Developmental Variation of Sugars, Carboxylic Acids, Purine Alkaloids, Fatty Acids, and Endoproteinase Activity during Maturation of *Theobroma cacao* L. Seeds

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The changes of mono- and oligosaccharides, carboxylic acids, purine alkaloids, and fatty acid composition, and of aspartic endoproteinase activity, were analyzed during seed development in two varieties of cacao (*Theobroma cacao*). The majority of the components examined either decreased or accumulated steadily in concentration during the second half of bean development. Sucrose is the major sugar in the mature embryo, whereas fructose and glucose are at higher concentrations in the endosperm tissue. Considerable amounts of malate are found in the endosperm, whereas citrate is the dominant carboxylic acid in the embryo. A major change in the fatty acid composition occurs in the young embryo when the proportion of stearic acid increases rapidly at the expense of linoleic acid, which is reduced from about 18 to 3%. Theobromine is the dominant purine alkaloid (ca. 80%), and caffeine appears only toward the end of seed maturity. Aspartic endoproteinase activity increases rapidly during embryo expansion, reaching a maximal activity before final maturity. The results are discussed in conjunction with physiological changes in developing seeds, and the potential contributions of the compounds analyzed for cocoa quality.

Keywords: *Cocoa; Theobroma cacao; seed development; sugars; acids; alkaloids; fatty acid composition; aspartic endoproteinase*

INTRODUCTION

The analysis of the levels of the principal components occurring in mature and fermented cocoa beans has been reviewed in the literature (*1*). Most studies have considered the components in fermented cocoa beans from an industrial perspective as precursors of cocoa flavor and aroma (*1, 2*). Particular attention has been paid to the analysis of proteins (*3*) and polyphenols (*4*). The structure and composition of seed storage reserves of other plants has been reviewed elsewhere (*5*). Little information regarding the evolution and accumulation of constituents during cacao seed maturation is available. The morphological changes have been described (*6*), but only a few studies have dealt with the biochemical changes occurring during maturation. Essentially, these include work done on the accumulation of starch (*7*), the changes of proteins (*8*), the composition of ripening beans (*9*), the content of purine alkaloids (*10*), the changes in vicilin content (*11*), and the determination of peroxidase activity (*12*). Advanced stages of maturing seeds (between 105 and 160 days after pollination (DAP)) were analyzed in these studies, but their genetic origins frequently were not well defined. Developmental biochemical information is, however, an essential base for the elucidation of mechanisms of transport and metabolism in cacao seed and should provide targets for the improvement of technological and agronomic properties of cacao.

In this study we have investigated changes in concentrations or activity in two *T. cacao* varieties during seed maturation of the following components that are presumably implicated in cocoa quality: (a) glucose and fructose, together with sucrose, which are the main sugars for Maillard reactions that take place during drying and roasting (*13*); (b) inorganic and carboxylic acids, which contribute to acidity, an attribute that is associated with cocoa and chocolate flavor (*14, 15*); (c) theobromine and caffeine, which probably contribute to cocoa bitterness (*16*); (d) fat content and composition which are important parameters for the technological and nutritional quality of cocoa butter and chocolate products (*17, 18*); and (e) aspartic endoproteinase which is thought to be a key enzyme for cocoa aroma generation (*19*).

MATERIALS AND METHODS

Plant Material. Cacao pods from variety EET 95 (Nacional × Venezulano amarillo, auto-compatible) and ICS 95 (Trinitario type, parent unknown) were obtained from genetically identified trees grown on the experimental farm of Quevedo of Nestlé ex-R & D Center Quito, Ecuador. Both varieties were from the INIAP collection (Ecuador). On December 9, 1999, flowers of EET 95 were hand pollinated with EET 95 pollen, and those of ICS 95 were pollinated at random with available pollen. Pods were collected in intervals of about 20 days, starting at 62 days after pollination (DAP). The harvested pods were air-freighted under refrigeration to Nestlé Research Center Tours (France), and frozen at − 20 °C.

Cacao Powder Preparation. Powder was prepared from all the seeds of 10 (up to 125 DAP) and 5 (146 DAP and

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10.1021/jf010620z CCC: $20.00
Published on Web 09/22/2001

Table 1. Cacao Seed Development of Varieties EET 95 and ICS 95

DAP[a]	variety	FW[b] per pod	number of seeds per pod	endosperm FW per pod	seed FW per pod	mean DW[c] per seed
62	ICS 95	32 ± 13	20 ± 10	0.3 ± 0.2		0.0003
	EET 95	48 ± 8	43 ± 11	0.6 ± 0.2		0.001
83	ICS 95	182 ± 38	36 ± 8	4.2 ± 1.3		0.007
	EET 95	179 ± 39	34 ± 9	5.5 ± 1.9		0.012
104	ICS 95	382 ± 69	32 ± 6	14.9 ± 2.6	4 ± 4	0.072
	EET 95	482 ± 121	36 ± 11	10.9 ± 4	19.4 ± 8	0.097
125	ICS 95	710 ± 89	35 ± 4	2.4 ± 1	45.1 ± 4.5	0.445
	EET 95	744 ± 152	47 ± 3		71.3 ± 15	0.616
146	ICS 95	779 ± 51	37 ± 3		63.6 ± 8	0.918
	EET 95	741 ± 99	45 ± 4		96.9 ± 10	1.197
175	ICS 95	778 ± 84	39 ± 4		68.5 ± 2.3	0.979
160	EET 95	828 ± 66	47 ± 4		97.5 ± 8.8	1.207

[a] DAP, days after pollination. [b] Values (means ± SD) are given in g. FW, fresh weight. [c] DW, dry weight.

mature) pods per variety and stage, respectively. Cacao seed tissues were separated from the external mucilage tissue by dissecting the pods while they were in a frozen state. This facilitated the separation and isolation of the liquid endosperm tissue at 83 and 104 DAP. At 83 DAP, the seed tissue was essentially composed of endosperm. The endosperm and the developing embryo were separated into two fractions for the 104 DAP stage. The embryo (from 104 DAP onward) was considered to consist of the embryo axis and cotyledons. All the samples were homogenized in liquid nitrogen for 2 × 1 min in a cryo mill (model 6800, SpexCertiprep Inc, Metuchen, NJ). The obtained powder was freeze-dried for 24 to 48 h (Lyolab bII, LSL Secfroid, Lausanne, Switzerland). Samples were stored at −20 °C.

Fat Content and Fatty Acid Composition. Fat content was determined by extracting freeze-dried cacao powder (0.5 to 2 g) repeatedly in a Soxhlet apparatus with 3 × 500 mL of petroleum ether (60−80 °C). Fat content is expressed as % per g dry weight (DW). Fatty acids were determined according to an adapted version of the methodology described by Muuse et al. (*20*). Samples were prepared by mixing 100 mg of filtered oil with 2 mL of methyl tridecanoate (internal standard, 2.5 mg/mL hexane) and 200 μL of 2 M methanolic KOH solution for 2 min in a Vortex. After centrifugation (5 min, 700*g*), 30 μL of the upper layer was diluted in 10 mL of *n*-hexane, and 5 μL was injected onto a Chrompack CP-Sil88 column (100 m × 0.25 mm i.d., 0.25 μm film) run on a Carlo Erba 5300 gas chromatograph.

Sugar and Acid Determination. Freeze-dried powders were extracted in duplicate for 1 h in boiling water at a concentration of 20 mg/mL. Samples were centrifuged in Eppendorf tubes (10000 g, 10 min), and the obtained supernatant was separated for mono- and oligosaccharides by high-performance anion exchange chromatography coupled to pulsed electrochemical detection (HPAE−PED) using a Dionex PA 1 (250 × 4 mm i.d.) column (*21*). Dionex HPAE−PED was also used to determine the concentrations of carboxylic acids and inorganic anions (MA 1 column, 250 × 4 mm i.d.). Carboxylic acids and inorganic anions were separated by a gradient of 0.5−38.25 mM NaOH for 18 min. The injected sample volume was 20 μL and the flow rate was 1 mL/min for sugars and 1.5 mL/min for acids and anions. Standards of sugars, oligosaccharides, carboxylic acids, and inorganic anions were from Sigma (St. Louis, MO). All results are expressed as the mean of two or three repetitions.

Purine Alkaloids. Alkaloids were extracted in duplicate from 0.25 g samples of defatted cacao powder with 30−35 mL of boiling water for 30 min in weighed 125-mL flasks. After the extracts had cooled, the flasks were re-weighed, and the amount of water lost during boiling was replaced. Alkaloid extracts were filtered (Schleicher & Schuell paper 597 1/2) and passed through a 0.2-μm nylon membrane (Supelco, Bellefonte, PA) before being quantitated by HPLC. A Nucleosil 100-5 C_{18} column (250 × 4 mm i.d.) (Macherey-Nagel, cat. no. 720014, Germany) was used along a mobile phase of 0.05% phosphoric acid/acetonitrile (9:1). The separation was performed in isocratic mode for 15 min at room temperature at a flow rate of 1 mL/min. The diode array detector was operated at 274 nm.

Aspartic Endoproteinase Activity. Freeze-dried cacao powder (50 mg) was extracted in triplicate with 1.5 mL of sodium-borate buffer (25 mM, pH 9.0) according to a modified protocol of Hansen et al. (*22*) with constant shaking at 4 °C for 30 min, followed by centrifugation (2 × 10 min, 10000*g*). The enzyme activity was measured in the supernatant with acidified hemoglobin (Sigma, St. Louis, MO) followed by the *o*-phthaldialdehyde (OPA) reaction (*23*). The incubation mixture contained 400 μL of citrate buffer (200 mM, pH 3.0), 160 μL of enzyme extract, and 240 μL of acidified 2% bovine hemoglobin. Each extract was assayed four times. The control was run with pepstatin (2 μM final concentration) (Sigma, St. Louis, MO). The mixture was incubated for 60 min at 45 °C, and aliquots of 200 μL each were removed at 0, 5, 15, 30, and 60 min, and mixed with 200 μL of 8% aqueous trichloroacetic acid to stop the reaction. This mixture was incubated for 15 min at room temperature, and the supernatant was isolated by centrifugation (10000*g*, 10 min). The OPA reaction was carried out by mixing 50 μL of the supernatant with 250 μL of the freshly prepared OPA (0.8 mg/mL) reagent. The mixture was incubated for 15 min at room temperature in a microtiter plate, and the absorbance was read in a spectrophotometer at 340 nm (MR 5000, Dynatech). The absorbance values obtained for buffer and pepstatin were subtracted for the calculation of the enzyme activity from a standard curve which was generated by using the same procedure with porcine pepsin A (Sigma, St. Louis, MO). The enzyme activity is expressed as pepsin equivalent (U/mg extractable protein). Total protein content was determined according to the method of Church et al. (*23*).

RESULTS AND DISCUSSION

Pod and Seed Development. All pods were obtained from genetically identical trees (5 and 4 trees, respectively, for EET 95 and ICS 95) that were generated by vegetative propagation. Development of ripe pods and seeds of EET 95 required 15 days less than those of variety ICS 95 (160 versus 175 days) (Table 1). Endosperm and embryo tissues were carefully separated by dissecting the pods while they were in a frozen state, thus allowing the analysis of compositional changes in endosperm and the developing embryo. Stage-dependent developmental changes became apparent between 83 and 104 DAP, during which the endosperm reserves were reduced and utilized for the growth of the embryo. At 104 DAP, EET 95 seeds were clearly more advanced in development than seeds of ICS 95, with most of the dry weight already in the embryo (Table 1). Developmental differences between cacao varieties have not been described in detail, and information on this topic is scarce. The only comparable study, carried out in Malaysia on varieties ICS 95 and KKM 4 (*11*), indicated a similar growth pattern for ICS 95. Important differences between EET 95 and ICS 95 were also found for the number of isolated seeds per pod (42 versus 33, on

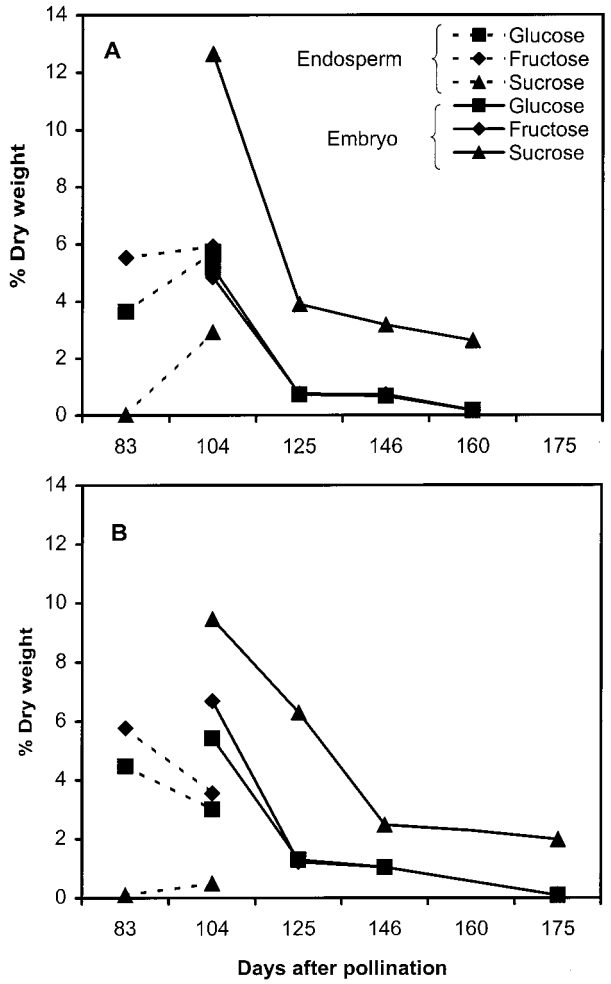

Figure 1. Changes in concentrations of sucrose, glucose, and fructose in seeds of cacao varieties EET 95 (A) and ICS 95 (B) during maturation. Developing seeds were divided into endosperm (at 104 DAP) and embryo tissue for analysis.

average for all seed stages), and seed weight (1.21 versus 0.98 g DW), respectively. The discrepancy found in the number of seeds early in development (62 DAP) could be due to asynchronous development. The average seed weight of EET 95 was considerably higher than that usually found for commercial cocoa beans from Ghana or Ivory Coast, which normally have a bean weight that is slightly below one gram. Regarding the biochemical composition, the two principal tendencies observed are a diminution for some components from an initial higher level in the young embryo, and a steady accumulation in the developing embryo for other components from a low to a high level in the mature seed.

Sugar Composition. Analysis of the free sugars glucose, fructose, and sucrose in the two varieties indicated that the maturation profiles evolved in a similar way during maturation. In the endosperm examined (83 and 104 DAP), glucose and fructose were the major free sugars, varying between 3 and 6% DW for both varieties (Figure 1). Sucrose levels were considerably lower, reaching at the most about 3% DW at 104 DAP (variety EET 95). In the newly forming embryo tissue at 104 DAP, the total amount of the three sugars glucose, fructose, and sucrose was considerable (22.7% and 21.6% DW for EET 95 and ICS 95, respectively). However, during embryo development, total sugar content decreased rapidly to 3 and 2.2%, respectively, at the final maturity stage. Total sugar content is known to vary between 2 and 4% DW (*1*). At the final maturity stage (Figure 1), concentrations of glucose and fructose had decreased to 0.1 to 0.2% DW, whereas sucrose remained the principal free sugar with 2.6% (EET 95) and 2% DW (ICS 95). Assuming that sucrose is the major carbohydrate transported in the phloem, the high levels of glucose and fructose in the endosperm would represent enhanced sucrose catabolism in this tissue. This is consistent with the requirement by this tissue of an increase of osmotic pressure to enable both the initial expansion within the locular space, and to allow the tissue to exert a sink function. A similar situation has been found for the developing coffee grain (*24*), however, with an important imbalance in the perisperm between glucose and fructose. Free sugars of cacao do not confer sweetness to cocoa; however, enzymatic (during fermentation) and chemical hydrolysis of sucrose (during roasting) generate sufficient amounts of reducing sugars (glucose and fructose) that react with free amino acids and peptides during roasting to produce characteristic aroma components (*2*, *13*).

The two oligosaccharides stachyose and raffinose were normally not detected in the endosperm, but did accumulate later in the developing embryo (Table 2). Interestingly, for both varieties, raffinose was at a maximum concentration at 125 DAP, whereas stachyose concentration was highest at the final maturity stage. Raffinose and stachyose have previously been shown to accumulate primarily in mature embryo axes of cacao, reaching concentrations of 2.1 and 1.5%, respectively (*25*). Their results indicated that recalcitrance of cacao axes to dessiccation cannot be caused by a lack of these oligosaccharides.

Acid Composition. Among the carboxylic acids analyzed, the predominance of citrate was confirmed, and shown to be part of a steady decline of carboxylic acid concentrations during maturation from the 104

Table 2. Content of Free Sugars, Quinate, and Inorganic Anions (% g/g DW) of Varieties EET 95 and ICS 95 during Seed Maturation[a]

variety	tissue	DAP[b]	galactose	xylose	raffinose	stachyose	quinate	chloride	nitrate	sulfate	phosphate
EET 95	endosperm	83	0.00	0.22	0.01	0.00	0.73	0.11	0.02	0.18	1.20
		104	0.01	0.14	0.21	0.00	1.40	0.05	0.05	0.37	2.28
	embryo	104	0.03	0.25	0.02	0.03	1.08	0.06	0.05	0.39	2.90
		125	0.04	0.09	0.60	0.01	0.44	0.08	0.04	0.14	0.40
		146	0.06	0.04	0.40	0.25	0.19	0.04	0.02	0.08	0.21
		160	0.07	0.01	0.27	0.48	0.28	0.04	0.03	0.08	0.22
ICS 95	endosperm	83	0.01	0.17	0.00	0.00	0.48	0.06	0.02	0.14	1.03
		104	0.00	0.17	0.00	0.00	0.96	0.07	0.03	0.23	1.58
	embryo	104	0.01	0.24	0.07	0.01	1.98	0.08	0.03	0.46	4.58
		125	0.02	0.10	0.53	0.00	0.68	0.10	0.01	0.12	0.88
		146	0.02	0.15	0.25	0.08	0.50	0.05	0.00	0.07	0.31
		175	0.03	0.02	0.24	0.45	0.43	0.05	0.01	0.14	0.20

[a] Results are means of duplicate or triplicate experiments. [b] DAP, days after pollination.

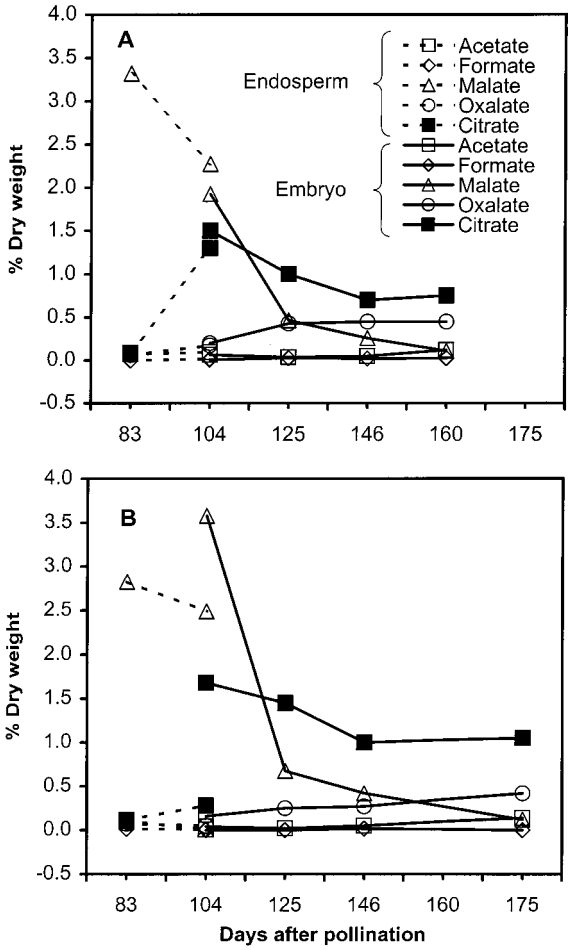

Figure 2. Changes in concentrations of carboxylic acids in seeds of cacao varieties EET 95 (A) and ICS 95 (B) during maturation. Developing seeds were divided into endosperm (at 104 DAP) and embryo tissue for analysis.

DAP stage onward (Figure 2). At final maturity, oxalate was the second most abundant carboxylic acid, followed by low amounts of malate, acetate, and formate. Oxalate was the only carboxylic acid that increased during seed development. These results are in agreement with data obtained on unfermented cacao from Malaysia (*26*). The high concentrations of malate in the endosperm and young embryo are possibly linked to asparagine and glutamine interconversion taking place in the newly forming seed (*5*). In addition, it might be implicated in the malate/aspartate shuttle involved in glyoxysome−mitochondria interactions, contributing to ATP synthesis via NADH oxidation. Of the carboxylic acids present in fresh cacao seeds, only oxalic acid has been linked to increased chocolate flavor (*15*). Cocoa acidity has been frequently associated with the presence of acetate and lactate, two compounds that are both formed by microorganisms during fermentation (*14*).

Inorganic Anions. Concentrations of chloride and nitrate did not vary much during maturation (Table 2). Concentrations of unbound phosphate were considerable (1 to 2.3% DW) in the endosperm and in the early development of the embryo at 104 DAP (2.9 and 4.6% DW), indicating its importance for the sugar phosphate pools that play a central role in the supply of carbon skeletons for biosynthetic reactions (polysaccharides, proteins, and others). Sulfate and quinate underwent similar changes.

Fat Accumulation and Fatty Acid Composition. Fat accumulation was found to proceed faster in the EET 95 seeds; however, similar values were reached (51−52%) for both varieties at seed maturity (Table 3). These fat levels make the two varieties not particularly suitable for cocoa butter production. The fat content of mature seeds of 51.8% for ICS 95 was comparable to that found for the same variety grown in Malaysia (*11*). During seed maturation, one major shift occurred in fatty acid composition between 104 and 125 DAP when the transiently high proportion (18%) of linoleic acid (18:2) dropped rapidly to about 3%. Presumably, linoleic acid synthesis catalyzed by oleate desaturase ceases during this period, as further indicated by a 6-fold bean DW increase during this period (Table 1). This change is paralleled by a steep increase in the amount and proportion of stearic acid (18:0), most likely by increased acyltransferase activity. From 125 DAP onward, the fatty acid composition did not change anymore as previously described (*9*), and was about the same for both varieties. Fatty acid synthesis in cacao has not been studied so far, despite the uniqueness of cacao fat for making chocolate. It is conceivable that the fatty acid composition of cocoa fat could be altered by variety selection or genetic modification, eventually leading to the manufacture of specific cocoa fat for the manufacture of chocolate with different rheological properties. For both varieties, linoleic (18:2) and α-linolenic (18:3)

Table 3. Fatty Acid Composition (%) and Fat Content of Varieties EET 95 and ICS 95 during Seed Maturation

		104 DAP[a]		125 DAP		146 DAP		mature	
fatty acid		EET 95	ICS 95	EET 95	ICS 95	EET 95	ICS 95	EET 95	ICS 95
C14:0	myristic	0.30	0.50	0.18	0.17	0.08	0.12	0.08	0.07
C15:0	pentadecanoic	0.13	0.22	nd[b]	0.06	0.02	0.02	nd	nd
C16:0	palmitic	31.21	31.93	27.04	30.67	27.07	30.51	27.36	30.54
C16:1	palmitoleic	0.65	nd	0.34	0.14	0.34	0.20	0.34	0.21
C17:0	margaric	0.22	nd	0.28	0.18	0.28	0.19	0.34	0.20
C18:0	stearic	14.72	15.00	34.00	28.31	36.24	31.27	36.00	31.86
C18:1	oleic & other *cis*	31.41	26.46	32.77	34.69	32.07	33.82	31.62	33.14
C18:2	linoleic	16.25	20.39	3.51	3.29	2.25	2.00	2.45	2.24
C18:3	alpha-linolenic	1.13	1.44	0.27	0.33	0.17	0.19	0.20	0.18
C20:0	arachidic	1.94	1.89	1.19	1.43	1.21	1.22	1.26	1.28
C20:1	eicosenoic	0.32	0.30	0.03	0.14	nd	0.08	0.05	0.02
C22:0	behenic	1.21	1.32	0.35	0.40	0.20	0.25	0.22	0.18
C24:0	lignoceric	0.51	0.55	0.04	0.19	0.07	0.13	0.08	0.08
total fatty acids		100	100	100	100	100	100	100	100
fat content of seeds (%)		16.6	28.0	44.8	37.2	53.9	49.7	51.5	51.8

[a] DAP, days after pollination. [b] nd, not detected.

Table 4. Concentrations of Theobromine and Caffeine in Cacao Seeds (% DW of defatted material) of Varieties EET 95 and ICS 95 during Seed Maturation

variety	DAP[a]	theobromine	caffeine	total	% theobromine
EET 95	104	0.01	nd[b]	0.01	100
	125	0.43	nd	0.43	100
	146	1.15	0.11	1.26	91
	160	1.97	0.55	2.52	78
ICS 95	104	0.06	nd	0.06	100
	125	0.89	nd	0.89	100
	146	1.60	0.19	1.79	89
	175	1.90	0.31	2.21	86

[a] DAP, days after pollination. [b] nd, not detected.

acid were found at all maturity stages. Interestingly, these two fatty acids were not detected in several Venezuelan cacao clones (*17*), indicating that genetic variation exists. Another difference was the presence of 1.3% of arachidic acid (20:0) in varieties EET 95 and ICS 95, although this fatty acid has not been detected in an Arriba cacao from Ecuador that is genetically related to variety EET 95 (*18*).

Purine Alkaloids. Analysis revealed that theobromine started to accumulate at 125 DAP, and increased thereafter to about 2% of defatted material (the cocoa powder fraction) (Table 4). For both varieties, caffeine was detected only toward the end of seed maturity. The proportion of theobromine content of the total purine alkaloids was higher for ICS 95 than for EET 95 (86 versus 78%, respectively). The data obtained on theobromine and caffeine evolution during maturation are in accordance with an earlier study (*10*). However, the information that caffeine is formed only toward the end of seed maturity, presumably by activation of the *N*-methyltransferase enzyme that methylates theobromine to caffeine, is new.

Aspartic Endoproteinase Activity. The changes in aspartic endoproteinase activity are shown in Figure 3. In the endosperm (essentially 83 and 104 DAP), activity values were very low. This changed rapidly in the developing embryo where aspartic endoproteinase activity expressed on a seed basis increased by 24-fold (EET 95) and 36-fold (ICS 95) between 104 and 146 DAP, before leveling out to a similar activity at final maturity. The specific endoproteinase activity was 4 times higher in mature seeds of EET 95, indicating a possible link to the more pronounced flavor of cocoa from variety EET 95. By probing the enzyme with a polyclonal antibody, maximal levels of endoproteinase protein were found between 110 and 130 DAP for variety ICS 95 (*11*). Therefore, maximal expression of aspartic endoproteinase seems to be reached before final seed maturity. A similar situation was described for wheat in which aspartic endoproteinase activity was detected at an intermediate stage of grain development (*27*). The presence of aspartic endoproteinase activity at the final seed maturity stage appears to be a prerequisite for the degradation of seed storage proteins that are induced during cacao fermentation, and the subsequent generation of free amino acids and peptides that react with reducing sugars during roasting to give the characteristic cocoa aroma (*19*).

This analytical study provides data, not previously available, on the evolution of sugars, carboxylic acids, fatty acid composition, and purine alkaloids, and of aspartic endoproteinase activity, during cacao seed development in two varieties. The elucidation of cacao seed maturation, and its underlying genetic and biochemical processes, could potentially lead to a better understanding of the quality of this interesting raw material, and its subsequent impact on the technological, nutritional, and sensory quality of cocoa products.

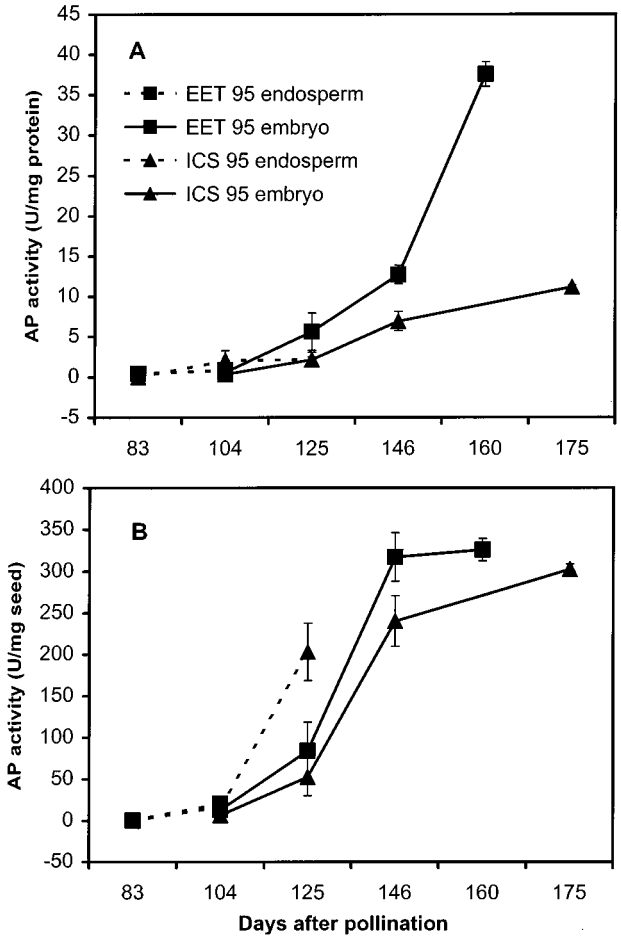

Figure 3. Evolution of the aspartic endoproteinase (AP) activities in seeds of varieties EET 95 and ICS 95 during maturation. AP activity is expressed as pepsin equivalents (U/mg protein). (A) Specific AP activity; (B) AP activity per seed. Seeds were divided into endosperm (up to 125 DAP) and embryo tissue for analysis. Error bars represent the standard deviation from the mean.

ACKNOWLEDGMENT

The authors thank F. Dionisi (fatty acids), S. Michaud (sugars, acids, and anions), and A. Touché (alkaloids) for carrying out the respective analyses.

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Received for review May 14, 2001. Revised manuscript received August 6, 2001. Accepted August 8, 2001.

JF010620Z