PEROXIDASE AND SENESCENCE

by

Michael Jay Chaskes UC1979
14

****

Submitted in partial fulfillment
of the requirements for
Honors in the Departments of
Biological Sciences and Chemistry

UNION COLLEGE

April, 1979

ABSTRACT

CHASKES, MICHAEL JAY
April 1979

PEROXIDASE AND SENESCENCE
DEPARTMENT OF BIOLOGICAL SCIENCES AND DEPARTMENT OF CHEMISTRY

Kinetin and α, α'-dipyridyl prevented the rapid decrease of chlorophyll content in oat leaf intact segments senescing in the dark.

In light, detachment caused a 27 to 40% rise in peroxidase activity and kinetin enhanced the enzyme in the segments to about 80%. Darkness prevented any detachment-induced rise and decreased the stimulatin action of kinetin and mechanical injury. The effect of dipyridyl on peroxidase activity in the dark was similar to that of kinetin.

Kinetin enhanced the same distinctive isoperoxidases under light and dark conditions.

Neither horseradish peroxidase nor that extracted from oat leaves showed any ability to hydroxylate free proline in vitro. The system, which supposedly led to peroxidase-catalyzed proline hydroxylation, yielded small amounts of hydroxyproline in the absence of the enzyme.

Staining with Fast Blue BB salt in the presence of IAA as a substrate after electrophoresis indicated that all detected oat isoperoxidases had an IAA oxidase activity visually paralleling their peroxidatic activity. Crude extracts contained IAA oxidase inhibitors that could be partially or fully removed by dialysis.

The possible significance of the rise in peroxidase activity during senescence is discussed.

ACKNOWLEDGEMENTS

I wish to express my deepest gratitude to Dr. H. Birecka, whose wisdom, patience, knowledge, and encouragement has made this tremendously worthwhile learning experience not only possible but exciting.

I wish to thank Dr. W. R. Knapp, Crop Science Extension, Cornell University, for supplying the seeds of oats, cv. Garry.

INTRODUCTION

Peroxidase is an enzyme which catalyzes the oxidation of certain classes of organic compounds including phenols, aromatic primary, secondary, and tertiary amines, and certain heterocyclic compounds by hydrogen peroxide. Hydrogen peroxide serves as the oxygen donor while the organic compound serves as the oxygen acceptor. In addition, peroxidase may act as an oxygenase using molecular oxygen. It can also show a catalatic activity under certain conditions.

The action of peroxidase may be inhibited by an excess of hydrogen peroxide. Peroxidase and catalase work at relatively low concentrations of H_2O_2 because of their high affinity for hydrogen peroxide and because of the formation of an inactive form when H_2O_2 is present at high concentrations.

Peroxidase is an ubiquitous enzyme. It is found in all organs of higher plants. The sap of fig trees and the root of horseradish are particularly abundant in peroxidase. Peroxidase may also be found in human saliva, the adrenal medulla, blood plasma, liver etc.. Dwarf varieties of plants have a greater peroxidase activity per unit of weight than do corresponding normal or tall varieties. However, this may be do to the difference in the number of cells per sample between the two kinds of varieties. Dwarf varieties, with smaller cells, may have a greater number of cells per

unit weight therefore one would expect a higher activity of peroxidase to be found.

In 1855 Schonbein (41) reported that dilute solutions of H_2O_2 when mixed with "substances" from selected plants and animals resulted in the oxidation of certain organic compounds. In 1898 Linassier (28) used pus to prepare an oxidase-free preparation which he called "peroxidase". In the early part of the twentieth century many sources of peroxidase were reported. Willstatter, (49) in 1920, pioneered the purification of this enzyme and demonstrated its ferroporphyrin nature.

In 1942 Theorell (39) showed the existence of isoperoxidases in horseradish roots. The literature between 1945 and 1965 is chiefly concerned with the elucidation of the chemical, physical-chemical and biochemical properties which horseradish root peroxidase (HRP) exhibits in the presence of various substrates in vitro. Jermyn and Thomas (23) in 1954 used paper electrophoresis to show that peroxidase in the analysed extracts could be separated into several isoforms (anodic and cathodic) all exhibiting peroxidatic activity.

Wilkinson has defined isoenzymes as closely related molecular forms of proteins which can catalyze similar reactions within a single species (47). Differences among isoenzymes can be found in minor modifications of protein structure which may include: changes in amino acid sequence, amidation of carboxyl groups, conjugation with small

molecules, polymerization, variation in folding of the primary structure or variation in subunit structure. Isoenzymes may differ in substrate affinity, specific activity, sensitivity to inhibitors, and optimum pH. The isoenzymes are commonly seperated by electrophoresis, chromatography, gel filtration, and catalysis.

Peroxidase belongs to a family of proteins known as glycoproteins. The synthesis of this enzyme is genetically controlled by the transcription process as well as by the specificity of glycosyl transferases during the posttranscription stage of the enzyme synthesis. Studies of glycoproteins have been mainly done in mammalian systems with very limited studies in plants. It is not known whether sugar transferases, directing the assembly of the carbohydrate moiety, are exclusively located in the Golgi apparatus or whether they may also be located in the smooth ER as well as the plasma membrane. Shore and Maclachlan have shown that in the decapitated and IAA-treated pea plants β-1,4-glucan synthetase activity is associated with the smooth ER fraction as well as with the Golgi membranes; however in untreated plants the enzyme activity is found primarily in the Golgi apparatus.

Peroxidase has been shown to exist in many animal tissues and fluids (39). Myeloperoxidase (MW$\sim$155,000) is mainly found in leukocytes and tissues rich in leukocytes (39). Lactoperoxidase is found in milk, saliva, and the glands associated with their production (30). The activity of

glutathione peroxidase has been studied in leukocytes and erythrocytes, especially in leukemia cases, (18) muscles, liver, and Ehrlich ascites tumor cells (19).

Peroxidases from plant and animal tissues are capable of oxidizing NAD(P)H and glutathione. This leads to their implication in the control of the thiol group level and redox potentials in cells and also in that of the NADP-dependant hexose monophosphate pathway (19).

Evidence also suggests that peroxidase plays an important role in estrogen metabolism in the uterus. It is able to convert estradiol to an inactive, water-soluble, metabolite which is mainly bound to proteins (22). Peroxidase activity in mammary tumors may reflect the ability of tumor cells to differentiate in response to hormonal stimulation and be indicative of the degree of tumor progression (22). It has also been shown by Klebanoff (25) that HRP is capable of inactivating estradiol in the presence of H_2O_2.

Studies have illucidated the fact that peroxidases are very strong antimicrobial agents when combined with an oxidizable factor, such as a halide, and H_2O_2 or an H_2O_2 generating source. This peroxidase system may then be involved in intracellular killing of microbial organisms and in the extracellular destruction of different kinds of target cells. Myeloperoxidase of blood monocytes and lactoperoxidase of milk and saliva were extremely effective against bacteria, fungi, viruses, and mycoplasma (10). The antimicrobial activity of the myeloperoxidase H_2O_2-Cl^- systems appears to

be due to decarboxylation and deamination of amino acids. The myeloperoxidase-H_2O_2-I^- system's antimicrobial activity was not accompanied by amino acid decarboxylation (45).

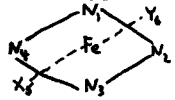
It has been suggested that the lacrimal gland peroxidase may be responsible in human tears for antibacterial activity which cannot be attributed to the action of lysozyme (12).

Isoglycoenzymes may not differ significantly in their amino acid composition, but mainly in their carbohydrate portion. In horseradish roots twenty isoperoxidases (IP's) have been identified. These twenty isoforms have been classffied into three groups, each group being composed of isoforms with similar primary structure. Isoforms within a group showed similar catalytic properties in both peroxidatic and oxidatic reactions. Each IP was comprised of a single polypeptide with 3-4 intrapeptidal S-S bridges. In no IP was there found a free thiol group or a hydroxyproline residue. Although in each isoenzyme qualitatively the same sugars could be found, the carbohydrate moiety varied from 21 to 37% of the molecular weight.

The removal of the carbohydrate moiety from the major IP with pI 9 had little or no effect on the peroxidatic activity of the enzyme. It should be noted that the heterogeneity in the composition of the isoforms may be due to others factors besides genetically controlled polypeptide synthesis. Changes may be induced in the protein at the posttranscriptional stage (eg. aggregation, modifications of

the polypeptide chain(s), or modification of the carbohydrate moiety in the case of glycoenzymes, etc...).

HRP has a molecular weight of approximately 40,000. Its composition is carbon ≈ 47.0%, oxygen ≈ 32.0%, hydrogen ≈ 7.35%, nitrogen ≈ 13.2%, sulfur ≈ 0.43%, and iron ≈ 0.127%. It is a colorless protein (apoenzyme) combined with an iron-porphyrin. The iron has six coordination positions, of which four are occupied by porphyrin nitrogen atoms and a fifth by a protein group. The sixth can be variously occupied by H_2O, CN^-, etc... It is through this sixth position that the peroxidase appears to operate.



(38)

HRP can react with RO-OH as well as with HOO-H but not with structures of the general formula ROOR (where R=alkyl or acyl groups).

Phenols are readily oxidized by the peroxidase system. Guaiacol is often used as a substrate as it is easily oxidized and forms colored products. The optimum pH for the reaction is 5.0 - 6.0. The red-brown oxidation product is partly due to the 3',3'-dimethoxybiphone - 4,4'-quinone produced. Peroxidase reacts with cyanide, sulfide, flouride, azide, hydroxylamine and nitric oxide to form stable compounds. In these forms the enzyme activity is lost.

The activity of oxidative enzymes, including peroxidase, have been shown to be significantly affected in plants by trauma of various kinds. Gamma radiation (32), cold (37),

boron deficiency (35) and infection by various pathogens (40) have increased the activity of the enzyme in plant tissue. Increased enzyme activity due to mechanical injury such as slicing or decapitation has been reported by various authors in a variety of tissues (4,40). In many of the tissues a lag period was reported. Using 3mm slices of tobacco pith, Birecka found increasing activity from 12 to 24 hours after excision; aeration was a requirement for increased activity. A number of investigators have reported that wounding caused the appearance of new isoenzymes as well as increased activity of certain isoenzymes found in the intact plant.

Many peroxidases are able to catalyze the aerobic oxidation of dihydroxyfumeric acid using molecular oxygen. Purified HRP is known to be able to oxidize IAA (indole-3-acetic acid) aerobically, although IAA oxidase activity is not found in some instances in all IP's as revealed by polyacrylamide gel electrophoresis. The oxidation of IAA occurs in the presence of some cofactors such as Mn^{++} and certain monophenols. Under aerobic conditions, and in the presence of dihydroxyfumaric acid, HRP is able to catalyze the hydroxylation of certain aromatic compounds. The peroxidase behaves as an oxidase towards dihydroxyfumaric acid.

There is considerable evidence that IP's may show significant differences between their IAA oxidase and peroxidase activities (16). It is also reported that some IPs or IP fractions lack IAA oxidase activity (16) while some IAA oxidases or IAA oxidase fractions lack peroxidase activity(31).

Monophenols are regarded as stimulatory whereas diphenols inhibit the IAA oxidase activity. Monophenols having one or two methoxy groups ortho to the hydroxyl group are considered inhibitory. Parish found that when inhibitory diphenols are oxidized in the presence of H_2O_2 and peroxidase, the inhibitory properties are lost. These studies indicate that phenolic compounds may be important modifiers of the in vivo oxidation of IAA. Peroxidases not only oxidize IAA but also appear to be involved in the oxidation of coniferyl alcohols (and other hydroxyphenol propanes) leading to the formation of lignin in plant cells. Lignins are highly branched, rigid organic polymers of aromatic units which are deposited in the walls of differentiated plant cells where they serve to resist compressional forces. Evidence suggests that lignin formation occurs in the presence of a polymerization matrix such as cellulose, suitable precursors, hydrogen peroxide, and peroxidase (42). Due to its ability to oxidize IAA and its involvement in the formation of lignin peroxidase has been linked to the senescence of plants. IAA oxidase activity of peroxidase is not only controled by phenolics, but also by proteinaceous inhibitors ("auxin protectors") which are primarily found in young tissue, wound tissue, and crown gall tumors. This function links peroxidase to processes that are controlled by IAA or hormonal balance, ie. cell growth and differentiation, membrane permeability, cell wall elasticity, senescence, etc...

An increase in the peroxidatic activity of this enzyme, often due to enhancement of selective IPs, has been observed with aging. This is independent of whether the senescence is naturally occuring or ethylene-induced.

Ethylene is a plant hormone which, in contrast to IAA, inhibits cell elongation and promotes lateral cell expansion and also accelerates senescense. It has been suggested that peroxidase may be involved in the synthesis of ethylene from methionine. At the same time exposure to exogenous ethylene enhances peroxidase activity in many plant species (36). The stimulation of endogenous ethylene production by mechanical injury is also well documented. In sweet potatoes the degree of ethylene production was correlated to the degree of cutting (21) and the increase in peroxidase activity (20).

In experiments with tobacco leaves and pith no qualitative difference in the IP patterns were revealed in relation to the organ, its age or position, mechanical injury, or infection with TMV (1,3). However, significant age-related quantitative differences were found between some distinctive anodic IP's which did not react to mechanical injury or infection. The same distinctive IPs which reacted to mechanical injury also reacted to infection. Exposure of leaf or pith sections to ethylene did not affect their peroxidase reaction to mechanical injury. Neither was there any significant effect of ethylene on peroxidase activity in pith of intact plants, contrary to earlier reports (2).

Birecka has found that in tobacco mainly cathodic IPs

synthesized at the early stage of cell growth (juvenile IPs) are secreted into the walls, where they are bound ionically and covalently. With aging the synthesis of the "juvenile" IPs slows down whereas that of "mature" anodic isoforms increases. This is accompanied by a partial efflux of IP's into the cell wall and also into the intercellular spaces. Golgi vessicles seem to be involved in secretion of the "juvenile" isoforms. Injury may initially enhance the synthesis of IPs synthesized typically in very young cells and lead to their secretion into the walls. The secretion of peroxidase into the vacuole may also be an indirect effect of injury.

Yip (50) has claimed that HRP in the presence of Fe^{++}, EDTA, and 50mM H_2O_2 catalyzes in vitro hydroxylation of free proline. According to Yip this reaction is an enzymatic conversion of proline to its hydroxy form controlled by peroxidase, in particular by HRP.

Hydroxyproline can inhibit cell division (43) and elongation (8). This process is proline reversible.

Hydroxyproline is considered to be an amino acid antagonist, interfering with protein synthesis. Lamport (26) as well as Cleland and Olson (9) have produced evidence that free hydroxyproline is not normally incorporated directly into proteins but rather that hydroxylation of proline catalyzed by proline-4-monooxygenase occurs after it is bound to the polypeptide chain. Holleman (17), on the other hand, has shown that hydroxyproline at growth inhibiting

concentrations could be incorporated directly into proteins.

Parish (34) reported that the delaying effect of kinetin on the rate of senescence could be countered by hydroxyproline. This could then be reversed by proline. Hydroxyproline alone enhance proteolysis in the senescing discs.

Detachment and darkness are two factors which accelerate senescence in leaves (46). Kinetin, a plant hormone, can delay the rapid increase in the rate of catabolic processes. The rate of senescence is most easily measured by the degree of breakdown of chlorophyll and proteins, the accumulation of free amino acids, and the increase in oxygen uptake, not accompanied by an increased production of ATP. The cytokinin-induced repression of the increased rate of oxygen uptake was ascribed to tightening of coupling or maintanance of a tight coupling between respiration and phosphorylation.

In spite of the view of peroxidase being one of the most reliable indicators of senescence, no firm evidence of a cause-effect relation between them is available. Neither is any information available on the catalytic properties or function of distinctive IP's whose activities increase with aging.

In view of the above mentioned implications of peroxidase, this study deals with changes in activities of its isoforms in detached oat leaves in relation to kinetin and dipyridyl action. Proline and IAA were included as substrates in testing the enzyme.

MATERIAL AND METHODS

Avena Sativa, a local cultivar of oats was used in a preliminary experiment. In all other experiments cv.Garry, a cross of Victory x (Victoria x Hajira-Banner) was used. All seedlings were grown in a vermiculite and peat mixture in a growth room under a 16 hr. light and 8 hr. dark cycle. Nine day old plants were used in all experiments. Apical four -cm long segments were excised from the first leaves and floated on water, kinetin (3 mg/l), α, α' -dipyridyl (0.3 mM) or EDTA (50 mM) in Petri dishes under conditions of continuous light or darkness for 28 to 74 hrs. After exposure, one -cm basal portions were cut off to eliminate the effect of excision-induced mechanical injury on peroxidase. In addition three-cm apical segments were excised at various intervals from attached first leaves of intact plants grown in light or darkened with foil allowing for good aeration.

To study the effects of mechanical injury, the apical segments were cut into five -mm pieces perpendicular to the veins. The segments (intact and injured) were exposed to light or dark conditions in Petri dishes on filter paper moistened with water.

In each experiment two or three replicates per treatment and ten leaves per replicate were used. Chlorophyll content was measured using additional replicates.

In order to extract peroxidase the tissue segments were ground in mortars using 0.02M phosphate butter, pH 6.0, and

the homogenates were centrifuged at 20,000g for 25 min. The first supernatant was taken as the protoplasmic fraction, which also included the free fraction present in the walls and intercellular spaces. The pellets were washed to remove any remaining cytoplasm by resuspension in buffer and centrifugation, then treated with a one percent solution of Triton x-100 (a non-ionic detergent) and rewashed. The pellets were treated with 0.5 M NaCl to remove ionically bound cell wall IP's (3). This solution yielded the same results as did 1 M NaCl.

The total peroxidase activity was determined at 470 nm using 15 mM guaiacol and 5 mM H_2O_2 as substrates. In most cases, starch gel electrophoresis at pH 8.3 (borate buffer) in a gradient of 10mv/cm was used for IP examination. The presence of NaCl at high concentrations in extracts significantly affected the mobility of isoforms during electrophoresis at pH 8.3. Dilution of the extracts prior to electrophoresis eliminated this effect. In some cases appropriate amounts of NaCl were added to the protoplast fraction, extracted with 20 mM phosphate buffer, to permit comparative analysis of the isoenzyme patterns(17). The relative differences in total enzyme activity found between the different treatments before electrophoresis and that of all the individual IP activities summed after electrophoresis was very similar.

Hydroxyproline determination was done colorimetrically (15). Among the reactants in this procedure is

p-dimethylaminobenzaldehyde, which may be purified but is not a necessary step. One milliliter of the solution containing 5-15 ug of hydroxyproline is transfered to a test tube followed by 1 ml each of a 0.01 M copper sulfate solution, 2.5 N NaOH, and 6% hydrogen peroxide solution. The mixture is shaken for about 5 min. and then placed in a water bath at 80°C for another 5 minutes with further shaking. The later step assists in the destruction of excess peroxide; this can also be effected by adding 0.1 ml of 0.05 M ferrous sulfate in 0.5% (V/V) sulfuring acid. The mixture is chilled to 5°C and treated successively with 4 ml of 3 N H_2SO_4 and 2 ml of p-dimethylaminobenzaldehyde solution (5% in redistilled n-propanol). A red color is developed by heating the mixture in a bath at 70°C for 16 minutes, after which the mixture is cooled to room temperature and the absorbancy measured at a wavelength of 540 mu. Standard solutions containing 5,10, and 15ug each of hydroxyproline are used simultaneously. Using this method the hydroxylation of proline by peroxidase in the presence of added H_2O_2 was tested. In the absence of added H_2O_2 under the conditions described by Buhler amd Mason (6) the hydroxylation of proline by peroxidase was again tested. The incubating solution was aerated with pure oxygen.

The IAA oxidase activity of peroxidase was assayed using Salkowski reagent (14). The enzyme extracts were incubated for one hour in 2 mM pH 6.0 buffer solution. This solution also contained 0.2 mM IAA, 0.1 mM $MnCl_2$, and

0.05 mM 2, 4-dichlorophenol. The residual IAA was determined using 1 ml aliquots at various intervals of time.

The IAA oxidase activity of individual IPs after electrophoresis was tested in a buffer solution (pH 5 or 6) containing 0.2-1 mM IAA plus cofactors and 0.2% Fast Blue BB salt (11). Peroxidatic and IAA oxidase activities were determined using one slab gel. The slab gel was divided into two halves with each half containing one of the duplicated samples.

All enzyme extract activities were compared with that of "Sigma" HRP (RZ 3.2; 300 purpurogallin units/mg) when proline and IAA were used as substrates. Horseradish and sweet potato root peroxidases with or without polyvinylpolypyrrolidone added during tissue grinding, were also tested in some cases. Similar comparisons were applied to oat leaf peroxidase extracts. Oat leaf extracts were also dialyzed overnight using a 20 mM buffer, pH 6.0.

Mackinney (29) determined the specific absorption coefficient, K, of pure chlorophyll samples a and b in 80% aqueous acetone extracts at a number of wavelengths. From the valves at 645 mμ and 663 mμ he derived two equations for the calculation of the chlorophyll concentrations. Using Mackinney's results, Arnon developed a formula for expressing the amount of total chlorophyll in mg/l extract:

$$c=20.2\ A_{645} + 8.02\ A_{663}$$

where A is the absorbancy of the extract, measured in a 1cm cell at the appropriate wavelength. Plotting the absorption

curves Arnon found an intersection to occur at λ = 652 mμ at the K value of K=34.5. Therefore another equation for total chlorophyll concentration in mg/1, is:

$$c=1000\ A_{652}/34.5$$

In practice it turns out that the latter formula gives about 5% higher values than does the former. This small deviation may be due to the inaccuracy in the estimation of the K value at 652 mμ. Bruinsma (5) than drew these curves more precisely using a number of specific absorption coefficients. As a result he modified Arnon's equation since the curves actually intersect at 651.8 and a K value of K=36.0. The modified equation for the determination of total chlorophyll in mg/1, in 80% acetone extracts used in these studies is:

$$c=20.2\ A_{645} + 8.02\ A_{663} = 1000\ A_{652}/36$$

RESULTS

From Table 1 it can be seen that within the 60 to 144 hours of the experiments, attached leaves did not show any significant increase in total peroxidase activity. Under light conditions, detachment caused a rise in the total peroxidase activity of 27-40%. Kinetin further enhanced this rise in activity to 70-80%. Within 28 hours after detachment the rise in peroxidase activity becomes significant. Under dark conditions, there was no detachment-induced rise in total peroxidase activity. Infact, after 84 hours darkness may have slightly lowered the enzyme activity. Moreover, the stimulating action of kinetin was reduced to 30-50%. In the dark the effect of dipyridyl was similar to that of kinetin.

During the first 28 hours after detachment there was no observable effect caused by treatment with 50mM EDTA. However, there was a decrease in the total enzyme activity during the next 44 hour period. This decrease in activity was more pronounced under light than dark conditions. When the excised segments were floated on EDTA solutions in light or when floated on water in dark for 84 hours there was a partial loss of turgor as well as an increase in weight due to the water entering the intercellular spaces.

One might expect the chlorophyl content to decrease with senescence. The rate of the decrease would be expected to be greater in dark than in light, and with kinetin this process would be delayed. As clearly shown in Table 2 these

predictable results were in fact observed. If the rate of senescence is measured by the change in chlorophyll content, then from Tables 1 and 2 it is clear that there is no correlation between the peroxidase activity and the observed changes.

The peroxidase enzyme activity was enhanced by injury. The results of this injury induced rise in activity are presented in Table 3. The dark related partial inhibition of enzyme enhancement found in experiments II and III was also found in the case of injury-induced rises in peroxidase activity in experiment IV. This rise in activity due to injury occured most dramatically in the first 24 hours after cutting.

There were no qualitative differences found in the IP spectrum of the buffer soluble fraction, that is the cytoplasmic fraction, and that extracted with 0.5M NaCl from wall debris. The peroxidase fraction covalently bound to the walls was only 8-9% of the total enzyme activity and did not change significantly in response to treatments. The ionically bound cell wall peroxidases did contribute about 25-30% to the total enzyme activity. As seen in Figure 1 nine cathodic and five anodic IP's were detected, with A5 being the major contributor. This IP spectrum is similar to that reported for coleoptiles of oats, cv. Victory (13).

IP's C7, C5 and A1, mainly in the buffer soluble fraction, were increased in activity by detachment alone under light conditions. When the segments were treated with

kinetin these isoperoxidases were further stimulated in addition to the enhancement of C8, C4, C2, and A5 (Table 4). EDTA decreased the activity of all IP's, especially under light conditions. (In light a decrease of the chlorophyll content of the segments was more pronounced than in the dark). As can be seen from Table IV, mechanical injury may be associated with a rise in the activity of all IP's especially the cathodic ones.

Under dark conditions the enhancement of the IP's due to detachment, kinetin and injury was partially inhibited. In addition, dark exposure decreased the activity of A5. There was a similar decrease found in attached leaves exposed to darkness for 60 hours as well as in the physiologically senescing leaves sampled six days after the beginning of the experiment. Kinetin and dipyridyl both showed similar effects. No qualitatively new isoforms were found in either treatment.

In 1964 Yip indicated conditions under which he claimed peroxidase had the ability to hydroxylate proline. This claim was tested under conditions identical to those indicated by Yip. However, since 50mM H_2O_2 proved to inhibit the enzyme when quaiacol was used as the substrate, a medium containing 5mM H_2O was included in the assays. HRP "Sigma" preparation had a slowly moving cathodic isoperoxidase, whose activity represented 90% of the total quaiacol oxidizing activity. The results obtained were in direct contrast to those reported by Yip:

(1) Even in the absense of enzyme, hydroxylation of proline did occur, although in relatively small amounts. The rate was higher in the medium with 50mM H_2O_2 than that with 5mM H_2O_2

(2) As indicated in Table 5 the amount of hydroxyproline formed did not increase when the proline was incubated with HRP or enzyme extracts from oat leaves subjected to various treatments. The amount of hydroxyproline found in the medium without peroxidase was similar to that reported by Yip to be formed in the presence of the enzyme. Peroxidase was not found to catalyze proline hydroxylation in the presence of dihydroxyfumarate or ascorbate without the addition of H_2O_2.

When IAA was used as a substrate in the presence of molecular oxygen, HRP "Sigma" preparation alone showed significant activity. A crude extract of the enzyme from horseradish or sweet potato roots with a slightly higher or similar peroxidatic activity, respectively, also showed a significant IAA oxidase activity. However, as evidenced by the results in Table VI, a lag phase could be observed. The use of polyvinylpolyrrolidone to remove phenolic compounds had a very small effect if any at all. The activity of sweet potato root peroxidase was additive with that of HRP when mixed together. However, crude extracts from control or treated oat leaves with higher peroxidatic activities than that of HRP showed a very small IAA oxidase activity

regardless of the type of treatment. By adding polyvinyl-polypyrrolidone during grinding the activity was somewhat increased. This increase was even greater when dialysis of the extract was applied. In the presence of a larger volume of crude extracts from oat leaves, the IAA oxidase activity of HRP was significantly inhibited.

After electrophoresis and exposure to IAA as the substrate, the gels were stained with Fast Blue for seven to twelve hours. All IP's revealed IAA oxidase activity, the intensity of the staining in individual bands paralleling that of staining by quaiacol oxidation products as visually assessed.

Table I. Effects of Detachment, Kinetin, α, α'-Dipyridyl, and EDTA on Peroxidase Activity in Leaf Segments of Oat cv. Garry in Light and Darkness.

Apical 4-cm segments were excised from the first leaves of 9-days old seedlings (time 0) and floated on water, kinetin (3 mg/l.), dipyridyl (0.3 mM), or EDTA (50 mM) solutions in continuous light or darkness. After exposure, 1-cm basal portions were cut off and the 3-cm segments were frozen; 3-cm apical segments excised at various times from attached leaves were frozen immediately after excision. Peroxidase activity was determined using guaiacol and H_2O_2 as substrates.

Treatment	Experiment I[1]					Experiment II						Experiment III				
	Light			Dark		Light				Dark		Light			Dark	
	H2 of Exposure															
	0	28	72	28	72	0	30	60	144	30	60	0	36	84	36	84
	ΔA/min • g fresh wt[2]															
Leaves attached	302	297	305			314		322	328		301	299				
Leaves detached at time 0																
Water		369	430	317	321		352	395		330	326		385	418	312	273
Kinetin		443	557	422	460		414	525		385	418		453	538	391	417
Dipyridyl		451	538	414	443											
EDTA		328	205	308	268											

[1]Similar results were obtained with leaf segments of a local oat cultivar.

[2]Peroxidase activity comprises the protoplast fraction and the ionically bound fraction extracted from wall debris. The peroxidase fraction covalently bound to the walls amounted to 8-9% of the total enzyme activity and did not change significantly in response to treatments.

Table II. Effects of Detachment and Kinetin on Total Chlorophyll Content in Oat Leaf Segments in Light and Darkness

Details as in Table I.

Treatment	Experiment II						Experiment III				
	Light				Dark		Light			Dark	
	Hr of Exposure										
	0	30	60	144	30	60	0	36	84	36	84
	mg/g·fresh wt										
Leaves attached	2.00		1.85	1.28		0.99	1.92				
Leaves detached											
Water		1.92	1.65		1.42	0.71		1.64	1.35	1.28	0.24
Kinetin		1.98	1.81		1.91	1.85		1.96	1.82	1.92	1.64

Table III. Effect of Mechanical Injury on Peroxidase Activity in Oat Leaf Segments in Light and Darkness

Apical 4-cm intact segments or 3-cm segments cut into 5-mm pieces were exposed on water-moist filter paper to light or darkness. After exposure 1-cm basal portions were cut off from the intact segments and all samples were frozen. For further details see Table I.

Experiment IV

Segments	Light			Dark	
	Hr of Exposure				
	0	24	72	24	72
	ΔA/min·g fresh wt				
Intact	317	380	431	331	329
Injured		1057	1163	502	539

Figure 1. Isoperoxidase pattern in the first leaf of oat cv. Garry. Slab gel electroporesis at 8.3, 10/cm, A:anodic; C:cathodic isoenzymes revealed using guiacol - H_2O_2 as substrates.

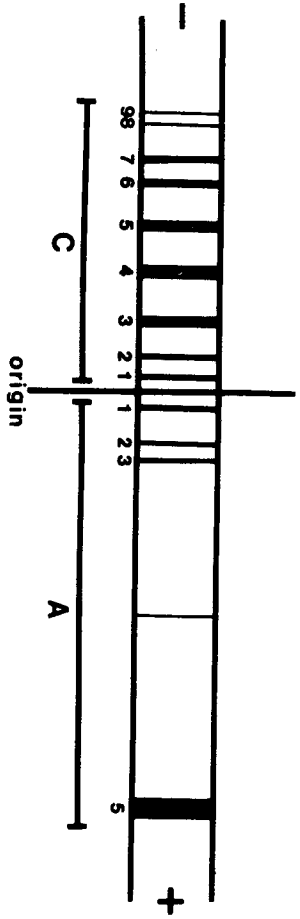


Table IV. Activity of Isoperoxidases in Oat Leaf Segments in Light and Darkness

Details as in Tables I and III.

Isoperoxidases	Experiment II								Experiment IV				
	Segments excised at hr								Segments				
	0					60		144	Intact			Injured	
	Hr of exposure												
	0	60						0	0	72			
		Light		Dark		Light	Dark			Light	Dark	Light	Dark
		H_2O	Kinetin	H_2O	Kinetin					H_2O			
	ΔA/min·g fresh[1]												
Cathodic													
C8-C9[2]	3	4	18	4	6	3	4	4	5	6	6	80	11
C6-C7[2]	32	52	75	46	54	33	35	45	29	66	43	208	94
C5	21	47	79	40	40	22	20	29	18	57	32	104	67
C4	36	38	49	35	50	35	37	38	35	36	34	60	31
C2-C3	28	30	50	27	43	28	29	28	32	35	34	217	85
C1	21	24	24	22	23	22	20	21	24	25	26	152	78
Total	141	195	295	174	246	143	145	165	143	225	175	821	365
Anodic													
A1-A4[2]	29	42	50	37	41	31	39	38	33	59	40	159	65
A5	144	158	180	115	161	148	117	125	141	147	114	177	108

[1]The activity includes both the buffer and 0.5M NaCl soluble enzyme fractions. In the latter, the total activity of cathodic and anodic isoforms amounted to 50-60 and 35-40 ΔA/min·g fresh wt, respectively with the exception of (a) kinetin-treated segments in which the ionically bound cathodic isoforms amounted to about 100 ΔA/min·g fresh wt, and (b) injured segments in which the activity of these isoforms amounted to 120-250 ΔA/min·g fresh wt. In all treatments, the activity of A5 ranged from 12 to 17 ΔA/min·g fresh wt.

[2]Increases in activity as compared to the control were mainly due to C7; C8; and A1.

Table V. Hydroxylation of L-Proline in the Absence or Presence of Peroxidase

The complete 2 ml buffered (pH 5) incubation medium contained: 20μmoles of L-proline (2306μg); 5μmoles of EDTA; 0.5μmoles of $FeSO_4$ 10 or 100μmoles of H_2O_2 (5 or 50mM); and HRP (Sigma) or peroxidase extracts from oat segments.

H_2O_2 mM	Experiment I				Experiment II			
	Peroxidase		Incubation-min		Peroxidase		Incubation-min	
	Source	Activity[2] ΔA/min	30 μg Hyp	60 formed	Source	Activity ΔA/min	15 μg Hyp	60 formed
5					-	-	4.4	5.0
50	-	-	7.8	9.2	-	-	7.0	8.2
5	HRP	2,040	3.6	3.6	HRP	8,160	4.2	3.2
50			8.0	10.4			8.6	8.6
5		4,080	4.4	3.2	Oat[3]	14,400	4.6	5.4
50			7.5	8.3			6.9	8.1

[1]Incubation of L-hydroxyproline solutions, used as standards, in the medium had no effect on the absorbance readings at 540nm.

[2]The activity was determined using guaiacol and H_2O_2 (5mM) as substrates at 470nm.

[3]Similar results were obtained with oat peroxidase extracts with activity 20,400 ΔA/min.

TABLE VI. Peroxidatic and IAA Oxidase Activities of Perosidase from Horseradish Roots, Sweet Potato Roots, and Oat leaves

The enzyme was extracted without or with polyvinylpolypyrrolidone added. Peroxidase activity was determined using quaiacol and H_2O_2. IAA oxidase activity was assayed with Salkowski reagent after incubation of extracts in 10 ml medium containing 0.2 mM IAA; 0.1 mM $MnCl_2$; and 0.05 mM dichlorophenol in the presence or absence of HRP (Sigma).

Peroxidase Source	Activity							
	Peroxidatic Δ A/min		IAA oxidatic % IAA oxidized					
	Polyvinylpolypyrrolidone							
	-	+	-			+		
			Incubation - min					
			15	30	60	15	30	60
HRP	1,800		53	88	91			
Horseradish roots	2,128	2,320	22	65	75	26	71	82
Sweet potato roots	1,724	1,740	17	45	75	21	50	78
plus HRP			69	94		76	95	
Oat leaves	2,850	2,900	2	4	14	0	15	21
plus HRP			42	90		56	91	
dialyzed			20	35	51			
Oat leaves (triple vol)	8,550		1	0	12			
plus HRP			0	4	49			

DISCUSSION

When subjected to those factors which are known to accelerate or delay senescence the oat leaves reacted in a manner similar to that reported by Tetley and Thimann (46). The chlorophyll content of the oat leaves was used as the indicator of senescence. There was no increase in the total peroxidase activity observed in attached first leaves during the experimental period, however during the later period of plant growth (data not reported here) there was a rise in peroxidase activity as senescence progressed. This increase in enzyme activity was due to an increase in the contribution of distinctive IP's to the total activity as previously observed by Birecka, Shih and Galston (4) and Birecka, Catalfamo, and Urban (2) in tobacco plants.

The results indicate a presence of IAA oxidase inhibitor(s) in untreated and treated leaves. They may resemble the "auxin protectors" described by Stonier and Yang (44). It is due to the presence of the inhibitor(s) that a fully reliable comparison of peroxidatic and IAA oxidase activities of the enzyme can not be made. If there was no inhibition after dialysis then the ratio between the peroxidatic and IAA oxidase activities of oat peroxidase may be higher than that of HRP. After the peroxidase samples underwent electrophoresis and were stained with Fast Blue in the presence of IAA all indications suggested that the inhibitor(s) either have a different mobility than that of the IPs or were unstable at room temperature during incubation of the

gel. After the IP's were extracted from the gel they were tested for IAA oxidase activity. Unfortunately the results obtained were not conclusive. The problem of IAA oxidase inhibition in oat leaves is one which should be the subject of another study.

All those IPs detected by staining with Fast Blue were found to have an IAA oxidase activity visually parallelling that of their peroxidatic activity. These results are similar to those reported by Whitmore (47) for buffer soluble IPs of wheat coleoptile and those reported by Lee (27) who found that the activity of IPs in tobacco callus cultures were enhanced by low and inhibited by high concentrations of Kinetin.

Detachment, mechanical injury, or kinetin caused an increase in peroxidatic activity by stimulating individual IPs. As seen from the reported results, the extent to which the IPs activity was increased in oat leaves is clearly a light dependent process. There are many factors that might be indirectly involved in the light effect. However, the experiments do not supply appropriate information for any reliable speculation.

Kinetin and α,α'-dipyridyl are known to delay senescence. As senescence progresses one would expect to observe an increase in peroxidase activity if it were linked to senescence due to its ability to oxidize IAA. One would therefore expect a decrease or no change in the enzyme activity in those leaf segments where senescence was delayed

by treatment with kinetin or α,α'-dipyridyl. An opposite effect was observed.

Kinetin is a plant hormone, while α,α'-dipyridyl is a chelator of Fe^{++} and Zn and an inhibitor of proline hydroxylation. As reported by Tetley and Thimann (46) α,α'-dipyridyl also inhibits senescence of leaf segments in the dark. It is not clear why both of these compounds produce a similar effect on peroxidase in the dark, as their mode of assumed action is so different. Parish (34) reported that kinetin-treated 10-mm tobacco leaf discs had a slightly lower activity than the control discs in which the enzyme activity increased 15 fold. This is only one example of the cases reported in literature indicating that the kinetin-induced changes in peroxidatic activity are more related to the effects of cutting than to senescence. In 8-cm long segments of old rice leaves enzyme activity increased by 70% when treated with kinetin in the dark as compared to a 30% increase in the water control (24). The data for light conditions have not been reported.

Yip (50) has claimed that HRP was a necessary catalyst for the in vitro hydroxylation of free proline. If this were in fact true, speculation concerning a relationship between the enzyme, proline hydroxylation, and senescence could be justified. However, the tests made trying to link the enzyme to proline hydroxylation were all negative. Hydroxylation of proline took place in the absense of all enzyme.

Thus, there is an extremely small probability that peroxidase is linked to senescence through proline hydroxylation unless peroxidase was involved in hydroxylation of bound proline, not tested here.

It is known that the production of H_2O_2 in cells increases with aging. Any increase in peroxidase activity with progressive senescence may be mainly related to the elimination of H_2O_2. This would then indicate a protective role of the enzyme, perhaps delaying senescence, rather than a causal role, i.e. an increase in peroxidase activity causing senescence. The possible protective function of peroxidase may be of significance because in senescing leaves catalase activity decreases. This decrease in catalase activity may be prevented by cutting (33) or kinetin (22).

Rudolph and Bukatsch (38) have shown that peroxidase has a stabilizing effect on chlorophyll. This would then lend further support to the speculation that an increase in peroxidase activity rather delays progressive senescence.

This interpretation requires further supports, especially with regard to the location of the IP's in the cells in relation to the location of H_2O_2 production.

REFERENCES

1. Birecka, Briber, and Catalfamo 1973 Plant Physiol., Lancaster, 52, 43-49
2. Birecka, Catalfamo, and Urban 1975 Ibid., 55, 611-19
3. Birecka, and Miller 1974 Ibid., 53, 569-74
4. Birecka, Shih, and Galston 1972 J.Exp. Bot., 23, 655-66
5. Bruinsma 1961 Biochim. Biophys. Acta, 52, 576-78
6. Buhler, and Mason 1961 Arch. Biochem.Biophys., 92, 424-37
7. Catalfamo, Fienberg, Smith, and Birecka 1978 J. Exp. Bot., 29,347-58
8. Cleland 1967 Plant Physiol., Lancaster, 42, 271-74
9. Cleland, and Olson 1967 Biochemistry, 6, 32-36
10. Edelson, and Cohen 1973 J. Exp. Med., 138, 318
11. Endo, T., 1968 Plant Cell Physiology, 9, 333-41
12. Essner 1971 J. Histochem. Cytochem., 19, 216
13. Gardiner, and Cleland 1974 Phytochem., 13, 1701-11
14. Gordon, and Weber 1951 Plant Physiol., Lancaster, 26, 192-95
15. Greenstein, and Winitz 1961 Chemistry of the Amino Acids Wiley, New York Vol.3 p. pp. 2027-28
16. Grove, and Huyle 1975 Plant Physiol., Lancaster, 47,312
17. Holleman, J. 1967 Proc. Nat. Acad. Sci. U.S.A., 57, 50-54
18. Hopkins and Tudhope 1973 Brit. J. Haematol., 25, 563
19. Husoda, and Wataru 1970 Biochim. Biophys. Acta, 222, 53
20. Imaseki 1970 Plant Physiol., Lancaster, 46, 172-74
21. Imaseki, Uritani, and Stahmann 1968 Plant and Cell Physiol., 9, 757-768
22. Jellinck, Smith, and Cleveland 1975 Steroids, 26, 329

23. Jermyn, and Thomas 1954 Biochem. J., 56, 63 (Saunders)

24. Kar, and Mishra 1976 Plant Physiol., Lancaster, 56.140-42

25. Klebanoff, J.J. 1960 J. Biol. Chem., 235, 52-55

26. Lamport 1964 Nature, 202, 293-93

27. Lee, T.T. 1971 Plant Physiol., Lancaster, 47, 181-85

28. Linassier, M.G. C.R. Soc. Biol. Paris 50, (1898) 333; of Loew O. Rep. U.S. Dept. Agric. No. 68

29. Mackinney, D.G. 1942 J. Biol. Chem., 140, 315

30. Morrison, and Allen 1970 Z. Zellforsch, 107, 403

31. Nanda, Bhattacharya, and Kaur 1973 Plant Cell Physiol., 14, 207

32. Ogawa, and Uritani 1970 Radiat. Res., 41, (2), 342-57

33. Parish 1968a Planta, 82, 1-13

34. Parish 1968b Planta, 82, 14-21

35. Parish 1969 Z. Pflanzenphysiol. Bd., 60 S., 211-216

36. Ridge, and Osborn 1970 J. Exp. Bot., 21(68), 720-734

37. Roberts, D.W.A. 1968 Can. J. Bot., 47, 263-65

38. Rudolph, and Bukatsch 1968 Flora, A158, 443-57

39. Saunders, Holmes-Siedle, and Stark 1964 Peroxidase Butterworths. Wash.

40. Schoeber, B. 1971 Potato Res., 14 (1), 39-48

41. Schonbein, C.F. Verh naturf. Gen. Basel 1(1855) 339 (Saunders)

42. Siegel, S.M. 1957 J. Am. Chem. Soc., 79, 1628-32

43. Steward, and Pollard 1958 Nature, 182, 828-32

44. Stonier, and Yang 1973 Plant Physiol., Lancaster, 51, 391-95

45. Strauss, et. al. 1971 Infect. Immunity, 3, 595

46. Tetley, and Thimann 1974 Plant Physiol., Lancaster, 54, 294-303

47. Whitmore 1971 Ibid., 56, 140-42

48. Wilkinson, J. Henry 1966 Isoenzymes J.B. Lippencott Co. Phil.

49. Willstatter, and Pollinger 1932 Physiol. Chem., 130, 281

50. Yip, C.C. 1964 Biochim. Biophys. Acta, 92, 395-97

END