Oxidizing Enzymes of Sugarcane: Peroxidase

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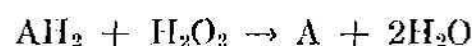
INTRODUCTION

Although a century has passed since peroxidase was first recognized in plants (*34*),[2] its specific roles have been obscured by the large number and diversity of potential substrates. By the type reaction



$$AH_2 + H_2O_2 \rightarrow A + 2H_2O$$

where AH_2 represents an electron donor and hydrogen peroxide the acceptor, numerous phenols, aromatic amines, and other compounds can be oxidized, themselves becoming potential oxidants of additional substances. Thus peroxidase has been put forward as a constituent of terminal oxidation (*1*), as an agent in the oxidation of metabolites by means of H_2O_2 by product (*16*, p. 364), and as a key component of the indoleacetic acid oxidase system (*33,27*). More recent findings implicate peroxidase in the formation of amides (*18,28*).

Peroxidase has been found in numerous plant species which include the fig tree (*34*), corn (*27*), cucumber (*18*), citrus (*28*), tomato (*7*), cauliflower (*3*), and sugarcane (*1,2*). However, the greatest advances toward understanding the physical and chemical properties of peroxidase have been made with the horseradish enzyme purified by Willstatter *et al.* (*40,39*), and crystallized by Theorell (*38*). It is a metalloporphyrin-protein with a molecular weight of about 40,000. The protein portion is itself inactive unless conjugated with hemin, which, in turn, makes up 1.47 percent of the horseradish enzyme. Peroxidase has been likened to methemoglobin (*16*, p. 363), both proteins bearing iron in the ferric state.

As an iron-requiring enzyme, peroxidase belongs to a "family" of metal-containing catalysts broadly classified as "oxidases".[3] Bearing a common capacity to stimulate the passage of electrons from donor to acceptor, the enzymes catalase (iron), cytochrome-c oxidase (iron), ascorbic acid oxidase (copper), and tyrosinase (copper) join peroxidase as potential catalysts of respiratory reactions. Many dehydrogenases also fall under the heading of oxidases. Those that are best known do not contain specific metals,

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[2] Italic numbers in parentheses refer to Literature Cited, pp. 50–2.

[3] Oxidation can be defined as: 1, Addition of oxygen or a hydroxyl; 2, loss of hydrogen; and 3, loss of electrons. Since the oxidation of one substance usually means that another substance is being reduced, the "reductases" may be viewed as catalyzing the reverse of oxidation.

although some have common requirements for riboflavins or for coenzymes I and II.

The necessity of iron for normal peroxidase activity has led to the study of this enzyme as a possible indicator of iron deficiency in agricultural crops. Brown and Hendricks (*7*) found that plants grown on iron-deficient soil exhibited a greatly weakened peroxidase activity as compared with that of plants receiving an adequate iron supply. Brown and Steinberg (*6*) reported low peroxidase activity among iron-deficient tobacco plants. Similar iron-peroxidase relationships were observed with cauliflower grown in sand culture (*3*).

Peroxidase of sugarcane has not received the close attention given to enzymes more obviously related to sugar synthesis and degradation. Nevertheless, a strong peroxidase reaction has been observed in cane, and, curiously enough, this enzyme exhibited an almost perfectly inverse relationship with changing sucrose levels induced by variable nitrate supply (*1*). A similar peroxidase-sucrose correlation was later encountered among plants treated with indole-3-acetic acid (*2*). This paper summarizes *in vitro* properties and behavior of cane peroxidase as an initial effort toward clarifying the relationships between sucrose content and oxidizing enzymes of cane.

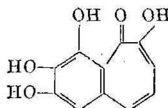
MATERIALS AND METHODS

Enzyme preparations were made with leaves +1 to +4,[4] from the variety M.336, which had been frozen, freeze-dried, and ground to pass a 60-mesh screen in accordance with procedures describes earlier (*1*). Six grams of the dried powder, representing about 30 gm. of fresh material, were extracted for 30 minutes at 20° C. in distilled water or phosphate buffer. The extraction was facilitated with a mechanical shaker operating at high speed. Extracts were clarified by expression through four layers of cheesecloth, followed by centrifuge at 3,000 r.p.m. The pH was adjusted to 7.0 with 0.2 *N* NaOH and sufficient solid ammonium sulfate was added to achieve 95-percent saturation. This was accomplished over a 10-minute period with constant stirring of the solution. The precipitated protein was removed by centrifuge and taken up in 5 ml. of distilled water. The protein solution was clarified and refrigerated at 2° C. Appropriate dilutions were made just prior to running peroxidase assays. Maximum activity was obtained without dialysis.

The peroxidase assay is based upon the formation of purpurogallin in a buffered mixture of pyrogallol, peroxide, and enzyme.[5] The resultant yellow color is measured spectrophotometrically at 430 mμ.

[4] Peroxidase was also obtained from meristem, leaves −1 and 0, leaves +2 and +3, leaves +6 and +7, sheaths +6 and +7, 8 to 10 nodes, 8 to 10 internodes, nodes 1 to 3, and internodes 1 to 3.

[5] This procedure is derived from the standard assay of Willstatter *et al.* (*40*, *39*),



Purpurogallin

The standard reaction mixture for peroxidase was composed as follows: One milliliter of 0.1 M (molar throughout) phosphate buffer, pH 7.2, was placed in duplicate 1-cm. pyrex cuvettes, followed by 0.5 ml. of 0.3-percent hydrogen peroxide. Approximately 5 units of enzyme were added to each cuvette. Reference compositions received distilled water in place of peroxide. After adding 1 ml. of 0.5-M pyrogallol to the control vessel, the optical-density reading was adjusted to zero, pyrogallol was added to the test cuvette, and a stopwatch started immediately. Readings were again taken for both test and reference vessels after 1 minute had passed. Since pyrogallol is slowly oxidized by the enzyme preparation in the absence of peroxide, the final peroxidase reading was obtained by subtracting the control value from that of the test composition.

One peroxidase unit was arbitrarily defined as the amount of enzyme causing an optical density increase of 0.10 per minute under the prescribed conditions of the assay. Peroxidase action is expressed as specific activity, *i.e.* units per milligram of protein. Protein content of the enzyme preparations was measured colorimetrically by the method of Sutherland *et al.* (*35*), or by dry-weight determinations of ovendried samples.

Before continuing with a discussion of cane peroxidase, it must be emphasized that the enzyme values recorded here are relative, or "apparent," rather than absolute activity measurements. Peroxidase forms at least three compounds with hydrogen peroxide (*21,9,10,11*). An intermediate enzyme-peroxide complex is formed enzymatically prior to the reaction with the electron donor molecule, pyrogallol. Two reactions must therefore be considered, and, since the first can be completed before all of the product has been formed (*14*, p. 770), the appearance of purpurogallin is not actually a direct measure of peroxidase activity.

RESULTS AND DISCUSSION

EXTRACTION, FRACTIONATION, AND DISTRIBUTION

Some success has been gained at this laboratory by pretreating leaf and sheath suspensions by sonification prior to extraction. Yields of Q enzyme

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who developed one of the few satisfactory techniques for measuring peroxidase. The procedure described here employs a smaller volume of reagents, direct measurement of product, and a shorter reaction period.

(4) and invertase (5), in particular, were increased by subjecting the chilled suspensions (0–14° C.) to sonic disintegration for several minutes. However, similar treatments were negative with regard both to peroxidase and total

TABLE 1.—*Extraction of cane peroxidase and protein following pretreatment of leaf-powder suspensions by sonic disintegration*

Data classification	Treatment	Data for solvent indicated[1]			
		Acetate buffer (pH 4.65)	Acetate buffer (pH 5.50)	Distilled water	Phosphate buffer (pH 7.0)
Peroxidase[2]	With sonifier	9.5	25.7	6.4	6.1
	Without sonifier	12.8	27.4	31.4	21.7
Total protein[3]	With sonifier	2.74	2.80	1.66	1.70
	Without sonifier	2.90	3.76	2.90	6.60

[1] Buffer concentrations were 0.1 M.
[2] Expressed as specific activity.
[3] Expressed as milligrams per gram of dry weight.

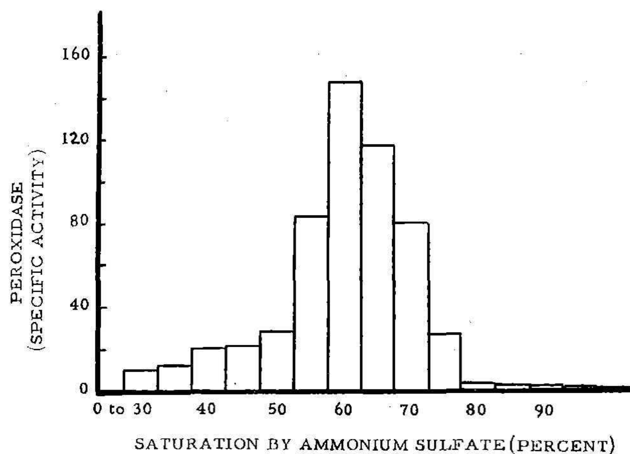


FIG. 1.—Peroxidase activity among protein increments precipitated by ammonium sulfate from extracts of sugarcane leaves.

protein during the present study (table 1). The sonifier was apparently quite destructive of protein when distilled water or phosphate buffer was used for solvents, as evidenced by low protein yield and retarded enzyme activity. Phosphate buffer was the most satisfactory solvent tested.

Fractionation of the cane-leaf extracts by ammonium sulfate revealed that most of the peroxidase was precipitated between 50- and 70-percent saturation (fig. 1). This fraction, which was employed during the remainder of the study, also contained large quantities of amylase and phosphatase.

Distribution of peroxidase within the plant was ascertained with a series of tissue samples taken from 10-month-old cane of the variety Uba Marot (table 2). Easily the greatest specific activity was recorded with preparations from leaves +6 and +7, and from sheaths +6 and +7. However, activity expressed on a dry-weight basis showed that meristem tissues have the highest peroxidase content. The same observation has often been made with sugarcane, the meristem having extremely high enzyme activity, albeit only a small fraction of the plant is represented by this tissue.

TABLE 2.—*Peroxidase activity among different tissues of 12-month-old sugarcane of variety Uba Marot*

Data classification	Data for tissue source indicated								
	Meristem	Leaves (−1 & 0)	Leaves (+2 & +3)	Leaves (+6 & +7)	Sheaths (+6 & +7)	Nodes (8–10)	Internodes (8–10)	Nodes (1–3)	Internodes (1–3)
Specific activity	107.9	107.8	142.9	202.1	216.4	71.3	56.2	78.9	96.4
Peroxidase units per gram of tissue	192.0	44.8	70.0	95.0	72.5	28.5	18.5	28.0	26.5

PROPERTIES OF SUGARCANE PEROXIDASE

Stability

Stability of cane peroxidase was tested at several temperatures, including room (27–29° C.), laboratory (19–21° C.), and refrigerator (2° C.). All samples were stored under toluene. Table 3 indicates that the enzyme lost much of its catalytic capacity within 48 hours of extraction, but that it was still readily measureable after 12 days. This decline was more pronounced among refrigerated samples than those standing at laboratory temperature. This is unusual behavior for an enzyme. Henceforth peroxidase samples were prepared daily.

Peroxidase preparations revealed a definite recuperative capacity after having been boiled for 5 minutes. Whereas no activity was evident immediately after boiling, 13 percent of the original activity returned after 10 minutes, 32 percent within 30 minutes, and a maximum of 42 percent after 3 hours. According to Sumner and Somers (*34*, p. 223), boiled crude preparations may regain some activity upon standing, whereas purified horseradish peroxidase is not seriously affected at all by boiling. Regenera-

tion of activity has been linked with the presence of cytochrome-c in the tissues (*31*).

Optimum Temperature and pH

Cane peroxidase was tested at temperatures ranging from 16 to 52° C. Since no satisfactory method is available for regulating temperatures of test solutions within the spectrophotometer employed, the following procedure was adopted: Duplicate test tubes containing all components of the standard digest, except substrate, were equilibrated for 5 minutes in a water bath adjusted to the desired temperature. Pyrogallol solution was equilibrated separately. The reaction was initiated by transferring 1 ml. of substrate to the test and reference solutions. Two minutes were allowed for the reaction to proceed. Contents of the test tubes were rapidly transferred to 1-cm. cuvettes where optical-density differences between control and

TABLE 3.—*Specific-activity decline of cane peroxidase preparations stored at variable temperatures*[1]

Storage temperature	Specific activity on days following preparation indicated				
	0	2	4	8	12
Room (27-29°C.)	26.2	17.5	15.0	13.7	11.2
Laboratory (19-21°C.)	26.2	22.2	20.0	16.3	12.2
Refrigerator (2°C.)	26.2	18.7	16.2	10.0	10.0

[1] The standard peroxidase procedure was used for all tests.

test solutions were determined as usual. About 2 minutes and 15 seconds elapsed for each reaction. Although some error was unavoidable, the results are nevertheless relative and showed that the optimum temperature for this enzyme is about 36° C. (fig. 2,A). The laboratory temperature usually employed for peroxidase (20° C.) permitted an activity somewhat less than half of that recorded at 36° C.

Optimum pH is about 7.4 (fig. 2,B).

Km and Optimum Peroxide Concentration

A practical definition of the "Michaelis constant" (*Km*) is that it is the substrate concentration at half maximum velocity (*29*, p. 58). Sugarcane peroxidase achieved a linear reaction up to 5 µmoles of pyrogallol per milliliter of digest (fig. 3,A). Insofar as it is applicable to the peroxidase reaction, the apparent *Km* thus becomes 2.5×10^{-3} moles of pyrogallol per liter.

Maximum peroxidase velocity was obtained with 1 µmole of hydrogen

peroxide per milliliter of digest (fig. 3,B). However, some activity increase could be gained by raising the peroxide concentration to as much as 140 μmoles/ml. Possibly a number of peroxidases exist in cane, some of which

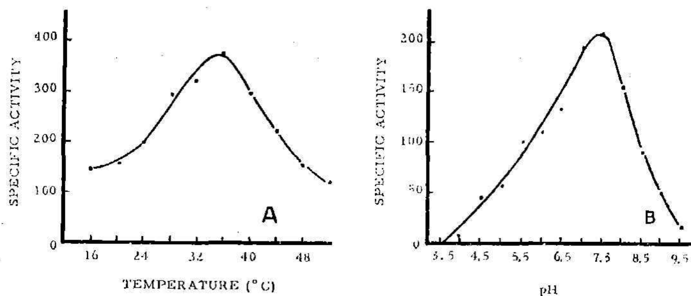


FIG. 2.—A, Effects of temperature on cane peroxidase activity; phosphate buffer (pH 7.2) was used with standard assay. B, Effects of pH on cane-peroxidase activity; all reactions were run at 22° C., according to standard assay, in conjunction with 0.1-M concentrations of the following buffers: Acetate, pH 3.5–5.5; succinate, pH 6.0–7.0; and tris (trishydroxymethylamino methane), pH 7.5–9.5.

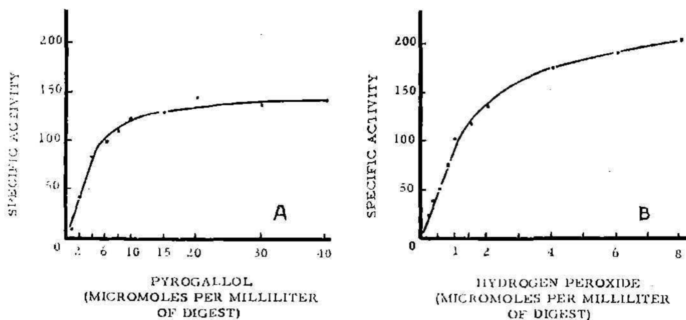


FIG. 3.—A, Pyrogallol required for optimum activity of cane peroxidase. Standard digest was used with variable pyrogallol. B, Saturation of cane peroxidase by hydrogen peroxide. Standard digest was employed throughout with variable peroxide.

continue to strive for saturation with peroxide long after this has been achieved for the principal enzyme.

Reaction Velocity vs. Time and Enzyme Concentration

The standard assay herein employed measures peroxidase from the time pyrogallol is added to the digest. Since both enzyme and peroxide were

previously combined in the reaction mixture, the initial phases of the peroxidase reaction are already underway, *i.e.* the formation of enzyme-peroxide complexes. For this reason, plus the fact that the initial reactions are supposedly rapid and not considered to be rate-limiting (*34*, p. 222), pyrogallol formation should be plotted as a typical diphasic reaction. This does in fact occur for cane peroxidase (fig. 4,A). Under conditions of the standard assay, a zero-order reaction is maintained slightly less than 2 minutes, with a first-order reaction continuing at least up to 5 minutes.

Peroxidase preparations were diluted during this study so that between 4 and 6 units were ordinarily present in the reaction mixture. A linear relationship existed between reaction velocity and enzyme content to a concentration of at least 12 units per digest (fig. 4,B).

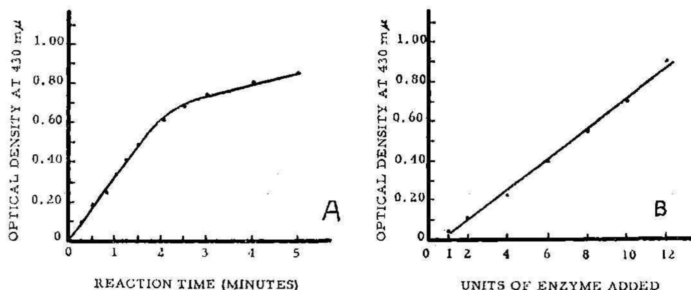


FIG. 4.—A, Proportionality of peroxidase activity with time; five units of enzyme were used. B, Proportionality of activity with enzyme concentration; units of enzyme are indicated.

Substrate Specificity

As pointed out earlier, a large number of compounds may be oxidized by various peroxidases, while the products, in turn, may oxidize other substances. Sumner and Somers (*34*, p. 221) list 14 compounds subject to the action of plant peroxidase in the presence of H_2O_2. Sugarcane peroxidase gave positive reactions with the following compounds: Orthocresol, hydroquinone, catechol, pyrogallol, benzidine, analine, tryptophane, guaiacol, and gum arabic. Other substances can undoubtedly serve in the cane peroxidase reaction.

Activation and Inhibition

A series of cations and anions were tested for possible regulatory effects upon peroxidase (table 4). Also tested were β-glycerophosphate, thiourea, glucose, and hydroxylamine. All additives were employed at the rate of 1

μmole/ml. of digest. When tested in accordance with the standard assay, the additives copper and molybdenum each appeared to stimulate peroxidase. This was actually caused by a nonenzymatic oxidation of pyrogallol in the presence of additive and hydrogen peroxide. Iodide more than doubled peroxidase activity as the only true activator encountered.

Calcium, manganese, and zinc each caused a moderate suppression of the sugarcane enzyme. Hydroxylamine markedly inhibited this reaction, in agreement with Sumner and Somer's statement regarding horseradish peroxidase (*34*, p. 223). In contrast to the horseradish enzyme, however, neither thiourea nor an excess of hydrogen peroxide (180 μmoles/ml. of digest) caused inhibition.

TABLE 4.—*Effects of additives upon the specific activity of sugarcane peroxidase*[1]

Additive	Specific activity	Additive	Specific activity	Additive	Specific activity
H_2O (control)	142.5	I	350.0	Zn	116.3
Mg	127.5	As	150.0	Thiourea	141.3
Ca	115.0	Cu	-	Nitrate	132.5
B	126.3	Mn	103.8	β-glucerophosphate	147.5
F	121.3	Mo	-	Glucose	146.3
Br	130.0	KCN	151.3	Hydroxylamine	58.8

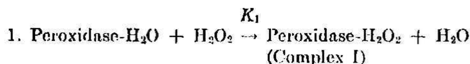
[1] Each additive was supplied at the rate of 1 μmole/ml. of digest. The standard reaction was employed throughout with supplemental materials taking the place of distilled water.

Dialysis

No effect was noted upon cane peroxidase activity following 36 hours of dialysis against distilled water. No dialysis effects were anticipated, since the nonprotein complement (hematin) is tightly bound to the protein and cannot be removed by other than extraordinary means (*36*).

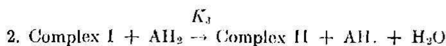
Mechanism of Peroxidase Action

It was mentioned earlier that at least two phases are involved in the peroxidase reaction, including the formation of enzyme-peroxide complexes, and the transfer of electrons from a donor molecule to peroxide. Whereas the chemical changes undergone by peroxidase (horseradish) during its catalytic processes have not been clarified in detail (*13,17*), the work of Chance (*9,10,11,12*) points with certainty toward the formation of at least three enzyme-peroxide complexes. Peroxidase reactions can thus be summarized as follows[6]:

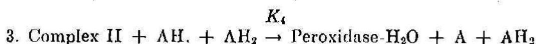


$$1.\ \text{Peroxidase-}H_2O + H_2O_2 \xrightarrow{K_1} \text{Peroxidase-}H_2O_2 + H_2O \quad \text{(Complex I)}$$

[6] AH. denotes a half-oxidized electron-donor molecule.



$$2.\ \text{Complex I} + AH_2 \xrightarrow{K_3} \text{Complex II} + AH. + H_2O$$



$$3.\ \text{Complex II} + AH. + AH_2 \xrightarrow{K_4} \text{Peroxidase-}H_2O + A + AH_2$$

Complex III is formed from complex II in the presence of excess hydrogen peroxide. It is stable and does not oxidize the substrate. When observed spectroscopically, complex I is green, complex II is pale red, and complex III is bright red.

It is assumed that cane peroxidase takes part in reactions similar to those given above for the horseradish enzyme. This can be demonstrated by

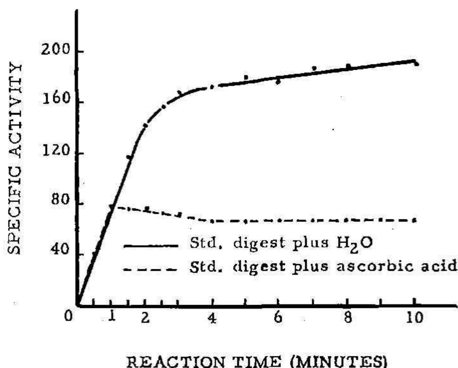
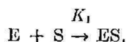


FIG. 5.—Inhibitory effects of ascorbic acid on cane peroxidase: ascorbic acid was added to 1 of 2 digests, after 1 minute had elapsed, in sufficient concentration to make 10 μmole/ml. of digest.

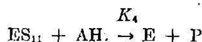
uncoupling the reaction sequence with a readily oxidizable substance. For example, each of the three compounds should rapidly disappear in the presence of ascorbic acid, with the concurrent formation of free enzyme plus dehydroascorbic acid. Stabilization of purpurogallin content in the reaction mixture would follow immediately. Figure 5 illustrates the retarding effect of ascorbic acid upon sugarcane peroxidase, including a slight loss of purpurogallin during the 2 minutes following ascorbic acid incorporation. This was possibly caused by a moderate reversal of reaction 3 above, which would necessitate the constant K_5, although enzymatic oxidation of the donor molecule is not generally regarded as reversible.

Two rate constants need to be distinguished in order to evaluate the catalytic potential of peroxidase, including the velocity constant for the formation of an enzyme-substrate complex,



$$E + S \xrightarrow{K_1} ES,$$

and the velocity constant for the reaction of a secondary complex (complex 2) with the electron donor molecule,



$$ES_{II} + AH_2 \xrightarrow{K_4} E + P$$

K_1 and K_4 of sugarcane peroxidase were estimated by the "guaiacol test." Developed by T. M. Devlin and described in detail by Chance and Maehly (*14*), the procedure is based on the principle that peroxide utilization (dx/dt) depends upon the respective concentrations of peroxide and guaiacol in the following manner:

$$\frac{dx}{dt} = \frac{e}{\frac{1}{K_4 a_0} + \frac{1}{K_1 x_0}}$$

where e = concentration of enzyme.
a_0 = initial concentration of donor.
x_0 = initial concentration of substrate.

K_1 is determined under carefully prescribed conditions in which $K_4 a_0 \gg K_1 x_0$, and K_4 is determined by adjusting the assay so that $K_1 x_0 \gg K_4 a_0$. Enzyme concentration was estimated colorimetrically (*35*) and by dry-weight determinations of ovendried samples. Dry weight of enzyme used was divided by the molecular weight of peroxidase (40,000)[7] to obtain e. Where x = 7.5 μmoles of H_2O_2, and t = the measured time interval to achieve an optical density increase of 0.050 at 470 mμ, K_1 and K_4 were calculated as follows:

$$K_1 = \frac{1}{x_0 e} \times \frac{\Delta x}{\Delta t} = \frac{7.5 \times 10^{-6}}{3.3 \times 10^{-5}} \times \frac{1}{e\Delta t} = \frac{0.22}{e\Delta t}$$

The experimental value for K_1 was $0.13 \times 10^7 M^{-1} \times sec^{-1}$ at 20° C.

$$K_4 = \frac{1}{a_0 e} \times \frac{\Delta x}{\Delta t} = \frac{7.5 \times 10^{-6}}{3.3 \times 10^{-4}} \frac{1}{xe\Delta t} = \frac{2.2 \times 10^{-2}}{e\Delta t}$$

The computed K_4 value was $0.46 \times 10^5 M^{-1} \times sec.^{-1}$ at 20° C.

Under identical test conditions, pure horseradish peroxidase has K_1 and K_4 values of 0.89×10^7 and 2.2×10^5, respectively (*14*), or, in other words, these constants are about 7 and 5 times greater, respectively, than those for unpurified cane peroxidase. This difference is undoubtedly attributable to

[7] Theorell (*37*) placed the molecular weight of horseradish peroxidase at 40,200, whereas Cecil and Ogston (*8*) reported a value of 39,800.

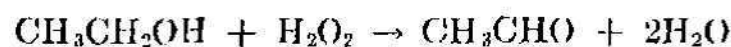
the fact that a mixture of enzymes composed the sugarcane preparation rather than a pure catalyst. It is also important that the assay conditions of the guaiacol test have been worked out with precision only for horseradish peroxidase.

Nevertheless it is evident that oxidation of the donor molecule proceeds far less rapidly than the initial formation of an enzyme-peroxide complex. Hence, complex II may be regarded as the "Michaelis compound" (rate determining) of the peroxidase catalysis, and oxidation of donor as the true rate-limiting reaction.

Physiological Significance of Peroxidase

The potential of peroxidase to affect plant physiology is so broad that, for years, little more than generalizations were suggested to account for this enzyme. Thus Sumner and Somers (*34*, p. 222) suggested that, through the oxidation of phenols and ferrocytochrome-c, peroxidase might play a role in terminal oxidation, but little experimental evidence was available to support this point.

Keilin and Hartree (*22*) demonstrated that, if catalase and ethanol are added to a system in which H_2O_2 is produced, the alcohol is oxidized to acetaldehyde:



$$CH_3CH_2OH + H_2O_2 \rightarrow CH_3CHO + 2H_2O$$

This corresponds to the type of reaction catalyzed by peroxidases, indicating a similarity of action between these and the catalases.[8] The two types of enzymes are thus considered to belong to a single group, the "hydroperoxidases," with each of the enzyme types exhibiting specificity preferences for the electron donor molecule.

Peroxidase has been implicated strongly in the maintainance of auxin levels in plants. It is regarded as the effective component of indoleacetic acid oxidase (*33*). McCune (*27*) reported that corn contains several peroxidases which differ in their capacity to take part in the indoleacetic acid oxidase system. Investigations by Halevy (*18*), and by Monselise and Halevy (*28*), revealed that the growth-retarding compound "Amo-1618" increased peroxidase activity in seedlings of cucumber and citrus. It was proposed that growth retardants affect the auxin level of plant tissues by promoting auxin destruction. This concept was vindicated during later studies by Halevy (*19*), when it was found that five growth-retarding compounds stimulated indoleacetic acid oxidase and peroxidase in hypocotyl tips and cotyledons of cucumber seedlings grown in darkness. This effect was inversely correlated with hypocotyl elongation effects of the same

[8] Since H_2O_2 is toxic to living systems, it was long thought that the principle function of catalases was to destroy H_2O_2 .

chemicals. Halevy suggested that growth retardants exert their effect on plant growth by interacting with gibberellin in indoleacetic acid oxidase, or its cofactors and inhibitors.

A different but highly important function of peroxidase was suggested by the work of Mazelius and Ingraham (*26,25*). These investigators studied the oxidative decarboxylation of methionine by horseradish peroxidase and found that the product was an amide (3-methylthiopropionamide). Traditionally, the enzymatic formation of primary amides has involved the activation of NH_3 (*24*) or the transfer of an amino (*23*) or amide group (*30*). Ureido compounds, having amide character in view of their $-CONH_2$ groups, likewise occur by NH_3 activation (*20*) or by pyrimidine and purine degradation (*15,32*). The report of Mazelis and Ingraham (*25*) was the second to review amide formation directly from an amino acid without involving transfer of the nitrogenous group or cleaving of a heterocyclic ring, and the first to directly implicate peroxidase.

Peroxidase activity of sugarcane leaves has shown a striking inverse relationship with sucrose content (*1,2*). However, even if one considers that all known roles of peroxidase are being assumed by the sugarcane representative, no clear connection can yet be drawn between peroxidase activity and sucrose content. The relationship must certainly be an indirect one and will have to await additional findings for its ultimate clarification.

SUMMARY

A study was made of the distribution and properties of peroxidase in sugarcane. The enzyme was extracted with phosphate buffer (pH 7.0) from freeze-dried tissues which had been ground to pass a 60-mesh screen. Peroxidase was assayed spectrophotometrically at 430 mμ, the procedure being based upon the formation of purpurogallin in a buffered solution of hydrogen peroxide, enzyme, and pyrogallol.

Fractionation of cane extracts with ammonium sulfate showed that peroxidase is precipitated between 50- and 70-percent saturation. The richest source of this enzyme was meristematic tissue, whereas greatest specific activity was recorded with preparations from leaves +6 and +7, and from sheaths +6 and +7.

Preparations of peroxidase were not affected by dialysis against distilled water, but considerable activity loss was experienced within 2 days following preparation. Refrigeration did not help preserve the enzyme. Activity of the preparations was totally lost after boiling for 5 minutes. However, much of the activity returned upon standing, a property also known for horseradish peroxidase.

Sugarcane peroxidase acted upon a number of substrates, including orthocresol, hydroquinone, catechol, pyrogallol, benzidine, analine, trypto-

phane, guaiacol, and gum arabic. Optimum pH for the standard reaction was about 7.3, and optimum temperature was 36° C. Apparent K_m was 2.5×10^{-3} mole of pyrogallol per liter. Maximum velocity was obtained with 1 µmole of hydrogen peroxide per milliliter of digest. Under standard conditions, cane peroxidase maintained a zero-order reaction for about 2 minutes.

Peroxidase was moderately inhibited by calcium, manganese, and zinc, each at a concentration of 1.0 µmole/ml. of digest. Iodide stimulated the enzyme. Hydroxylamine markedly suppressed the reaction. Neither thiourea nor an excess of peroxide (180 µmoles/ml. of digest) caused inhibition, although the latter substances, as does hydroxylamine, greatly retard horseradish peroxidase.

Cane peroxidase behaves like other plant peroxidases in that several enzyme-peroxide complexes are formed prior to oxidation of the electron-donor molecule. This reaction sequence was readily uncoupled with ascorbic acid. The velocity constant for the formation of the initial enzyme-substrate complex, K_1, was $0.13 \times 10^7 M^{-1} \times sec.^{-1}$ at 20° C. The velocity constant for the oxidation of the donor molecule, K_4, was $0.46 \times 10^6 M^{-1} \times sec.^{-1}$ at 20° C.

Known roles of plant peroxidases were briefly reviewed.

RESUMEN

Se hizo un estudio de la distribución y las propiedades de la peroxidasa en la caña de azúcar. La enzima se extrajo usando un fosfato con un pH de 7.0, como agente amortiguador, de tejidos congelados y disecados que se maceraron y pasaron por una criba de malla número 60. Se analizó la peroxidasa mediante el procedimiento espectrofotométrico a 430 mµ, a base de la formación de purpurogalina en una solución amortiguada de peróxido de hidrógeno, enzima y pirogalol. Al fraccionarse los extractos de caña con sulfato amónico, la peroxidasa se precipitó a una saturación de 50 a 70 por ciento. La más rica fuente de esta enzima fue el tejido meristemático, mientras que la mayor actividad específica tuvo lugar en las preparaciones hechas con las hojas +6 y +7, y las yaguas +6 y +7.

La acción dializadora no afectó las preparaciones de peroxidasa en presencia de agua destilada, aunque sí redujo su actividad considerablemente a los dos días de haberse hecho las preparaciones. La refrigeración no ayudó a preservar la enzima. La actividad de las preparaciones cesó totalmente después de hervir por 5 minutos. Sin embargo, la actividad se reanudó pasado algún tiempo, propiedad que también posee la peroxidasa del rábano picante.

La peroxidasa de la caña de azúcar actúa sobre numerosos sustratos que incluyen el orto-cresol, la hidroquinona, el catecol, el pirogalol, la bencidina,

la analina, el triptofano, el guayacol y la goma arábiga. El pH óptimo para la reacción "stándard" fue de alrededor de 7.3 y la temperatura óptima de 36° C. La constante Michaelis (Km) fue 2.5×10^{-3} mol de pirogalol por litro. La velocidad máxima se obtuvo con 1 µmol de peróxido de hidrógeno por mililitro de digesto. Bajo condiciones normales, la peroxidasa mantuvo una reacción de orden-cero en unos 2 minutos.

La peroxidasa fue inhibida moderadamente por el calcio, el manganeso y el zinc, a concentraciones de 1.0 µmol/ml. de digesto. El yoduro estimuló la enzima. La hidroxilamina redujo la reacción marcadamente. Ni la tiurea ni un exceso de peróxido (180 µmol/ml. de digesto) causaron inhibición alguna, aunque esas mismas sustancias, al igual que la hidroxilamina, retardan grandemente la peroxidasa del rábano picante.

La peroxidasa de la caña actúa de igual manera que las peroxidasas de otras plantas, formando varios complejos de enzima-peróxidos antes de la oxidación de la molécula que provee el electrón. Esta secuencia reactiva se desconectó rápidamente con el ácido ascórbico. La constante de velocidad para la formación del complejo inicial de la enzima-sustrato, K_1, fue $0.13 \times 10^7 M^{-1} \times sec.^{-1}$ a 20° C. La constante de velocidad para la oxidación de la molécula proveedora, K_4, fue $0.46 \times 10^5 M^{-1} \times sec.^{-1}$ a 20° C.

Se hizo un ligero análisis del papel que desempeñan las peroxidasas en las plantas.

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