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COCONUT PEROXIDASE ISOENZYMES: ISOLATION, PARTIAL PURIFICATION AND PHYSICOCHEMICAL PROPERTIES*

CESAR V. MUJER, EVELYN MAE T. MENDOZA and DOLORES A. RAMIREZ

Biochemistry and Genetics Laboratories, Institute of Plant Breeding, University of the Philippines at Los Baños College, Laguna, Philippines 3720

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Key Word Index—*Cocos nucifera*; Palmae; coconut; peroxidases; enzyme purification; IAA oxidase; mutant endosperm.

Abstract—Peroxidases (donor: hydrogen peroxide oxidoreductase; EC 1.11.1.7) from normal and mutant (makapuno) coconut endosperms were compared in terms of their specific activities and isoenzyme patterns, physicochemical properties and ability to oxidize IAA. Makapuno peroxidases have significantly higher activity during the early stages of development and significantly lower activity at maturity when compared with the normal. Six anodic isoenzymes were detected at pH 8.9 from both sources by polyacrylamide gel electrophoresis. Both normal and makapuno crude peroxidases have optimum activity at pH 5.5 and are thermostable over a wide range of temperature. The V_{max} was obtained at 1×10^{-2} M hydrogen peroxide while the apparent K_m was at *ca* 1×10^{-3} M hydrogen peroxide. In general, no marked difference was noted between the physicochemical properties of peroxidases from both sources. Isolation and partial purification of the isoenzymes were performed by conventional methods including ammonium sulfate fractionation, DEAE Sephadex A-50 ion exchange chromatography and Sephadex G-200 gel filtration. Peroxidase activity was localized in the 50–95% ammonium sulfate precipitate. Gel filtration resolved three protein peaks with peroxidase (Px) activity, the electrophoretic mobilities of which corresponded to Px3, Px4 and Px5. Px5 was purified 17 600-fold and found to be a tetramer with a native MW of 196 000 and a subunit MW of 55 000. Px3 and Px4 were monomers with MWs of 36 300 and 46 800, respectively. IAA oxidase activity was detected in the partially purified peroxidases from developing normal endosperms but not from mature endosperms.

INTRODUCTION

Peroxidases are widely distributed among higher plants and isoenzymes of peroxidase are known to occur in a variety of tissues in several species [1]. One of the important roles attributed to this enzyme is its ability to oxidize the plant growth hormone, indole-3-acetic acid (IAA) [2]. This ability has been demonstrated *in vitro* [3–5] and peroxidase may also function similarly *in vivo* [6]. In addition, peroxidase has also been implicated in the biosynthesis of IAA [7]. Since IAA level was shown to effect the trigger for tumorous growth in certain *Nicotiana* hybrids [8, 9], a study of peroxidases in coconut endosperms may yet provide clues on the regulation of IAA metabolism in makapuno. We, therefore, developed a purification scheme for coconut peroxidases in order to test for their possible IAA oxidase activity.

The makapuno endosperm (Fig. 1) results from continued cell proliferation and uncontrolled growth, characteristic of neoplastic tissues or tumors [10]. Early studies on the inheritance of this trait shows that it is controlled by a single Mendelian recessive factor [11]. The monohybrid segregation of nuts from self-pollinated and from intercrossed makapuno-bearing trees (Mm) indicates that the normal and makapuno endosperms differ in only one gene [12]. However, the three types of makapuno [13] and its different nuclear behavior when compared with the normal [14] indicate that this character may have a more complex genetic behavior.

Biochemical studies to elucidate the genetically controlled apparatus responsible for the expression of this trait has been barely started. Early investigations in this laboratory [unpublished] dealt mostly with the comparison of isoenzyme patterns with the hope of detecting possible alteration of gene products in the course of development. More recently, the enzyme systems involved in IAA metabolism were studied. It may be possible that a misregulation in IAA biosynthesis and/or degradation leads to an altered state, phenotypically visible as makapuno.

This paper reports on the ontogeny of peroxidase activity and isoenzyme patterns, the scheme for peroxidase isolation and purification, some properties of peroxidases and the determination of IAA oxidase activity of the purified enzyme.

RESULTS AND DISCUSSION

Statistical analysis of peroxidase activity between the normal and makapuno endosperms showed that at stages II and IV the activity in makapuno was significantly higher than in the normal (Table 1). However, at stage VI the activity was lower in the makapuno. No marked difference was observed at stages III and V between the two endosperms.

Among the normal endosperms, peroxidase activity was significantly greater at stage VI as compared to all the other stages of its development; no significant difference

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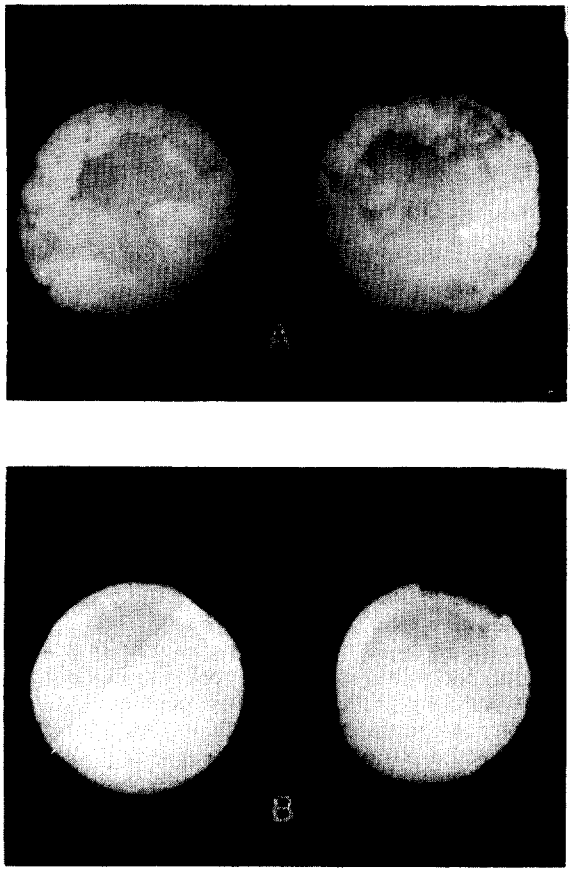

Fig. 1. Mature endosperms of makapuno (A) and normal (B) coconut (11–12-months-old).

in stages II–V. In contrast, peroxidase activity among the makapuno endosperms was significantly greater at stage II as compared to stages V and VI; and at stage IV as compared to stages III, V and VI. There was no significant difference between stages II and IV and among stages III, V and VI.

The difference in activity in the early and late stages of development between the two endosperms may be related to the genetically controlled tumorigenesis in makapuno. The enhanced peroxidase activity in some stages of development is a characteristic alteration in mutants of several species. Dwarf mutants of tomato [15], peas [16], corn and sorghum [17] exhibited greater activity than the normal controls in peroxidase and other enzymes. Gupta and Stebbins [18] compared peroxidase activity in the hooded (mutant) and awned genotype of barley at successive stages of development and found a higher activity at most stages in the hooded variety.

Polyacrylamide gel electrophoresis (PAGE) resolved several isoenzymes in all stages of growth. The crude extract from the fresh samples contained five anodic isoenzymes at stage I, designated as Px1, Px2, Px3, Px4 and Px5 with R_f values of 0.75, 0.72, 0.31, 0.28 and 0,20, respectively. However, Px1 and Px2 could not be resolved after a few days of freezing at $-10°$. It is possible that they are cold labile. Stages II–VI have no Px1 and Px2, and contained only Px3, Px4 and Px5.

Except at stages I and IV, the crude extract from both lyophilized normal and makapuno endosperms contained an additional isoenzyme, designated as Px6, with an R_f value of 0.14. In general, there was no marked difference in the number of isoenzymes between the normal and makapuno endosperms ontogenetically as previously reported [19].

Purification

Peroxidase activity was detected in the 50–95% ammonium sulfate cut. Above 95% saturation, a minimal amount of activity was detected in some samples. A summary of the procedures used in the extraction and partial purification of peroxidase in the normal and makapuno endosperms is shown in Table 2.

Gel filtration on Sephadex G-100 resolved two peaks of peroxidase activity. The first major peak came out at the void volume indicating a high MW of *ca* 200000. However, three peaks of peroxidase activity were separated after gel filtration on Sephadex G-200. Peak 1, which corresponded to Px5 after PAGE, has a MW of *ca*

Table 1. Peroxidase activity of coconut endosperms at various stages of development

Stage†	Age‡(months)	Specific activity (units/mg protein)* Normal§	Makapuno	Genotype × age‖
II	7–8	0.098^{c}	0.50^{ab}	s
III	8–9	0.27^{b}	0.26^{bc}	ns
IV	9–10	0.12^{b}	0.79^{a}	s
V	10–11	0.18^{b}	0.082^{c}	ns
VI	11–12	0.67^{a}	0.13^{c}	s

*Average of three replications.

†Stage I was omitted because the makapuno endosperms could not be distinguished from the normal at this age.

‡Age is given in months after pollination.

§Means followed by the same letter are not significantly different from each other at the 0.05 level of significance (Duncan's Multiple Range Test).

‖s, Significant; ns, not significant at 0.05 level of significance (DMRT).

Table 2. Purification of makapuno and normal endosperm peroxidase from 11–12-month-old coconut

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Purification factor	Recovery (%)
Normal					
Crude total extract	1770	531	3.3	1	100
50–95% $(NH_4)_2SO_4$ cut	1490	150	9.9	3	84
Sephadex G-200 gel filtration	780	16.1	48.4	14.6	44
Makapuno					
Crude total extract	376	1150	0.32	1	100
50–95% $(NH_4)_2SO_4$ cut	273	180.2	1.5	4.7	73
Sephadex G-200 gel filtration	151	36.8	4.1	12.8	40

196 000, as compared to peak 2 and peak 3 which have MWs *ca* 46 800 and 36 300, respectively. The last two peaks corresponded to Px4 and Px3, respectively. Three zones of activity were resolved when all the peaks were pooled and subjected to PAGE. When run separately, Px5 migrated anodally as a single band (R_f 0.21), whereas Px3 and Px4 migrated as two separate bands with R_f values of 0.30 and 0.28, respectively. After gel filtration, peroxidases from the normal and makapuno endosperms were purified up to 14.6- and 12.8-fold, respectively. No appreciable difference in their elution patterns were noted.

The pooled active fractions of Px5 from Sephadex G-200 were purified up to 182-fold after ion exchange chromatography. However, the small amount of enzyme protein obtained after this step was not enough for its further characterization. Another procedure was developed by reversing the chromatographic steps that were used in the process of purification (Table 3). Peroxidases were purified up to 320-fold after running them first on a DEAE-Sephadex A50 ion exchange column. The major peak was eluted at 0.03 M sodium chloride and contained Px5 as resolved by PAGE. Several other protein bands were still present in this peak. Px3 and Px4 were eluted as a separate minor peak at 0.045 M sodium chloride. After ion exchange chromatography, Px5 was applied on a Sephadex G-200 column and its elution pattern is shown in Fig. 2. The isoenzyme was purified 17 602-fold. PAGE of the pooled active fractions resolved three protein bands which coincided with peroxidase activity. The first major band corresponded to Px5, whereas the other two fast moving bands corresponded to Px1 and Px2. The presence of these two fast moving bands was not expected because they could not be detected from the crude extract, except at stage I of endosperm growth. SDS-PAGE of purified Px5 resolved only one protein band with a MW of *ca* 55 000. Px5, therefore, could be a tetramer with subunits of identical MW. Previous reports indicated only a monomer [20–23] or dimer [24] structure for peroxidase. The physicochemical properties of Px5 will be reported elsewhere in succeeding papers.

Catalytic properties

Two pH optima were obtained for the peroxidases from both crude extracts of mature normal and makapuno endosperms; one at pH 4.5 and another at pH 5.5, with the activity at pH 5.5 several times greater than that at pH 4.5. At pH 5.5, the enzyme activity was found to be independent of the buffer concentration between 0.1 and 0.5 M KPi buffer. The presence of two pH optima suggests the heterogeneity of coconut peroxidases or the existence of isoenzymes as shown above. On further purification, the pH optimum shifted to 5.0 for Px3, Px4 and Px5. Similar pH optima were previously reported from other plant sources [25, 26]. The acidic pH optimum of 4.5 was similar to that reported for atypical peroxidase. Beaudreau and Yasunobu [21] reported a pH optimum of 4.2 for pineapple peroxidase B which is an atypical peroxidase.

A continuous increase in enzyme activity was obtained even at assay temperatures above 70°. This thermal stability of peroxidase was confirmed by incubating the enzyme in a boiling water bath at different time intervals (Table 4). After 2 min of heating, more than 50% of the activity was retained. The enzyme was thermally inactivated in both normal and makapuno only after 20 and 40 min of incubation, respectively. The heat stability exhibited by coconut peroxidases suggests the probable presence of a large number of cysteine residues in the polypeptide chains. Peroxidase was reported to be one of

Table 3. Purification of Px5 from 11–12-month-old normal endosperm

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Purification factor	Recovery (%)
Total crude extract	409	2990	0.14	1	100
50–95% $(NH_4)_2SO_4$ cut	228	1170	0.20	1.4	56
DEAE-Sephadex A50	113	2.5	44.8	320	27.6
Sephadex G-200 gel filtration	83	0.034	2460	17 600	20

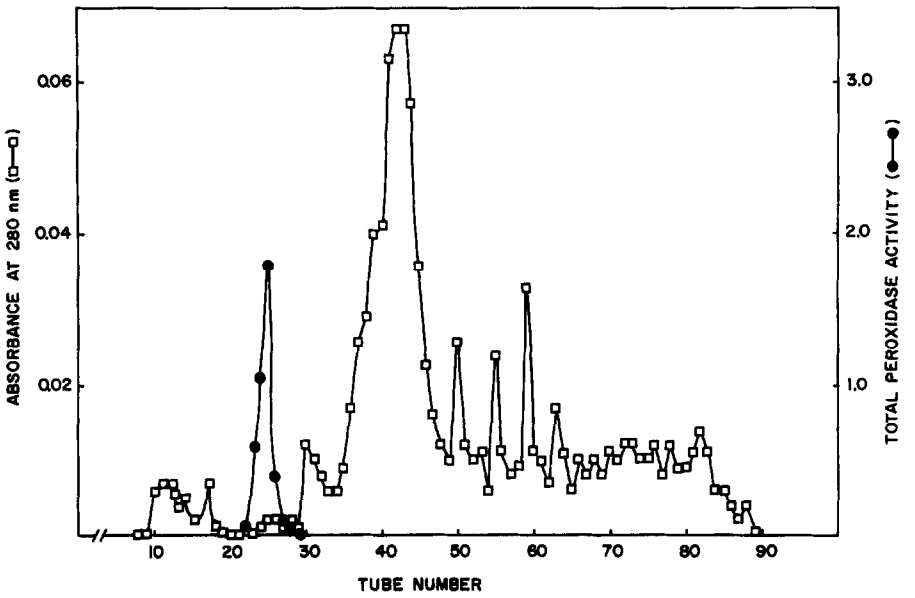

Fig. 2. Protein elution pattern and peroxidase activity obtained after final chromatography of Px5 on Sephadex G-200.

Table 4. Thermal stability of peroxidase

Heating time (min)	Normal		Makapuno	
	Total activity	Activity retained (%)	Total activity	Activity retained (%)
0	0.74	—	0.40	—
1	0.70	95	0.28	70
2	0.43	58	0.21	52
5	0.17	23	0.11	28
10	0.12	16	0.06	15
15	0.09	12	0.06	15
20	0	0	0.02	5
40	0	0	0	0

1 ml of the enzyme solution was incubated in a boiling water bath at the times shown. The solution was allowed to cool for 10 min at room temp. (28°) before it was assayed.

the most heat stable enzymes in plants [27, 28]. Farkas *et al.* [29] reported that 6 min at 121° was needed to inactivate peroxidase in green peas. Heat treatments of less severity have resulted in reactivation of the enzyme upon storage at various intervals.

The effect of hydrogen peroxide concentration on peroxidase activity is shown in Fig. 3. The maximum velocity was obtained at 1×10^{-2} M hydrogen peroxide while the apparent K_m was at *ca* 1×10^{-3} M hydrogen peroxide. Both normal and makapuno peroxidases exhibited sigmoidal curves perhaps due to Px5, which is multimeric, and the major peroxidase.

IAA oxidase activity

The partially purified peroxidases from mature endosperms of both makapuno and normal coconut have no

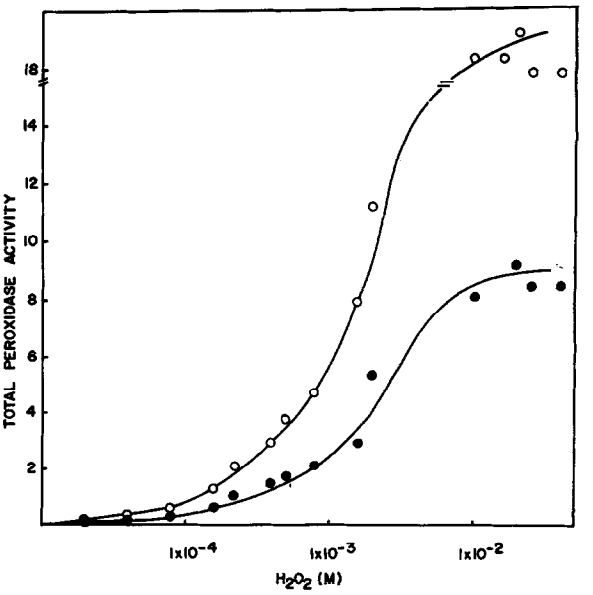

Fig. 3. Effect of hydrogen peroxide concentration on peroxidase activity. (●) Makapuno; (○) normal.

detectable IAA oxidase activity. However, high activity was detected in the Sephadex G-200 pooled fractions of Px3, Px4 and Px5 of developing normal endosperms (stage I). Px5 and the pooled Px3 and Px4 catalysed the decomposition of IAA at 0.113 and 0.32 mM, respectively. The difficulty in distinguishing normal from makapuno at this early age limited our enzyme activity detection only among the normal endosperms. The detection of high IAA oxidase activity from the peroxidases of very young endosperms (stage I) suggests its possible role in the metabolism of IAA at this age. Thus, the significantly higher peroxidase activity among makapuno at stages II

and IV possibly indicates an altered hormone metabolism at these ages. Tumorigenesis has been reported to be associated with the metabolism of IAA in certain plant systems. It was shown that its onset is triggered by a low level of this hormone [8, 9]. The transformation of IAA to another intermediate, methylene oxindole, is catalysed by an IAA oxidase, the enzymatic activity of which was reported to be due to a peroxidase [3, 21].

EXPERIMENTAL

Endosperms. Makapuno (mmm) and normal (M—) coconut endosperms at five stages of development were collected from embryo-cultured makapuno trees (*Cocos nucifera* L. var. laguna) at the Davao Research Center, Philippine Coconut Authority, Davao City. Normal (MMM) coconut endosperms were obtained from true-breeding normal trees. 1–3 nuts from three makapuno and two normal trees were collected at each stage of development. The endosperms were frozen, lyophilized and stored at −10° until use.

Enzyme extraction. Freeze-dried coconut endosperm (20 g) was homogenized twice with 100 ml cold Me_2CO in a prechilled Waring blender. 8 g of defatted sample was homogenized with 320 ml 0.05 M KPi buffer, pH 6. The slurry was centrifuged at 10000 *g* for 20 min at 4° and the supernatant was filtered through eight layers of cheesecloth. The crude extract was stored at −10° for several months without any appreciable loss of enzyme activity. Further purification steps were conducted at 4° unless specified otherwise.

$(NH_4)_2SO_4$ *precipitation.* The crude peroxidase extract was brought to 50% satn by adding solid $(NH_4)_2SO_4$. After standing for 2 hr the soln was centrifuged at 10000 *g* for 20 min and the inactive ppt was discarded. The supernatant was brought from 50% to 95% satn and centrifuged as described above after standing 18 hr. The ppt was dissolved in 4 ml 0.05 M KPi buffer, pH 6, and dialysed twice against 4 l. of the same buffer for 24 hr. A small amount of inactive ppt was removed by centrifugation.

Sephadex G-200 gel filtration. 2ml of the concd $(NH_4)_2SO_4$ fraction from normal endosperm containing 150 mg of protein was applied to a column (3 × 100 cm) of Sephadex G-200 (Pharmacia) equilibrated with 0.05 M KPi buffer, pH 6. In a separate run, 3 ml of the enzyme from makapuno endosperm containing 180 mg of protein was also loaded on the same column. The proteins were eluted with the same buffer at a flow rate of 11.4 ml/hr. MW was estimated by using appropriate standards.

DEAE-Sephadex chromatography. The active fractions from Sephadex G-200 gel filtration were pooled, dialysed overnight against 0.05 M Tris buffer, pH 9, and concd by lyophilization. The major peak (Px5), obtained after gel filtration, was subjected to ion exchange chromatography on a column (2.2 × 45 cm) of DEAE-Sephadex A-50 equilibrated with 0.05 M Tris buffer, pH 9. Likewise, Px5 from makapuno endosperm was loaded separately into another column equilibrated under similar conditions. The enzyme was eluted with a linear gradient of from 0 to 0.2 M NaCl in the same buffer at a flow rate of 24.2 ml/hr.

PAGE. Electrophoresis was performed on 7% polyacrylamide gel (10 × 0.5 cm i.d.) at 4° and at a current of 4 mA/gel cylinder as described in ref. [30]. Peroxidase bands were detected by immersing the gels in a soln of KPi buffer, pH 5.5, containing 0.1% H_2O_2 and 0.016% *ortho*-dianisidine. The gels in the reaction mixtures were immersed in H_2O at 100° for 2 min to hasten the color development. Protein band patterns were visualized by incubating the gels with 1% amido black in 7% HOAc for 30 min and destaining in 7% HOAc for 18 hr.

SDS-PAGE of peroxidase was done as described in ref. [31]. MW was determined by using appropriate standards.

Peroxidase assay. Peroxidase activity was determined by following the procedure described in ref. [32] but with some modifications. 3 ml with 0.1% H_2O_2 in 0.1 M KPi buffer, pH 5.5, and 50 μl with 1% *ortho*-dianisidine in MeOH were introduced in a test cuvette. The reaction was started by adding 50 μl of the enzyme. The change in *A* per min at 460 nm was recorded. 1 unit of activity is defined as the amount of enzyme decomposing 1 μ mol H_2O_2 per min at 30°. Sp. act. is defined as unit activity per mg total protein. Total protein was estimated either by the method ref. [33] with BSA as standard or by *A* at 280 nm.

IAA oxidase assay. To each tube containing 0.5 ml of a freshly prepared soln of IAA, 2,4-dichlorophenol and $MnCl_2$ at a final concn of 0.15, 0.1 and 0.05 mM, respectively, and 100 μl of the enzyme soln were added. The tubes were incubated in a shaking water bath at 37° for 1 hr, after which 5 ml of modified Salkowski reagent was added. The *A* at 530 nm was read after 10 min [34]. 1 unit of enzyme activity is expressed as mmol of standard IAA destroyed per ml of the enzyme soln.

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