Quantitative Changes in Polyphenol Oxidase and Protein during *Theobroma cacao* Seed Development[1]

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ABSTRACT

Cacao trees, PSU-3 and PSU-20, were grown under greenhouse conditions and fruited green-colored and red-colored pods, respectively. Amounts of cocoa bean polyphenol oxidase (PPO) increased during the development of seeds in both. PPO activity of seeds from PSU-3 pods increased 60.7-fold from 81 days after pollination (DAP) to 163 DAP. Seeds from PSU-20 pods increased 44.0-fold from 120 to 175 DAP. Different seed colors from red pods were also examined for PPO activity. PPO activity of light puple-colored seeds (1.59×10^4 units/g seed) was similar to dark purple-colored seeds (1.56×10^4 units/g seed) at 144 DAP. Protein concentrations increased 30.2-fold from 81 to 163 DAP for seeds from PSU-3 but remained about the same for seeds from PSU-20 during seed development. Furthermore, protein concentration of light purple-colored seeds was greater than dark puple-colored seeds by 2.8-fold at 144 DAP.

COMPENDIO

Arboles de cacao, PSU-3 y PSU-20 cultivado en un invernadero, produjeron mazorcas verdes y rojas, respectivamente. Las cantidades del polifenol oxidasa (PPO) aumentaron durante la madurez de las semillas en ambos. La actividad de polifenol oxidasa de semillas de PSU-3 aumentó 60.7 veces desde 81 días después de la polinización hasta 163 días. El polifenol oxidasa de las semillas de PSU-20 aumentó 44.0 veces desde 120 hasta 175 días. Semillas de diferentes colores de mazorcas rojas también fueron analizadas para la actividad de polifenol oxidasa. La actividad en semillas color violeta claras (1.59×10^4 unidades/grano de semilla) de 144 días fue similar a la actividad de las semillas violeta oscuras de 144 días (1.56×10^4 unidades/grano de semilla). Las concentraciones de proteína aumentaron 30.2 veces desde 81 hasta 163 días en las semillas de PSU-3, pero quedaron al mismo nivel en las semillas de PSU-20 durante su madurez. Además, las concentraciones de proteína en las semillas violeta claras fueron 2.8 veces más altas que en las semillas violeta oscuras de 144 días después de la polinización.

INTRODUCTION

Pod color normally is a measure of maturation and ripening of cacao pods. The number of days to maturity varies and is within the range of 140-180 DAP. Green or dark red-purple colors can change to yellow, orange, or red depending on variety. Within cacao seeds, however, many changes can occur during maturation and ripening. As the seeds enlarge, they fill with a liquid endosperm (5). The liquid endosperm starts to harden by 110 to 120 DAP and continues to harden until the entire seed solidifies. During the enlargement phase, pigment (anthocyanin content) changes from white to dark purple in certain varieties (5, 9). Furthermore, lipid content increases dramatically during ripening (5). Although caffeine accumulates slowly until the later stages of development. Wright *et al.* (9) observed that the alkaloids, theobromine and caffeine, increase after an initial lag period. Fritz *et al.* (3) found that protein content increases in the soluble fraction from 112-151 DAP before leveling-off at 166 DAP. In contrast, microsomal protein concentration decreases with seed development. During the final days of pod ripening, seed protein concentration decreases (10) and there is a build-up of sugars in the pulp, which show an increase in acidity, tannins, and carbohydrates (6).

The use of greenhouses to grow cacao trees increases the availability of samples in non-growing regions while affording the advantage of environmental control. Due to self-incompatibility among cacao trees, cross-pollinations are often performed. Hence, the resulting hybrid fruit pods vary in color, shape, and size. Within any one pod, seed color may range from white to dark purple. Different degrees of pigmentation indicate different levels of polyphenols which may influence PPO activity, and thus, chocolate flavor. Furthermore, Criollo cacao seeds, which are white or pale pink in color and possess the finer flavor, have been compared to the varied purple colors of the bulk-grade Forastero cacao, although a

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link between seed color and flavor has yet to be proven. The purpose of this study was to determine the PPO activity in two cultivars in order to relate the amount of enzyme with the development of the cacao seeds and cacao proteins and perhaps infer the importance of PPO to the time of harvest of pods and chocolate flavor development.

MATERIALS AND METHODS

Samples

Hand pollinations of flowering cocoa trees (*Theobroma cacao* L.) were carried out in greenhouses at The Pennsylvania State University. This study involved trees which were PSU-3 and PSU-20 derived from seeds of open pollination EXQ-100 and UF-667, respectively, collected in the field at the USDA Agricultural Station, Mayaguez, Puerto Rico. They were given PSU (Pennsylvania State University) numbers because they are unique clones. PSU-3 and PSU-20 fruited green and red-colored pods, respectively. Numerous flowers were used in pollination in order to maximize the number of fruit and, thus, samples for extraction and assay. During pod development, pod length was monitored montly. After harvesting, pods were weighed and measured; and the seeds were removed and the testa and adhering pulp peeled off. Dark purple seeds were separated from light purple seeds. The seeds were quickly frozen in liquid nitrogen and stored at −30°C.

Extraction procedure

Initial extraction. Crude PPO extract (Fig. 1) was prepared by grinding 10 g of frozen cocoa in liquid nitrogen with a Janke and Kunkel model A10 S1 mill (3 x 15 sec bursts). Portions of 1 to 2 g were mixed with pH 8.0 extraction buffer (10 ml buffer/g seed) containing 350 mM mannitol, 40 mM Tris-HCl, 5 mM ethylenediamine-tetraacetate (EDTA), 15 mM β-mercaptoethanol (β-ME), 2% polyvinylpyrrolidone (PVP)-10 000, and 100 mM diethyldithiocarbamate (DIECA). Phenylmethylsulfonyl fluoride (PMSF) and leupeptin were delivered to each extract resulting in final concentrations of 1 742 mg PMSF/g seed and 0 100 mg leupeptin/g seed. Initial extracts were centrifuged for 20 min at 12 000 x g at 4°C and the supernatants were used for PPO assay.

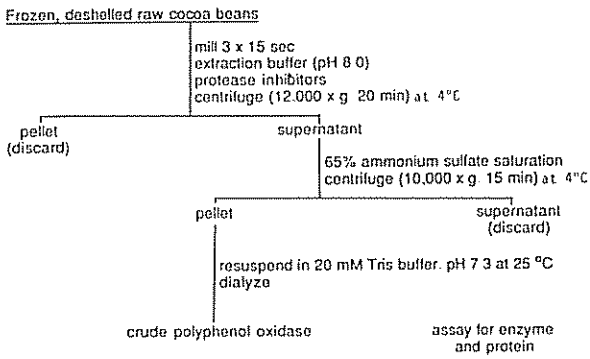

Fig. 1. Protocol for extraction of crude polyphenol oxidase from raw cocoa beans.

Preparation of crude PPO. The supernatant volumes from the initial extracts were measured and saturated to 65% with ammonium sulfate (430 g/l). The solutions were cooled on ice for 15 min before centrifuging 15 min at 10 000 x g. The pellets were resuspended in a minimum volume of 20 mM Tris, pH 7.3 at 25°C (Buffer A) for dialysis (3 500 MW cutoff, Bio-Rad, Richmond, CA) against Buffer A for 2 x 1 h. Retentate volumes were measured and then assayed for enzyme activity and protein concentration.

Enzyme assay

Assays for PPO were performed using Kim's (4) modification of procedures described in the Worthington Enzyme Manual (1) and by McCord and Kilara (7). The determination is based on the PPO-catalyzed oxidation of (−)-epicatechin to o-quinone which causes an increase in absorbance at 400 nm. Two ml 0.5M KH_2PO_4, pH 6.5 were mixed with 0.5 ml 0.001 M (−) epicatechin and 50 μl 0.005 M $CuSO_4$ in a glass cuvette and oxygenated for 5 min. After addition of sample, the cuvette was inverted to mix and then inserted into the spectrophotometer. Change in absorbance was calculated from the exponential portion of the curve. A Gilford (Oberlin, OH) Response UV-Vis spectrophotometer and printer was used to monitor change in absorbance.

Protein assay

The Bradford (2) method was used for protein determinations. Standard curves were made each day of a protein assay from samples of standard BSA at concentrations of 5, 10, and 15 μg/ml.

RESULTS AND DISCUSSION

Investigations of seed proteins from higher plants have been performed in order to understand the mechanisms which underly gene expression. In particular, significant results have been shown by quantitating protein during seed development (3). In this study, a contrast in soluble and microsomal protein concentrations was demonstrated. Soluble proteins

increased during development of cacao seeds then leveled off with the onset of maturity, whereas microsomal protein concentrations decreased with seed development. In the present study, quantitative protein analysis of the crude PPO extract represented microsomal and soluble protein contents combined. Protein content was measured in order to express specific activity of PPO.

Based on daily observations, green pod development appeared more advanced than red pod development. Green pods were noticeably larger than red pods of the same age (Table 1) with a commensurate increase in the size and number of seeds. Based on visual observations, both red and green pods resembled Trinitario variety. The seeds after 144 days post-pollination from green pods were dark purple and numbered from 45 to 61 seeds per pod (Table 2). Seed weight and size remained essentially unchanged throughout the period studied. Furthermore, seeds from green pods exhibited more extensive hardening and coloring than seeds from red pods.

In Fig. 2 the increase in PPO activity is shown to be nearly parallel between seeds from green and red pods. The PPO activity of seeds from green pods is slightly greater than activity of seeds from red pods of similar ages. The PPO activity of seeds from green pods increases 60.7-fold from 81 to 163 DAP compared to 44.0-fold from 120 to 175 DAP for seeds from red pods. The enzyme activity continues to increase with age, although seed sizes and weights reach a plateau at 144 days. Also, dark (1.56×10^4 units/g seed) and light (1.59×10^4 units/g seed) demonstrate similar activity, indicating that pod or seed color, i.e.

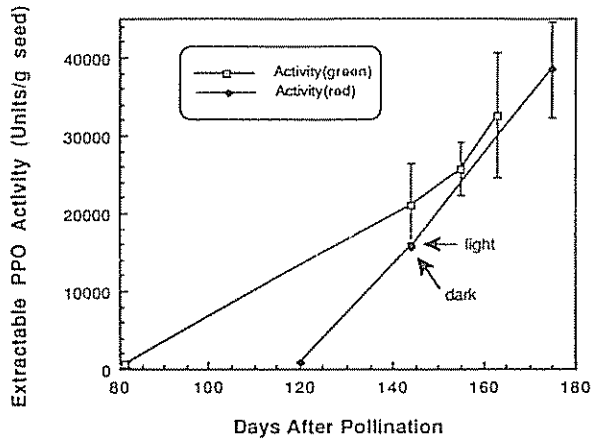

Fig. 2. Extractable polyphenol oxidase (PPO) activity of developing cacao seeds from green (EQX-100) and red (UF 667) colored pods with labeled values for light and dark purple seeds. Those points without standard deviation bars indicate lack of sufficient replicates.

the concentration of anthocyanins, does not influence the amount of PPO enzyme.

Clearly, pigmentation increases, seeds solidify, and folds develop throughout the cotyledon as seed development progresses. Younger seeds are opaque, contain a liquid endosperm, and lack any pigmentation. The hardening process appears to begin at the hypocotyl (placental) end of the seed, when growth has stopped, and spreads, causing an increase in mass but little change in size (length and width). Indeed, the light and dark purple colors of cacao seeds develop at different rates within any one pod with mechanisms which are not yet understood. However, it is known that the fine-flavored Criollo seeds are normally white

Table 1. Shapes and sizes of cocoa pods harvested in this study at various ages.

	Pod dimensions[1]						
Days after pollination	Weight (g)	Length (cm)	Circum. (cm)	Apex shape number	Base shape number	Wall thickness (cm)	Dia. (cm)
PSU-3							
81	NA[2]	6.7	8.0	NA	NA	NA	2.3
144	410.0	16.7	25.2	1–2	0–1	1.2	7.9
155	465.8	18.3	25.2	1–2	0–1	0.9	8.0
163	392.3	16.8	25.5	1–2	0–1	1.1	7.8
PSU-20							
120	320.2	14.4	23.1	1	1	0.9	7.0
144	293.7	14.0	22.4	1	0–1	1.2	NA
175	244.6	14.0	22.1	1	1	1.0	7.0

1 Apex shape number refers to the form of the point opposite to the stem on a scale of 1 to 6. A value of 1 implies the most pointed form. Base shape number refers to the degree of basal constriction on a scale of 0 to 4. As the number becomes larger the degree of constriction near the stem becomes greater.

2 NA = not available

due to the lack of the anthocyanins which color the bulk grade Forastero seeds purple. A distribution of light and dark purple-colored seeds was observed in red pods with no apparent pattern (Table 2). Seed weight slightly increased from 0.63 g/seed at 120 DAP to 0.77 g/seed at 175 DAP. Seed sizes, however, remained about the same during this period of development.

Protein assays of cacao seeds from green and red pods revealed a dramatic difference. Protein concentrations of seeds from green pods increased 28.8-fold from 81 to 155 DAP before beginning to level off at 163 DAP with a total increase of 30.2-fold (Fig. 3). In obvious contrast, protein concentrations of seeds from red pods remained about the same during development. Light purple-colored seeds had a 2.8-fold higher protein concentration than dark purple-colored seeds. It is known that during higher plant seed development proteins accumulate in a short period of time (3). Possibly, protein concentrations peaked between harvest dates and subsequently declined since microsomal proteins are known to decrease with seed development (3).

Predictably, specific activity (SA), which relates total PPO activity to total protein content, increased more dramatically for seeds from red pods (Fig. 4). Specific activity increased 566-fold from 120 DAP to 175 DAP. Although total PPO activities among seeds from the green and red pods were only slightly different, large differences were observed in protein content. High specific activities of PPO in seeds from red pods reflected low protein contents whereas relatively low specific activities in seeds from green pods only increased slightly since the total PPO activity closely paralleled the protein content. Specific activity increased 2.6-fold from 81 DAP to 144 DAP but reached a plateau at 155 and 163 DAP with increases in SA of 1.7-and 2.0-fold, respectively.

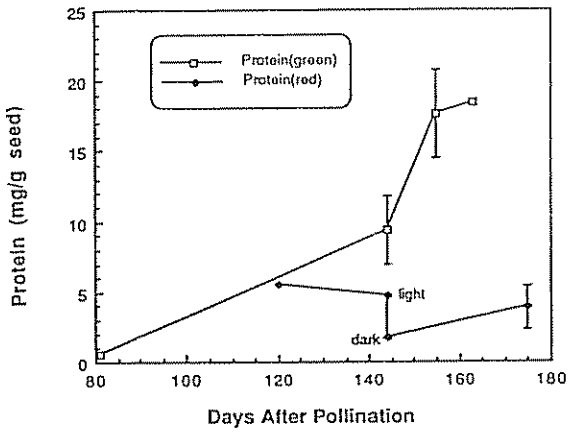

Fig 3 Protein of developing cacao seeds from green (EQX-100) and red (UF 667) colored pods with labeled values for light and dark purple seeds. Those points without standard deviation bars indicate lack of sufficient replicates.

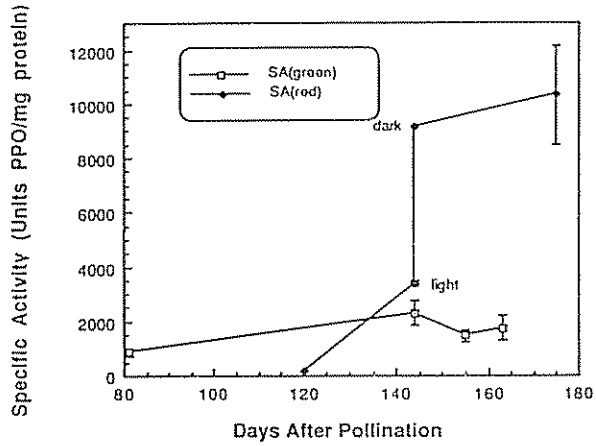

Fig 4 Specific activity (SA) of polyphenol oxidase of developing cacao seeds from green (EQX-100) and red (UF 667) colored pods with labeled values for light and dark purple seeds. Those points without standard deviation bars indicate lack of sufficient replicates.

CONCLUSIONS

An extraction procedure has been developed for cocoa bean PPO in order to quantitate PPO as rapidly as possible. The application of this method to obtain crude PPO is quite suitable for post-pollination studies in order to observe quantitative changes in PPO activity during seed development. Seeds from two separate trees which fruited green and red pods, respectively, were studied. PPO activity from each group of seeds increased with development. Light and dark puple-colored seeds were found in red pods only, but similar PPO activities were measured for each. Therefore, no relationship exists between amount of PPO activity and concentration of pigmentation. Larger differences were seen in protein contents. Protein concentrations of seeds from green pods increased with development. However, protein contents remained about the same for seeds from red pods. The results in this study represent two cultivars and further research is needed to determine if the trends found here occur in all cacao during development.

Ripe cacao pods are preferred for cocoa production. Certain biochemical changes are necessary, such as the build-up of pulp sugars and the increase in acidity, tannins, and carbohydrates (6). The combination of changes appears optimal, but it is not known whether an alteration of one of these would affect the quality of the resulting chocolate. Perhaps it is beneficial that PPO activity continues to increase until ripening since, in the ensuing fermentation, PPO activity declines rapidly (8).

Table 2. Pod color, seed counts and seed measurements of pods harvested at various ages.

	Seed count (%)			Seed measurements[1]		
Days after pollination	Dark purple	Light purple	Colorless	Weight (g/seed)	Length (cm)	Width (cm)
PSU-3						
81			51(100)	NA[2]	NA	NA
144	45(100)			1.12	2.2	1.1
155	61(100)			0.96	2.2	1.1
163	54(100)			0.99	2.2	1.1
PSU-20						
120	18(56)	13(41)	1(3)	0.63	2.0	1.1
144	8(24)	25(76)		0.72	2.0	1.1
175	29(100)			0.77	1.9	1.0

1 Measurements represent total weight of seeds without testa (g) divided by total number of seeds. Lengths and widths are the average of three representative seeds

2 NA = not available.

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