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PEROXIDASE ACTIVITY IN CITRUS TISSUES*

R. GOREN and E. E. GOLDSCHMIDT

Department of Citriculture, Faculty of Agriculture, Hebrew University, Rehovot, Israel

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Abstract—Peroxidase activity has been determined in various citrus tissues and varieties using four H-donors: pyrogallol, guaiacol, *p*-methylaminophenol-sulphate (metol) and *p*-phenylenediamine (PPDA). Results showed that fruits, leaves and roots, respectively, have increasing peroxidase activity, regardless of the H-donor used, indicating that the characteristics of the tissue used have a more pronounced effect on activity than the type of the H-donor. The relationship between H_2O_2 and H-donors which serve as substrate for the peroxidase was examined thoroughly. A wide range of combinations of H_2O_2 and different H-donors in various concentrations was tested in order to define the optimal conditions for each tissue. As for peroxidase from other sources, it was found that in all citrus tissues and varieties tested there exists an interdependence between both substrates.

INTRODUCTION

PEROXIDASE is believed to participate in various oxidative processes, including respiration,[1,2] lignification[1,3] and degradation of auxin.[2,4–9] In previous studies,[10–13] guaiacol and pyrogallol were used as H-donors, as different H-donors may show diverse results when tested with the same tissue. Recent reports[2,6,10,14–16] suggested that this is due to the fact that the peroxidase system consists of several active fractions, which differ from each other in their biochemical characteristics.

Peroxidase is a system which has two substrates[17,18]—the O-donor (H_2O_2) and the

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H-donor. The relationship between these two components was first studied by Mann.[19] He was able to demonstrate that an increase in the concentration of one substrate caused a shift in the optimal range for the concentration of the other substrate. Chance[20] concluded that in a system like peroxidase an optimum in the activity–substrate concentration relationship is revealed not only by a variation of the substrate concentration, but also by a variation of the donor concentration.

The peroxidase system, using a variety of H-donors, was studied in various tissues of various citrus varieties as part of a study on oxidative systems related to growth mechanism in citrus.[10–12, 21–22]

RESULTS

pH and Temperature

The optimal pH conditions were tested by means of pyrogallol and guaiacol, using phosphate–citrate and phosphate buffers in the range of pH 3·0–6·0 and 5·8–8·0, respectively, and were found to be between pH 6·0 and 6·6. No differences were found when using several

TABLE 1. PEROXIDASE ACTIVITY OF CRUDE EXTRACTS OF SWEET LIME ROOTS AND MATURE SHAMOUTI ORANGE LEAVES, WITH AND WITHOUT DIALYSIS, MEASURED BY USING PYROGALLOL AND GUAIACOL

		Activity*	
		No dialysis	Dialysis
Roots	Pyrogallol	218	190
	Guaiacol	4007	3578
Leaves	Pyrogallol	91	80
	Guaiacol	220	185

* Absorptivity $\times 10^3$ after 120 sec·mg fresh weight.

buffer molarities ranging from 0·01–0·1 M, and so phosphate buffer, 0·05 M, pH 6·6, has been used throughout this study. It was found that the peroxidase is not particularly sensitive to temperature within the range of 18–30°, thus, "room temperature" was considered to be sufficiently constant for the purpose of the present study.

The Influence of Dialysis on Peroxidase Activity

It has been reported[23] that dialysis of crude peroxidase extract, carried out for a short time, causes the removal of peroxidase inhibitors and increases therefore the enzymatic activity. Dialysis of crude preparations from sweet-lime roots and Shamouti leaves was carried out for 2 and 48 hr against 10 l. of tap-water at 15°. The results were similar in both cases. Table 1 presents only results for the 2-hr dialysis. The slight decrease in the activity

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after dialysis might be due to some changes in the enzyme, to partial removal of co-factors, or to both.

Behaviour of H-donors

Pyrogallol and guaiacol were previously used as H-donors in our studies.[10–12, 21] Two additional donors—*p*-methylaminophenol-sulphate (metol) and *p*-phenylenediamine (PPDA)—were selected on the basis of existing data[16] for further investigation of the donor-enzyme relationship.

Pyrogallol. The absorption curve (Fig. 1) of the product formed with an extract of Shamouti orange leaves shows a steady decrease in absorptivity from 400 mμ with no peak. Nevertheless, preliminary experiments and previous studies[10–12, 21] showed an obvious

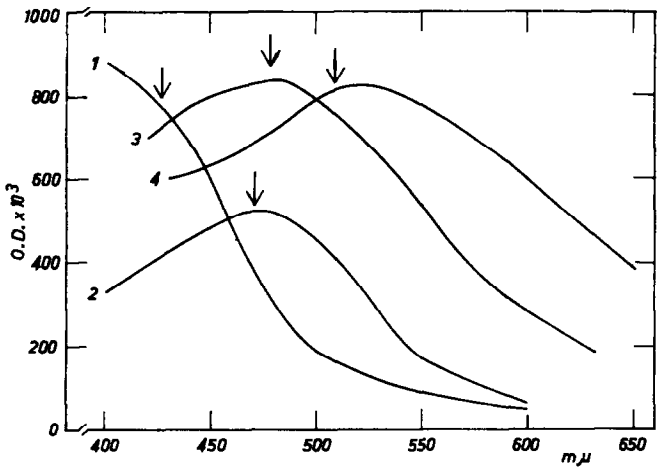

FIG. 1. TYPICAL ABSORPTION CURVES OF FOUR H-DONORS.

Arrows show wavelength at which optical density is usually read. 1—pyrogallol; 2—guaiacol; both tested with Shamouti orange leaves. 3—metol; 4—PPDA; the last two tested with sweet lime roots.

activity near 425 mμ, and since peroxidase is usually determined[2, 16, 24] with this H-donor by measurement in the range of 425–430 mμ this wavelength was chosen for the present study.

Guaiacol. The absorption curve (Fig. 1) with this substrate and the same extract, has a peak at 470 mμ as shown in previous studies.[2, 16, 24]

Metol. The absorption curves of the products from this donor were tested with crude extracts prepared from sweet-lime roots and with flavedo (the outer coloured layer of the fruit's peel), leaves and branches of Shamouti orange trees. It was found that with the extract from sweet lime roots different colours appear during the course of the reaction, ranging from brown (445 mμ) to crimson (525 mμ). The shifts in absorption peak are due to the decrease in the concentration of H_2O_2 from 0·73 M to $3{\cdot}7 \times 10^{-4}$ M. When comparing results obtained with extracts from Shamouti orange fruits and sweet lime roots it was noted that the different colours were also obtained by the two extracts. The curve in Fig. 1 was attained using $3{\cdot}7 \times 10^{-3}$ M H_2O_2. It appears that metol is not a suitable donor for determination of peroxidase activity in citrus tissues.

PPDA. This donor was tested with extracts from the same tissues as used for metol, and

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the oxidation product was found to be uniform in general. The colour developed was red-brown, and its peak shifted from 490 mμ (Shamouti leaves) to 520 mμ (in all other tissues tested).

A linear relationship was found between concentrations of fresh material used for preparation of the extracts and the intensity of colour developed in the course of the enzymatic reactions with all H-donors. However, as pyrogallol and guaiacol are the most commonly used H-donors for the determination of peroxidase activity,[1] most of the work has been performed with them.

TABLE 2. PEROXIDASE ACTIVITY OF CITRUS TISSUES, TESTED WITH DIFFERENT COMBINATIONS OF H-DONORS AND H_2O_2 CONCENTRATIONS

H-Donor and conc. mM	Activity* Conc. H_2O_2 mM							
Guaiacol†	0·3	1·5	2·9	5·9	11	59	290	590
270	70	110	180	250	270	195	105	103
110	33	137	255	370	460	205	10	14
50	65	232	405	525	525	170	17	0
21	90	270	370	435	395	95	0	0
11	95	225	270	300	228	30	0	0
5	90	163	160	135	98	10	0	0
1·1	26	35	22	0	0	0	0	0
0·1	0	0	0	0	0	0	0	0
Pyrogallol‡	0·3	1·6	3·1	6·2	12	6·2	310	620
140	235	600	980	1400	1500	1480	400	190
70	286	750	1100	1480	1800	1400	370	152
28	335	830	1250	1550	1700	1100	277	110
14	375	845	1200	1400	1400	780	215	82
7	345	765	1050	1200	1100	580	140	53
3·5	342	690	870	930	710	365	90	30
1·8	315	580	680	670	455	230	57	26
7	245	365	405	375	229	90	12	0

* Absorptivity $\times 10^3$ after 120 sec.
† Guaiacol, grapefruit leaves, 0·55 mg fresh material in the reaction mixture
‡ Pyrogallol, flavedo of lemon peel, 5 mg fresh weight.

Optimal Conditions for Peroxidase Assay in Different Citrus Tissues

In the following experiments peroxidase activity was tested at different combinations of H-donor and H_2O_2 concentrations and in various tissues with the object of finding out whether there exist different optimal conditions for maximum activity in different tissues. Two typical results of such experiments are presented in Table 2.

A careful study of Table 2 shows that maximum activity of the enzyme was not obtained at the highest concentration of H-donor and H_2O_2. On testing other citrus tissues, the same relationships were always found between the O-donor and each one of the H-donors tested. Therefore, it is obvious that the characteristics of the interrelationship enzyme–H-donor - O-donor are due to the nature of tissues tested. It has been found that changes in fresh

material (enzyme) concentration in the reaction mixture have no effect on the above mentioned relationships.

Catalase, if present in the homogenate, should not have interfered with the determination of peroxidase activity by pyrogallol and guaiacol since these two substrates appear to inhibit catalase activity almost completely.[24] Interference of catalase with the reactions of the other H-donors used seems also improbable since we know from previous studies[10–12,21] that filtering the homogenate through filter paper (as was the case in this study) greatly reduces the catalase activity. The filtering procedure did not affect peroxidase activity.[10]

TABLE 3. MAXIMUM PEROXIDASE ACTIVITY* IN DIFFERENT CITRUS TISSUES

Tissue	Variety	Activity † with			
		Pyrogallol	Guaiacol	Metol	PPDA
Fruit peel (flavedo)	Shamouti orange	68 (12:7)‡	100 (12:53)	174 (4:3)	250 (11:17)
	Sour orange	158 (9:3)	491 (11:110)		
	Marsh seedless grapefruit	158 (12:11)	509 (12:53)		
	Lemon	360 (12:7)	1455 (12:80)		
Mature leaves	Shamouti orange	248 (12:7)	191 (8:53)	419 (7:3)	1256 (370:17)
	Marsh seedless grapefruit	370 (12:14)	955 (8:53)		
	Lemon	375 (12:7)	709 (12:21)		
Roots	Sour orange	1000 (12:11)	2625 (6:21)		
	Sweet lime	1267 (3:7)	5000 (1:21)	2351 (7:3)	2200 (73:17)

* Tested at the optimum conditions for each H-donor separately, calculated from several sets of experiments involving different concentrations of H-donors and H_2O_2 (see Table 2).

† Absorptivity $\times 10^3$ in 120 sec/mg fresh wt.

‡ The figures in brackets give the concentration of H_2O_2 and H-donor giving this maximum activity (both in mM).

Effects of Tissue, Varieties and H-donors

The maximum values of peroxidase activity under its optimum conditions tested in different tissues and varieties of citrus are shown in Table 3. It is obvious that the highest activity of peroxidase, tested with all H-donors, is found in roots, and is stronger in the sweet-lime roots than in the sour orange roots. Activity in leaves is lower than in roots but higher than in the flavedo of fruits. Again there are differences in peroxidase activity when comparing several varieties of citrus. The content of dry matter in roots, leaves and fruit peel varies within the range of 25–40 per cent, therefore, the differences in the activity of peroxidase in these tissues is not related to changes in the percentage of dry matter.

The different conditions for each H-donor with which the peroxidase activity is measured prevent a simple comparison of the results obtained for various H-donors. It cannot be concluded, for instance, that peroxidase activity tested with pyrogallol in flavedo of Shamouti

orange fruit, is "lower" than that tested with guaiacol (Table 3). The molar concentrations of H_2O_2 and H-donors leading to maximum peroxidase activity in citrus tissues, also shown in Table 3, were derived from a series of experiments like those presented in Table 2.

DISCUSSION

The principal subject of the present paper was to study the characteristics of peroxidase in several varieties and tissues of citrus. It has been shown clearly that different citrus tissues give different activity values when peroxidase is being tested by means of different H-donors.

A fact worth noting is the higher order of magnitude of peroxidase activity in roots than in any other tissue tested, and it is well emphasized with all donors used (Table 3). The differences in peroxidase activity in roots as compared to leaves were previously reported.[10] The wide range of functions contributed to peroxidase seems to support the idea[2, 10, 16, 21] that this enzyme has different functions in different tissues which directly influence the intensity of its activity.

Repeated tests of the same tissues from different varieties (Table 3) indicate the fact that differences between tissues are of higher significance than differences between varieties. The higher activity in all tissues of the yellow coloured varieties: grapefruit, lemons and sweet-lime, as compared with the orange coloured: sweet and sour orange fruits, may be due to the taxonomic differences within the genus *Citrus*. These results are in agreement with a previous report.[25]

Recently,[2, 10–13, 16, 26] more significance has been attributed to the differences between H-donors when testing the same tissue. This difference is related to the presence of several active functions of peroxidase, capable of reacting differently with each H-donor. The question why in certain cases[6, 10–12] there exists a considerable difference in peroxidase activity when tested with several H-donors while in other cases[16, 21, 22] this difference is insignificant, is of some interest. It seems that when testing the same tissue, differences between H-donors are easily seen,[11] whereas when testing tissues of widely different activity (roots as compared with leaves, Table 3), the differences between H-donors are small in relation to the differences in the characteristics of the tissue compared.

While considering the interrelationship between H-donors and H_2O_2 it has been shown that for each tissue there exists a combination of optimum concentrations of H-donor and H_2O_2 (see Table 3). The comparison of tissues as shown in Table 3 is based on the principle of testing each tissue at its optimum conditions while pH, temperature, etc. remain constant, and has the advantage of characterizing each tissue at its maximum enzymatic activity. On the other hand, the fact that each tissue was tested under its own particular experimental conditions does not allow a comparison of several tissues. It seems that for the sake of general comparison of different tissues it is preferable to work under conditions of maximum enzymatic activity than to keep concentrations of H-donor and H_2O_2 constant, a procedure which does not allow an estimation of the potential activity of the tissue.

The present study was carried out over a very wide range of substrate concentrations, H-donors, varieties and tissues (Table 2). It is clearly demonstrated that there exists an interdependence between the two substrates (H_2O_2 and H-donor) of the peroxidase system as formerly discussed by Mann[19] and Chance.[20] The present study supports the theory that

[25] W. B. DAVIS, *Am. J. Botany* **29**, 252 (1942).
[26] C. L. MARKET and F. MOLLER, *Proc. Nat. Acad. Sci. U.S.* **45**, 753 (1959).

enzyme and H_2O_2 first form a complex that becomes, in fact, the "enzyme" to react with the H-donor.

EXPERIMENTAL

Determination of Perioxidase Activity

Fresh material, washed in twice-distilled water, was cut and transferred into phosphate buffer 0·05 M, pH 6·6, and homogenized in an ice bath by means of an Ultra Turrax homogenizer at 24,000 rev/min. The homogenate was filtered through filter paper Whatman No. 1.

The peroxidase activity was determined by means of four different H-donors: aqueous solution of pyrogallol and metol and alcoholic solution of guaiacol and *p*-phenylenediamine (PPDA).

The constituents of the reaction mixture were phosphate buffer (pH 6·6), H_2O_2 and one of the above H-donors; the concentrations of the last two varied as required for maximum activity (Table 2). The reaction mixtures contained 1 ml of H_2O_2 and 1 ml of one of the H donors, with the exception of pyrogallol which was added as 0·1 ml. The concentration of fresh material varied from 0·1 to 12·0 mg according to the intensity of the enzymatic activity, as determined by the different H-donors.

Fresh enzyme solutions were prepared daily. The H_2O_2 and the appropriate H-donor were mixed and added to a colorimetric tube containing the fresh material in a buffer solution and stirred vigorously. The final volume for pyrogallol, guaiacol, metol and PPDA was 7·1, 7·5, 6·0 and 6·0 ml, respectively. The absorbency was measured by a Bausch and Lomb "Spectronic 20" colorimeter at 425, 470, 480 and 510 mμ for pyrogallol, guaiacol, metol and PPDA, respectively. Blanks that contained H_2O_2 and the enzyme did not develop colour due to the presence of natural H-donors. The activity of peroxidase was measured by colorimetric readings taken at 15 sec intervals for 120 sec. The average of five differences in absorptivity per 15 sec over the period of 30–105 sec is calculated.

Another method[24] used was to determine the activity after a definite lapse of time, which varied in our experiment from 2 to 6 min after the beginning of the reaction, according to the features of the H-donor used. Most of the experiments described in this paper have been carried out by the last method at room temperature.

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