accompanied by IAA oxidase activity, however S t o n i e r et al. (1979) have found in enzymatic extracts of *Vigna* a factor masking IAA oxidase activity. This factor, most probably a complex polimer o-dihydroxyphenylpropanoid, referred to as a protector of auxin can be separated from enzymatic proteins by filtration through a gel column of Sephadex or Sepharose, which causes the appearance of IAA oxidase activity in the protein fractions. Most of the authors studying the relationship between PO, IAA oxidase and PPO consider the ability to oxidize IAA to be one of the many possible functions of an enzymatic PO molecule.

In the present study the enzymatic preparations have been separated electrophoretically and on acrylamide they formed a dosen or so fractions with a PO activity. These fractions catalysed the dehydrogenation of phenols in the presence of H_2O_2 into coloured chinones, or in the case of monophenols into condensing products forming in the gels a colourless precipitate (Fig. 1 and 2). Individual electrophoretic fractions differed in affinity to the substrates. Only some of the fractions with a PO activity have had the ability to oxidize IAA without the participation of H_2O_2. Oxidation of IAA in the presence of atmospheric oxygen is one of the properties of PO that was first observed by K e n t e n (1955).

L-tryptophan has been oxidized by enzymatic preparations of pine exclusively in the presence of H_2O_2 (Fig. 1), though HRP may catalyse the transformation of tryptophan into indolilo-3-acetamide in the presence of atmospheric oxygen, pirydoxal phosphate and Mn^{2+} (R i d d l e and M a z e l i s 1964, K l ä m b t 1964). The product of L-tryptophan oxidation by enzymatic extracts has the same colour and Rf position as the fraction oxidizing IAA. It is possible therefore that IAA is a transitional compound in the metabolic pathway of tryptophan synthesis. The transformation of tryptophan into IAA in pine extracts has been demonstrated earlier by W h i t e m o r e and Z a h n e r (1964). They have given tryptophan to extracts of cambium and phloem of pine and they have found the formation of IAA. In fresh extracts IAA formed rapidly, but it also formed, though much more slowly in boiled extracts. Since PO can maintain its activity even after boiling it is possible that it was this enzyme that catalyzed the transformation of tryptophan to IAA. Decarboxylation of IAA and L-tryptophan has been observed when labelled substrates were introduced into seeds and pine explants (H e j n o- w i c z and T o m a s z e w s k i 1969, T o m a s z e w s k i 1970a, 1970b).

One of the possible physioecological functions of PO associated with the cell wall could be the degradation of exogenous auxin. When penetrating into cell walls PO could be disturbing the processes controlled by endogenous auxin, as is the case in the mycorrhizal associations of pine (T o m a s z e w s k i and W o j c i e c h o w s k a 1974).

In the presence of atmospheric oxygen electrophoretic fractions of pine tissues have not been transforming any phenols into colour products, even though they have produced intensive colour reactions when the incubation took place in the presence of compounds coupling with products of the oxidation of phenols such as m-phenylenediamine, p-phenylenediamine or when the substrate was dissolved in a buffer-ethanol mixture (Fig. 2). S h e e n and C a l v e r t (1969) and v a n L o o n (1971) when using the factors associating themselves with the products of phenol oxidation have obtained an increase in the intensity of colour reactions catalyzed by PPO.

In the present study at no stage did it prove possible to physically

separate the PPO activity from that of PO. Enzymatic proteins with a PO activity have been found in soluble fractions and in those bound ionically or covalently with cell structures. Treating cell structures with a Triton X-100 solution after having washed away proteins soluble in a buffer has demontrated the presence of a very active PO (Fig. 4). Similar effects have been obtained by W a l k e r and H u l m e (1966) and by H a r e l and M a y e r (1971). According to some authors the strength of the bound beween oxidases and membranes depends on the type of tissue and the state of plant development. In none of the four cell fractions studied in the present investigation was the existence of a specific PPO activity demonstrated. Also the double fractionation of pine enzyme preparations first in anion-exchange cellulose and then electrophoretically on acrylamide (Fig. 5) did not disclose any protein forms with a specific PPO activity.

On the basis of results obtained in the present study one can suspect that PPO activity in pine is very low or that the studied enzyme preparations did not contain PPO and the reactions of dehydrogenating phenolic substrates can in some conditions take place irrespective of this enzyme.

Specific electrophoretic fractions with an exclusively PPO activity have been found in other plants for example in tobacco (S h i n s h i and N o g u c h i 1975) or in poplar bark (K i e l i s z e w s k a - R o k i c k a 1980). On the other hand, S r i v a s t a v a and van H u y s t e e (1973) have shown electrophoretically and chromatographically that tissue cultures of peanuts contain true PPO which however appears in the same fractions as PO and IAA oxidase.

Pine tissues have shown the same catalytic abilities as the commercial preparation of a purified PO of horse radish root (HRP). Figure 3 and Table 1 indicate that in some conditions HRP catalyzes oxidation reactions of phenolic compounds without the participation of H_2O_2, though it does not contain PPO. One can suspect therefore that also the pine tissue preparations studied here behave in the same way as HRP, that is they can in certain special conditions catalyze the oxidation with free oxygen.

In spite of the fact that PPO is common in nature, lack of this enzyme has been found in *Osmunda* sp. (R a y 1960), *Impatiens* (B a s t i n 1964) and in *Phaseolus* sp. (D i n a n t and G a s p a r 1967). In all these plants PO and IAA oxidase were active. The lack of PPO has been also found by C a m b i e and B o c k (1966) in 24 out of 44 studied *Gymnospermae* species. These authors have not studied the genus *Pinus*, but they have found the absence of PPO in *Cedrus libani* which belongs to the *Pinaceae*

S h a i n and M a c k a y (1973) have identified the activity of o-diphenoloxidase and p-diphenoloxidase in the heart-wood of *Pinus radiata*.

They have measured manometrically the absorption of oxygen by fresh and boiled heart-wood fragments incubated with phenolic substrates. Their results indicated that the activity of phenoloxidase originates rather from the pine tissues than from microorganisms. The occurrence in the heart-wood of *Pinus lambertiana* of brown stains suggesting a PPO activity has been observed by S t u t z (1959). However A n d e r s o n et al. (1963) and H i g u c h i et al. (1967) have suggested that the oxidation of phenolic substrates in the heart-wood is a nonenzymatic process (compare with T o m a s z e w s k i 1967).

It has long been known that besides oxidation in the presence of H_2O_2 in the reaction mixture PO is capable of oxidizing various compounds in the presence of air. So far such oxidation reactions have been described for dihydroxyfumaric acid (S w e d i n and T h e o r e l l 1940, C h a n c e 1952), IAA (K e n t e n 1955, R a y and T h i m a n n 1956), triose reductone (Y a m a z a k i et al. 1965), and naphtohydrochinon (K l a p p e r and H a c k e t t 1963a, 1963b). Besides PO is capable of hydroxylation of aromatic molecules in the presence of atmospheric oxygen and dihydroxyfumaric acid (DHF) (M a s o n et al. 1957, B u h l e r and M a s o n 1961). In this reaction PO may be replaced by some other heme system. The hydroxylation reaction can be also obtained in the presence of atmospheric oxygen without enzymatic participation. Such a reaction has been obtained by U n d e n f r i e n d et al. (1954) in the reaction mixture: inorganic iron (Fe^{2+} or Fe^{3+})+ascorbic acid+EDTA. In the presence of atmospheric oxygen PO may also catalyze the formation of ethylene from methional (Y a n g 1967). here similarly as in the case of IAA oxidation monophenols stimulate the reaction and polyphenols inhibit it (Y a n g 1969), and it is also known to have antiseptic activity (K l e b a n o f f 1967, 1968, L e h r e r 1969).

In view of these oxidative abilities one can suspect that the slow dehydrogenation of phenolic compounds in the presence of atmospheric oxygen is an unusual property of PO isolated from pine tissues.

There exists an opinion that one of the functions of PPO is to synthesize o-diphenols. V a u g h a n et al. (1969) have found that catecholase can hydroxylate naturally occurring phenols. However the hydroxylation of phenols as was mentioned earlier may be catalyzed by PO or have a nonenzymatic course. S t a f f o r d (1969) has found an active synthesis of diphenols from endogenous precursors and in the presence exogenous phenylenediamine or tyrosine, the PPO activity simultaneously declining.

Since PO may cause a change in the concentration of IAA, a growth hormone, it is suggested in the literature that there is a relation between the activity of PO and the growth of plants (O c k e r s e et al. 1966). Histochemical studies have allowed the conclusion that PO is strictly connected with the zone of actively dividing cells or with cells that are

just prior to division (V a n d e n B o r n 1963, V a n F l e e t 1959, D e
J o n g 1967).

In the present study, in the first week of embryo development after
seed imbibition, when the developmental processes are very intense, it
was observed that there is a dynamic increase in the number and acti-
vity of electrophoretic fractions of PO (Fig. 6). Similar increases have
been observed in the early development of *Pinus attenuata* (C o n k l e
1971) and *Pinus banksiana* (R a m a i a h et al. 1971), though G o r d o n
(1971) has shown that a high PO activity accompanied slow growth of
Populus deltoides (B a r t r.).

SUMMARY

Under investigation were electrophoretically fractionated oxidizing
enzymes of Scots pine. Substrate specificity for the enzyme complex
(peroxidase, polyphenoloxidase, and indole-3-acetic acid oxidase) was
studied and compared to that of a purified peroxidase of horse radish.
The studies have shown that plant tissues contain proteins with a pero-
xidase activity capable of dehydrogenation o-polyphenols and p-polyphe-
nols to coloured products in the presence of H_2O_2. Monophenols undergo
dehydrogenation and then condense forming a colourless precipitate.
With the help of colour reactions it was found that peroxidase of pine
tissues has the capacity to oxidize indole-3-acetic acid to 3-methyleno-
oxyindole both in the presence of H_2O_2 and in the presence of atmos-
pheric oxygen. L-tryptophan is oxidized by pine peroxidase exclusively
in the presence of H_2O_2. One can suspect that indole-3-acetic acid is
a transitional product of this reaction. In the absence of H_2O_2 purified
protein preparations from pine tissues dehydrogenate phenolic com-
pounds only in the presence of coupling factors (m- and p-phenylene-
diamines, ethanol).

A commercial purified preparation of horse radish root peroxidase
(HRP) has the same catalytical properties as the preparations from pine
tissues. In preparations of pine tissues the activity of polyphenoloxidase
cannot be physically separated from that of peroxidase. Enzymatic
proteins having an oxidase activity occur in soluble fractions and in
fractions that are ionically or covalently bound with cell structures.
Specific fractions have been demonstrated electrophoretically which ha-
ve peroxidase activity, peroxidase and IAA oxidase activity and peroxi-
dase and polyphenoloxidase activity.

The conclusion was reached that pine tissues do not have specific
polyphenoloxidases and that in special conditions proteins containing
a specific peroxidase can, similarly as peroxidase of horse radish root,
catalyze the oxidation of substrates with free oxygen.

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Wielopostaciowość i funkcja niektórych enzymów utleniających sosny zwyczajnej (*Pinus sylvestris* L.)

Streszczenie

Przedmiotem pracy było elektroforetyczne rozfrakcjonowanie enzymów utleniających sosny zwyczajnej. Badano specyficzność substratową kompleksu enzymów (peroksydaza, polifenoloksydaza i oksydaza kwasu indolilo-3-octowego) i porównywano ją ze specyficznością oczyszczonej peroksydazy korzenia chrzanu. Badania wykazały, że tkanki sosny zawierają białka o aktywności peroksydazy zdolne do odwodorowania w obecności H_2O_2 orto-polifenoli i para-polifenoli do barwnych produktów. Monofenole ulegają odwodorowaniu i następnie kondensują tworząc bezbarwny osad. Przy pomocy reakcji barwnych stwierdzono, że peroksydaza tkanek sosny ma zdolność utleniania kwasu indolilo-3-octowego do 3-metylenooksyindolu zarówno w obecności H_2O_2 jak i tlenu z powietrza. L-tryptofan jest utleniany przez peroksydazę sosny wyłącznie w obecności H_2O_2. Wydaje się, że kwas indolilo-3-octowy jest pośrednim produktem tej reakcji. Wobec braku H_2O_2 oczyszczone preparaty białkowe z tkanek sosny odwodorowują związki fenolowe jedynie w obecności czynników sprzęgających (meta i para-fenylenodwuamina, etanol). Handlowy preparat oczyszczonej peroksydazy korzenia chrzanu (HRP) wykazuje takie same zdolności katalityczne jak preparaty tkanek sosny.

W preparatach tkanek sosny nie można fizycznie oddzielić aktywności polifenoloksydazy od peroksydazy. Białka enzymatyczne o aktywności oksydaz występują we frakcjach rozpuszczalnych oraz we frakcjach związanych jonowo lub kowalentnie ze strukturami komórkowymi. Elektroforetycznie ujawniono specyficzne frakcje peroksydazy, frakcje o aktywności peroksydazy i oksydazy IAA oraz frakcje o aktywności peroksydazy i polifenoloksydazy.

Wprowadzono wniosek, że tkanki sosny nie posiadają specyficznej polifenoloksydazy, a w specjalnych warunkach preparaty białkowe zawierające specyficzną peroksydazę mogą, podobnie jak oczyszczona peroksydaza korzenia chrzanu, katalizować utlenianie substratów tlenem.

Полиморфизм и функция некоторых окислительных ферментов у сосны обыкновенной (*Pinus sylvestris* L.)

Резюме

Предметом исследований было фракционирование методами электрофореза окислительных ферментов сосны обыкновенной. Исследовали субстратную специфичность комплекса ферментов (пероксидаза, полифенолоксидаза, ИУК-оксидаза) и сравнивали ее со специфичностью очищенной пероксидазы корня хрена. Выяснено, что ткани сосны содержат белки с пероксидазной активностью, способные дегидрировать в присутствии H_2O_2, о-полифенолов и р-полифенолов до окрашенных продуктов. Монофенолы дегидрируют а затем конденсируются образуя бесцветный осадок. С помощью цветных реакций сбнаружено, что пероксидаза тканей сосны способна окислять ИУК до 3-метиленооксиндола так в присутствии H_2O_2 как и атмосферного кислорода. L-триптофан окисляется пероксидазой сосны исключительно в присутствии H_2O_2. Можно судить, что ИУК является промежуточным продуктом в этой реакции. При отсутствии H_2O_2 очищенные белковые препараты тканей сосны дегидрируют фенольные соединения исключительно в присутствии м- и р-фенилднамина и этанола.

Коммерческий препарат очищенной пероксидазы корней хрена характеризуется

сходными с препаратами с тканей сосны каталитическими способностями. В препаратах с тканей сосны практически не можно физически отделить активности полифенолоксидазы и пероксидазы. Энзиматические белки с оксидазной активностью встречаются в растворимых фракциях и фракциях с ионными и ковалентными связями с клеточными структурами. Методами электрофореза выявлены специфические фракции пероксидазы, а также фракции с пероксидазной и ИУК-оксидазной активностью и фракции с активностью пероксидазы и полифенолоксидазы.

Предполагается, что ткани сосны характеризуются отсутствием специфической полифенолоксидазы, а в специальных условиях белковые препараты содержащие специфическую пероксидазу могут также как и очищенная пероксидаза корней хрена, катализировать окисление субстратов кислородом.