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EFFECT OF GAMMA IRRADIATION ON POLYPHENOL OXIDASE ACTIVITY AND ITS RELATION TO SKIN BROWNING IN BANANAS

PAUL THOMAS and P. M. NAIR

Biochemistry and Food Technology Division, Bhabha Atomic Research Centre, Trombay, Bombay-85, India

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Abstract—A partially purified polyphenoloxidase preparation from three varieties of banana fruit showed both cresolase and catecholase activities. Enzyme preparations from pulp tissues showed more cresolase activity than skin whereas skin has more catecholase activity. Gamma irradiation had a differential effect on the cresolase and catecholase activities of the enzyme. The catecholase activity increased with increasing doses of irradiation whereas the cresolase activity showed an increase only up to 100 krad and decreased at higher doses. Results indicate that gamma irradiation augmented increase in polyphenoloxidase activity is due to an activation of the enzyme rather than a *de novo* synthesis. Among the various monophenolic substrates tested, para cresol showed maximum reactivity and dopamine was the most reactive substrate among diphenols. A good correlation between the polyphenol oxidase activity and skin discoloration in irradiated banana fruits has been observed.

INTRODUCTION

GAMMA irradiation of preclimacteric bananas at optimum dose levels, which varied with the variety of banana, delays the ripening process.[1] However, irradiation of bananas at dose levels higher than the optimum results in radiation injury, as manifested by skin browning and blackening. The extent of browning is proportional to the dose absorbed. Doses above 50 krad produce appreciable radiation injury and fruits turn brown to black during storage.

The blackening is mainly due to oxidation of phenolic substances to melanins catalysed by polyphenoloxidase.* (E.C. 1.10.3.1 *O*-Diphenol:O_2 oxidoreductase). There are only very few reports on the polyphenoloxidase from banana. Griffith[2] reported dopamine as the substrate for banana polyphenoloxidase and later Buckley[3] demonstrated that banana skin contained as much as 1·0–1·2 mg dopamine/g fresh skin at harvest. Palmer[4] studied the isolation and properties of polyphenoloxidase from ripe 'Gros Michel' fruits and showed that the enzyme catalysed only catecholase activity. More recently Marsh and Deacon[5] reported the presence of an enzyme in banana which catalysed the conversion of tyramine to dopamine. Smith and Krishner[6] isolated an enzyme from banana which converts dopamine to arterenol.

The present work was initiated in order to examine whether radiation induced browning in bananas was associated with any changes in polyphenoloxidase activity.

* The terminology used in this paper: polyphenoloxidase, enzyme which catalyse the oxidation of both mono- (cresolase activity) and diphenols (catecholase activity).

[1] P. THOMAS, S. D. DHARKAR and A. SREENIVASAN, in press.
[2] L. A. GRIFFITH, *Nature* **184**, 58 (1959).
[3] E. H. BUCKLEY, in Proc. of a Symposium of the plant phenolic group of North America, United Fruit Company, Mass. U.S.A. 1964.
[4] J. K. PALMER, *Plant Physiol.* **38**, 508 (1963).
[5] H. V. MARSH, JR. and W. DEACON, *Plant Physiol.* Suppl. 42, Abstract S-16 (1967).
[6] W. J. SMITH and N. KRISHNER, *J. Biol. Chem.* **235**, 3589 (1960).

RESULTS AND DISCUSSION

Palmer[4] reported that, polyphenoloxidase from ripe 'Gros Michel' bananas did not show any cresolase activity. He used 1 per cent nonionic, polyoxyethylated detergents in his extraction medium to solubilize the enzyme from cellular particles. When his extraction procedure was used in the present studies, the enzyme preparations were devoid of cresolase activity. Contrary to the above report, extraction of soluble polyphenoloxidase with both cresolase and catecholase activities was accomplished by the modified procedure adopted in the present investigation. The results given in Table 1 show that the cresolase activity of the

TABLE 1. EFFECT OF VARIOUS METHODS OF ENZYME EXTRACTION ON THE POLYPHENOLOXIDASE ACTIVITY IN BANANA PULP

Mode of enzyme extraction*	μM of oxygen consumed/ mg protein/min	
	p-Cresol	Dopamine
Frozen pulp extracted with cold 0·01 M phosphate buffer, pH 7·0 containing 1% Tween-80 in a chilled pestle and mortar for 10 min and acetone fractionated as detailed under Experimental.	35·0	938·0
Frozen pulp extracted with cold 0·02 M phosphate buffer, pH 7·0 without Tween-80 as above.	Nil	865·0
Frozen pulp extracted with cold 0·02 M phosphate buffer, pH 7·0 containing 1% Tween-80 in a waring blender for 5 min and acetone fractionated.	11·4	990·0
Fresh pulp extracted with cold 0·02 M phosphate buffer, pH 7·0 containing 1% Tween-80 in a chilled pestle and mortar and acetone fractionated.	3·5	874·0
Acetone powder extracted with cold 0·02 M phosphate buffer pH 7·0 with 1% Tween and acetone fractionated.	Nil	695·0
Method 1 but fractionated twice with acetone.	17·0	1010·0

* From variety—Red, unirradiated.

TABLE 2. POLYPHENOLOXIDASE ACTIVITY IN RIPE BANANA FRUITS

Substrate used	Activity in μM of oxygen consumed/mg protein/min	
	Giant Cavendish	Red
p-Cresol	48	81
Tryamine	21	40
Chlorogenic acid	12	15
DL-Dopa	23	30
L-Dopa	38	26
Catechol	52	62
DL-Noradrenaline	653	577
Dopamine	1011	865

In these studies two varieties 'Giant Cavendish' and 'Red' were used. Pulp tissues from ripe banana fruits were sampled and quickly frozen in liquid nitrogen. Enzyme extraction and assay conditions were as described under Experimental.

banana polyphenoloxidase is extremely labile and strict adherence to the conditions of extraction was required to obtain activity. Enzyme preparations with maximum cresolase activity were obtained by freezing the tissues and subsequent thawing in 0·02 M potassium phosphate buffer, pH 7·0, containing 1% Tween-80. A 4 to 5 fold purification of the polyphenoloxidase activity from the original detergent extract was obtained by acetone fractionation. However, prolonged exposure to acetone or higher temperatures during preparation

resulted in losing significant amounts of cresolase activity. So, for the present studies, the enzyme was purified only up to the acetone step. Using this procedure, polyphenoloxidase with both cresolase and catecholase activities could be isolated also from ripe bananas (Table 2).

The partially purified enzyme lost most of its cresolase activity in 24 hr at room temp. (24–29°) and a significant amount was lost after a weeks storage at −30°. A complete loss in cresolase activity was observed after a month's storage at −30° (Table 3). As compared to the

TABLE 3. EFFECT OF TEMPERATURE AND STORAGE TIME ON BANANA POLYPHENOLOXIDASE ACTIVITY

Time after enzyme extraction	μM of oxygen consumed/mg protein/min	
	p-Cresol	Dopamine
Initial—immediately after extraction	36	1260
24 hr at 24–29°	Nil	1120
7 days at −30°	15·5	1168
30 days at −30°	Nil	1078

Enzyme extracted from Green (unripe) 'Giant Cavendish' banana pulp and stored under different condition as shown above. Standard assay conditions were used.

cresolase activity the catecholase activity was more stable and the enzyme retained most of the original activity even after a months storage at −30° or for 24 hr at room temperature.

Polyphenoloxidase Activity in Irradiated Bananas

The effect of gamma irradiation ranging from 0 to 500 krad on the polyphenoloxidase variety in three varieties of bananas is shown in Table 4. It is discernible that in all varieties gamma irradiation had a differential effect on the cresolase and catecholase activities. While the catecholase activity increased with increasing doses, the cresolase activity showed an increase up to 100 krad and decreased at higher doses. The increase in cresolase activity was maximum in the variety 'Red' followed by 'Giant Cavendish' and 'Poovan'. It is also interesting that enzyme activities differed considerably among the three varieties (Table 4).

TABLE 4. POLYPHENOLOXIDASE ACTIVITY IN THE PULP TISSUES OF DIFFERENT VARIETIES OF BANANAS AFTER EXPOSURE TO GAMMA IRRADIATION

Dose (krad)	μM of oxygen consumed/mg protein/min					
	Giant Cavendish		Red		Poovan	
	p-Cresol	Dopamine	*p*-Cresol	Dopamine	*p*-Cresol	Dopamine
0	14	1260	35	938	6	2188
30	27	1575	108	1260	8	2938
100	30	1380	124	1276	10	3855
200	20	1422	90	1169	—	—
500	11	1807	29	1318	—	—

Enzyme extracted from pulp tissues 24 hr after irradiation to the above doses. Enzyme extraction and assay procedures were standard.

Enzyme extracts from 'Red' bananas showed maximum cresolase activity and least catecholase activity where as enzyme extracts from 'Poovan' bananas had more catecholase and less cresolase activity.

A comparative study of the enzyme activity in pulp and skin tissues of bananas (Table 5) revealed that the cresolase activity in the pulp was considerably higher than skin—whereas skin showed more catecholase activity compared to pulp. Irradiation at doses of 100 and 500 krad considerably increased both these activities in skin and pulp. However, the maximum

TABLE 5. COMPARISON OF POLYPHENOLOXIDASE ACTIVITY IN PULP AND SKIN TISSUES OF 'RED' BANANA FRUIT

	μM of oxygen consumed/mg protein/min					
	Skin			Pulp		
Substrate	0 krad	100 krad	500 krad	0 krad	100 krad	500 krad
p-Cresol	1·8	13·2	12·3	35·0	124·0	29·0
Pyrocatechol	81·4	193·6	122·5	56·0	94·0	69·0
DL-Noradrenaline	1406·0	2858·0	1862·0	475·0	542·0	685·0
Dopamine	1493·0	4395·0	3529·0	938·0	1276·0	1318·0

Enzyme extracted from pulp and skin tissues of 'Red' bananas 24 hr after irradiation to the above doses. Enzyme extraction and assay procedures were standard.

increase in activation was in the skin. 100 krad gamma irradiation caused a 6 fold increase in the cresolase activity and 3 fold increase in catecholase activity in the skin. The same dose resulted in a 3·5 fold increase in cresolase and 0·5 fold increase in catecholase activity in the pulp.

Substrates Studies

Various monophenolic substrates viz., *O*-cresol, *p*-cresol, tyramine, L-tyrosine and DL-tyrosine were tested for their activity. Monophenols such L-tyrosine and DL-tyrosine showed very low activity and *O*-cresol was inactive as substrate. *p*-Cresol showed maximum reactivity among the monophenols tested, followed by tyramine. With enzyme preparations from control fruits oxidation of *p*-cresol exhibited a lag period from 40 to 50 sec followed by a rapid reaction. Irradiation up to 100 krad shortened this lag period and changed the rate of oxidation considerably. Oxidation of tyramine did not show a lag period to the same extent.

When the reaction mixtures with *p*-cresol and tyramine as substrates were tested for diphenolic products (Fig. 1) it was found that *p*-cresol was hydroxylated much faster than tyramine. This corroborates the earlier observation that among the monophenolic substrates the enzyme was more active towards *p*-cresol. The data also provides additional evidence to the fact that banana polyphenoloxidase could catalyse the oxidation of the monophenols to give the corresponding diphenols.

From a comparative study of the rate of oxidation of various diphenolic substrates it was observed that dopamine was the most reactive substrate followed by DL-noradrenaline. Catechol was less active compared to former ones, whereas L-dopa, DL-dopa or chlorogenic acid were least active. These observations are in agreement with that reported by Palmer[4] for polyphenoloxidase from ripe 'Gros Michel' bananas. The substrate specificity of the enzyme extracts from gamma irradiated and unirradiated fruits were identical in all the varieties.

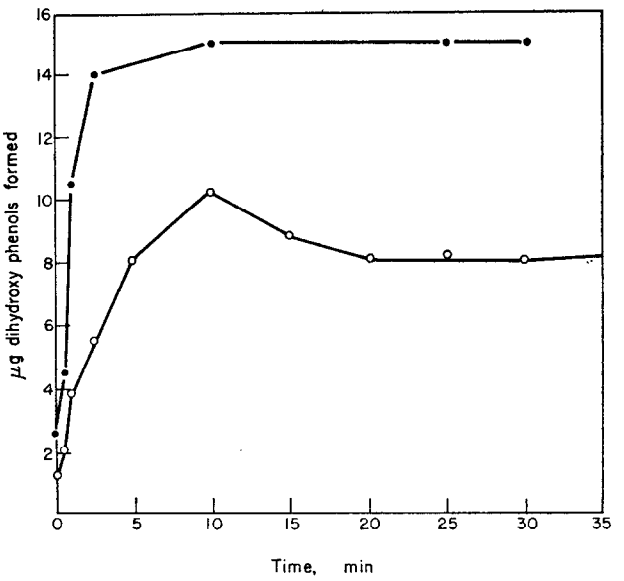

FIG. 1. THE RATE OF FORMATION OF ORTHODIHYDROXYPHENOLS FROM TYRAMINE AND *p*-CRESOL CATALYSED BY BANANA POLYPHENOLOXIDASE.

In this case the reaction was carried out employing a reaction mixture as described under Experimental. The reaction was stopped at different time intervals with 30% TCA and the diphenols formed were estimated colorimetrically. Data shows *p*-cresol was more reactive substrate then tyramine.

Similarly enzyme extracts from skin and pulp tissues did not show any change in their substrate specificities.

Effect of Time on the Increased Enzyme Activity after Irradiation

Increase in the enzyme activity just after irradiation and after different time intervals was studied. An increase in both cresolase and catecholase activities was observed just after irradiation and the activities did not show any significant increase as a function of time (Table 6). This sudden spurt observed in both the activities just after irradiation eliminated the possibility of a *de novo* synthesis of the enzyme protein as a result of irradiation. The data indicates that the increase in polyphenol oxidase activity on irradiation is due most probably to an activation of the enzyme rather than a *de novo* synthesis. This activation could be caused in many ways. It can be due to an increased extractability of the enzyme caused by irradiation damage of the cell wall in which the enzyme is known to be located.[4] Alternatively, it can be due to an activation of a pro-enzyme[7] or latent enzyme[8–10] or due to some changes caused at the active center. It has been shown that latent polyphenoloxidases are activated in consequence of fungus infections in beans[8] and rice leaves.[9] According to Robb *et al.*,[10] the prosthetic group of the 'latent' tyrosinase of broad bean is masked by virtue of its tertiary structure. Alternation of this structure by denaturing agents results in an active configuration of similar molecular weight. Whether these mechanisms have some

[7] J. B. FOX, J. B. BURNETT and S. FUCHS, *Ann. N.Y. Acad. Sci.* **100**, 840 (1962).
[8] B. J. DEVERALL, *Nature* **184**, 311 (1961).
[9] M. NAKAMURA and H. OKU, *Ann. Takamine Lab.* **12**, 266 (1960).
[10] D. A. ROBB, L. W. MAPSON and T. SWAIN, *Nature* **201**, 503 (1964).

role in the activation of polyphenoloxidase in irradiated banana fruits remains to be clarified. The result on the effect of different doses of irradiation on these activities (Table 4), which invariably show a differential effect, i.e. a decreased cresolase activity at higher doses indicates that increased extractability may not be the cause for activation.

TABLE 6. POLYPHENOLOXIDASE ACTIVITY IN 'RED' BANANA PULP ASSAYED AT DIFFERENT TIME INTERVALS AFTER IRRADIATION TO 100 krad

Time after irradiation	μM of oxygen consumed/mg protein/min			
	p-Cresol	Tryamine	Pyrocatechol	Dopamine
Control	49·0	28·5	54·0	855·0
0 hr	197·0	82·0	108·0	962·4
1 hr	165·0	63·0	102·5	902·4
5 hr	180·0	72·0	110·0	1010·0
24 hr	168·0	82·0	103·5	949·0
48 hr	162·0	82·0	102·0	1135·0

In these studies fruits were irradiated to 100 krad and incubated at 25° for different time intervals as above. Diced samples were frozen in liquid nitrogen soon after the incubation period. Enzyme extraction and assay procedures were as described under Experimental.

Table 7 shows the results of irradiation of isolated enzyme extracts. Though some increase in the cresolase activity in the detergent extract was observable, the magnitude of this increase was considerably less as compared to enzyme preparations from fruits of the same variety irradiated to same dose levels. Irradiation of the purified enzyme resulted in some inactivation of the cresolase activity. It is likely that some alterations at the enzyme active center occurred at higher doses, which is responsible for the decrease in cresolase activity.

There is standing controversy over the mechanistic view of polyphenoloxidase. At present it is agreed that these two activities are catalysed by one or many copper proteins in unison.[11] Copper and protein play vital role in the formation of enzyme–oxygen complex, which in

TABLE 7. EFFECT OF GAMMA IRRADIATION ON ISOLATED ENZYME EXTRACTS

Doses in krad	μM of oxygen consumed/mg protein/min			
	Crude extract		Purified extract	
	p-Cresol	Dopamine	*p*-Cresol	Dopamine
0	10	180	43	1011
30	11	175	37	1009
100	16	203	32	961
200	15	161	33	938
500	9	157	25	991

Enzyme extracts were prepared from pulp tissues of unirradiated 'Red' banana fruits. Both detergent extracts and partially purified (after acetone fractionation) enzyme were irradiated to different doses at 0° by keeping the enzyme extracts in ice, during irradiation.

[11] H. S. MASON, *Ann. Rev. Biochem.* **34**, 595 (1965).

turn hydroxylate and dehydrogenate the substrate species. The effect of irradiation in increasing the cresolase activity, may be a useful tool in solving some of the mysteries in the mechanism of polyphenoloxidase. Further work on this line is in progress.

A comparison of the cresolase and catecholase activities from skin and pulp tissues suggest that monophenolic substrates are hydroxylated at a faster rate in the pulp tissues and in all possibility translocated to the skin where they are readily oxidized by catecholase. It is known that irradiation affects the permeability of cell membranes[12,13] such that enzymes and substrates are brought into more intimate contact thus accelerating or even initiating reactions. It is quite conceivable that the increased polyphenoloxidase activity and the availability of substrates like dopamine[3] and arterenol[14] due to changes in cell permeability upon irradiation might contribute to radiation induced skin browning in bananas at higher doses.

EXPERIMENTAL

Banana (*Musa sapientum* L.) fruits of 75 per cent maturity[15] were used. Irradiation was carried out in a Gamma Cell 220 (Atomic Energy of Canada Ltd., 3991 c) in air at ambient temp. (25°) at a dose rate of 2·76 krad/min. Fruits were irradiated to 30, 100, 200 and 500 krad. Three varieties viz., 'Giant Cavendish', 'Red' and 'Poovan' ('Fill Basket') were selected for these studies.

Purification of the Enzyme

Banana fruits were incubated for 24 hr at 25° after they were irradiated to the appropriate doses. Skin and pulp tissues were separated and the diced tisues were immediately frozen in liquid nitrogen and stored at −30°. 10 g of the frozen tissue was thoroughly powdered in a pre-chilled (at −30°) porcelain pestle and mortar and extracted with 20 ml of cold 0·02 M K phosphate buffer, pH 7·0, containing 1% Tween-80. The mixture was ground for 10 min and the homogenate was passed through 2 layers of cheese cloth and centrifuged at 0° at 20,000 *g* for 15 min. To the clear supernatant 1·5 vol. of chilled acetone (−30°) was added and the precipitated material was collected at 20,000 *g* for 10 min. The precipitate was resuspended in 5–7 ml of cold 0·02 M K phosphate buffer, pH 7·0, without Tween-80 and homogenized in a Teflon tissue homogenizer for 2–3 min. The homogenate was further centrifuged for 10 min at 20,000 *g* and the supernatant was assayed for enzyme activity. Most of the polyphenol oxidase activity was in the supernatant. All operations were carried out at 0–3°.

Assay of Enzyme Activity

Enzyme activities were determined by measurement of O_2 consumption with various mono- and diphenols as substrates using a Teflon-covered Beckman oxygen electrode fitted into one side of a water-jacketed (25°) 3·0 ml cell equipped with a magnetic stirrer. The reaction was started by adding 0·5 mM of substrate to the reaction cell filled with air saturated K phosphate buffer, pH 7·0, containing an appropriate quantity of the enzyme (about 700 μg), in a final volume of 3·0 ml. The enzyme activity was calculated from the initial slope of the curve in the case of catecholase and from the slope after the end of lag period in the case of cresolase.

The ortho-dihydroxyphenols formed from *p*-cresol and tyramine as substrates were determined as follows. To a reaction mixture consisting of 0·95 ml air saturated, 0·02 M K phosphate buffer, pH 7·0 and 0·15 ml of enzyme solution (approximately 200–300 μg protein), 0·6 mM of *p*-cresol or tyramine was added. It was incubated at 25° for different time intervals, then trichloroacetic acid (0·25 ml of a 30%, w/v solution) added and the precipitate removed by centrifugation. An aliquot (1 ml) of the supernatant solution was used for the estimation of dihydroxyphenols formed by the procedure of Nair and Vaidyanathan.[16] The protein was estimated by Biurett[17] method.

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[12] J. P. Skon, *Physiol. Plantarum* **16**, 423 (1962).
[13] A. Forssberg, in *Advances in Radiobiology* (edited by C. G. Augenstein, R. Mason and H. Quastler), p. 117, Academic Press, New York (1964).
[14] T. P. Waalkers, A. Sjoerdsma, C. R. Creveling, H. Weissbach and S. Udenfriend, *Science* **127**, 648 (1958).
[15] P. Thomas, Ph.D. Thesis, Madras University, India (1969).
[16] P. M. Nair and C. S. Vaidyanathan, *Anal. Biochem.* **7**, 315 (1964).
[17] A. G. Gornell, C. J. Bardawell and M. M. Davis, *J. Biol. Chem.* **177**, 751 (1949).