Phospholipid Composition of Lipid Seed Crystal Isolates from Ivory Coast Cocoa Butter

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Seed crystals isolated from Ivory Coast cocoa butter were shown to differ in chemical and thermal characteristics from solidified Ivory Coast butter. Higher concentrations of complex lipids in the seed crystals have led to speculation on the role these polar molecules play in lipid crystallization events. Phospholipids separated from lipid seed crystal isolates were twelve-fold more concentrated than the original cocoa butter. Seed crystals contained 3.99% phospholipids while cocoa butter samples contained 0.34%. Phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, lysophosphatidylcholine, phosphatidylserine, and phosphatidic acid were identified in cocoa butter with phosphatidylcholine (37.7%), phosphatidylglycerol (27.3%) and phosphatidylethanolamine (15.6%) being the major phospholipid constituents. Two phospholipids not previously reported in cocoa butter were identified as phosphatidylglycerol and diphosphatidylglycerol based on co-migration of standards. Cocoa butter and its seed crystals contained the same phospholipid entities; however, individual phospholipids differed significantly in concentration. Phosphatidylethanolamine (30.4%) and phosphatidylcholine (30.2%) were the major phospholipids in seed crystal samples. Fatty acid composition of cocoa butter and seed crystal phospholipids were found to be similar, with the exception of myristic, stearic and oleic acids. Myristic acid was three-fold higher in phosphatidylglycerol and phosphatidylethanolamine in the seed crystals, whereas stearic acid was significantly lower in the seed crystals when compared to the cocoa butter. Concentrations of oleic acid were twice as high in seed crystal phosphatidylethanolamine and almost four times as high in seed crystal phosphatidylcholine than in corresponding cocoa butter samples. The possible role phospholipids play in seed crystal development and in crystallization events is discussed.

KEY WORDS: Cocoa butter, lipid crystallization, phospholipids, seed crystals.

Cocoa butter represents 28–30% of finished chocolate (1). Its narrow melting range (30–35°C) and smooth melt texture are thought to disperse flavor components across the palate, producing an even flavor and pleasant cooling sensation during consumption. These organoleptic properties result from the molecular arrangement of the lipid molecules that comprise the cocoa butter. Cocoa butter solidification into this crystalline form produces the glossy surface, hardness, melt properties and characteristic snap associated with quality chocolate. Cocoa butter can solidify into six polymorphic forms (2). The polymorph of value to the confectionery industry is initiated during the tempering process, which involves controlled cooling and agitation of the chocolate mass to form stable crystal nuclei (also termed "seed crystals") within the melt. The nuclei serve as growth points from which the high-melting-point crystals form during solidification. A sufficient concentration of small stable crystals promotes further crystallization of the cocoa butter into the desired, stable polymorph. Improper tempering or a loss of temper through temperature abuse results in recrystallization of cocoa butter and subsequent formation of multiple lower-melting-point polymorphs. This results in a soft, undesirable product. Proper tempering has been and, to a large extent, still is considered an art. A scientific understanding of nucleation and early crystallization events is slowly emerging. Research on isolated seed crystals of cocoa butter indicates major thermal (3) and compositional (4) differences between isolates and cocoa butter. High saturated triacylglycerol concentrations and a significantly high proportion of complex lipids exist in the seed crystal. Twelve-fold increases in glycolipid and eighteen-fold increases in phospholipid concentrations have been reported. Physically, seed crystals melt at temperatures as high as 70°C, twice the melting temperature of typical cocoa butter samples.

The present investigation considers the role of phospholipids in cocoa butter crystallization. The predominance of a particular phospholipid class, for example, might be thought to encourage the early aggregation of certain triacylglycerol species or may be important in nuclei stabilization. The following study is, therefore, one inquiry into an area that presents challenges to the understanding of organic lipid crystallization with specific reference to the importance of phospholipids in this process.

MATERIALS AND METHODS

Chemicals and reagents. Acetone, chloroform, ethyl ether, hexane and methanol were high performance liquid chromatography (HPLC) grade obtained from Fisher Scientific (Pittsburgh, PA), while all other solvents were certified American Chemical Society (A.C.S.) from Fisher Scientific. All other reagents used in these studies were reagent grade from either Aldrich Chemical Company (Milwaukee, WI) or Fisher Scientific unless otherwise noted. Water was distilled and deionized prior to use. Phospholipid standards were from Supelco, Inc. (Bellefonte, PA) and/or Sigma Chemical Company (St. Louis, MO). Silicic acid was from Mallinkrodt Analytical reagents (St. Louis, MO).

Samples. Approximately 90 kg of crude Ivory Coast cocoa butter was filtered in a EU-16 model, six-stage filter press (Ertel Engineering Co., Kingston, NY) containing grade 534-54, 30μ filter pads (Eimco Filter Media Division, Salt Lake City, UT) to remove shell fragments and other debris. The liquid cocoa butter (64–74°C) was passed through the press six times with an average time of 19 min per pass.

Seed crystals characterized in this study were obtained

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via static incubation of cocoa butter melts at 26.5°C, and isolated by centrifugation and acetone washings as previously described (3). When sufficient quantities of seed crystal were collected, samples were pooled and divided into three equal aliquots of 500 mg each, and assayed individually in triplicate.

Melting point determinations (onset transition temperatures) of the isolated seed crystals were performed with a Perkin-Elmer Model 4 Differential Scanning Calorimeter and a Model 3600 Thermal Analysis Data System with gallium as a standard (Norwalk, CT).

Chromatographic procedures. Three sets each of pure cocoa butter samples and seed crystal samples were separated into simple lipid, glycolipid, and phospholipid fractions by silicic acid column chromatography (5). Compositional analysis of the individual lipid classes was performed, and quantitative distribution of the classes was determined by gravimetric analysis. Thin-layer chromatography (TLC) was employed to monitor column separation efficiency. Purification of cross-contaminated fractions was performed on Analtech silica gel G preparative plates (200μ thickness) (Newark, DE), yielding pure lipid fractions. Separation and quantitative identification of individual phospholipid classes from cocoa butter and seed crystal samples were accomplished on Analtech silica gel G preparative plates (2000μ thickness) or Alltech Associates (Ontario, Canada) Adsorbasil hard-layer plates (200μ thickness). Plates were predeveloped in chloroform, air-dried and conditioned at 110°C for 1 hr. Separation of individual phospholipid classes proceeded as a single-dimension modification (6), and resolution was achieved by developing the sample plates in a chloroform:methanol:14% ammonia 65:35:5 (v/v/v) solvent system. Phospholipid classes were identified by comparison with phospholipid standards and visualized with iodine and the phosphate stain (7). Separated phospholipid classes were eluted, dried under nitrogen and subjected to an acid-catalyzed transesterification (8). Fatty acid methyl ester derivatives (FAMES) were run on a Hewlett-Packard Model 5730A gas chromatograph (Palo Alto, CA) equipped with a flame-ionization detector and a 1.8 m × 4 mm i.d. glass column packed with GP 10% PEGS PS on 80/100 Supelcoport (Supelco, Inc.). Separations were effected under isothermal conditions at 180°C. Low concentrations of phosphatidic acid and phosphatidylserine required the use of a Hewlett-Packard Model 5890 capillary gas chromatograph equipped with a flame-ionization detector. An 8 m × 0.32 mm i.d. nonbonded phase, narrow-bore fused silica column SPB-5 (Supelco, Inc.) was employed for these sample separations. Standard FAMES were injected for both qualitative and quantitative determinations. Phospholipid quantification was based on a gravimetric analysis after major lipid separation and also was calculated from integrated gas chromatography (GC) peak areas for phospholipid fatty acids with appropriate mass adjustments made for each phospholipid class. This method was utilized for quantification because the inorganic phosphorus concentrations of the seed crystals, obtained by acid digestion (9), were at the detection limits of the Technicon Autoanalyzer Model 2.

RESULTS AND DISCUSSION

Static incubation of Ivory Coast cocoa butter at 26.5°C for 8–10 hr yields seed crystals representing 0.01% of 15 kg of cocoa butter. Typical differential scanning calorimetry (DSC) thermograms of Ivory Coast cocoa butter and seed crystal samples are illustrated in Figure 1. Samples were scanned from 0° to 100°C and yielded melting points ranging from onset or transition temperatures of 55.35° to 61.91°C for seed crystals and from 32.33° to 38.52°C for cocoa butter. An onset melting point of 55.53° ± 0.17°C was observed for seed crystals, while cocoa butter exhibited a 32.87 ± 0.27°C onset melting point. Onset melting points were nearly reproducible for both cocoa butter and seed crystal samples, indicating homogeneous composition within each sample set. Similar thermal characteristics indicated that day-to-day variations in solidification and isolation processes for seed crystal samples were minimized by randomization of samples.

Quantitative distribution of separated lipids is presented in Table 1. As reported by Davis and Dimick (4), significant differences exist between cocoa butter and seed crystal lipid classes. Cocoa butter samples exhibited lipid distributions consistent with reported values (4,10). Simple lipids were the most abundant lipid class at 99.34% with glycolipids at 0.31% and phospholipids at 0.34%. A comparison of these results with the lipid distribution of seed crystals revealed significant differences in complex lipid concentrations. Seed crystals were found to contain only 87.65% simple lipid, 6.02% glycolipid and 3.99% phospholipid, representing a sixteen- and twelvefold increase in the complex lipids, respectively.

Phospholipid analysis procedures were applied to three sample sets of purified cocoa butter and seed crystal phospholipids. Phospholipid classes were designated PG—phosphatidylglycerol, PE—phosphatidylethanolamine, PC—phosphatidylcholine, LPC—lysophosphatidylcholine, PS—phosphatidylserine, PA—phosphatidic acid, DPG—diphosphatidylglycerol, PI—phosphatidylinositol, and LPE for some lysophosphatidylethanolamine contamination. A single-dimension TLC separation system was applied to cocoa butter and seed crystal phospholipid samples (Fig. 2). The present study is the first to identify the presence of DPG and PG in cocoa butter (refer to SCPL3 in Fig. 2).

Gas chromatographic analyses of fatty acid methyl ester (FAMES) derivatives obtained from phospholipids of cocoa butter and seed crystal samples are presented in Table 2. No significant differences in concentration were found in palmitic (16:0), palmitoleic (16:1), linoleic (18:2), linolenic (18:3) and arachidic (20:0) acid in the seven classes of phospholipids between cocoa butter and the seed crystals. Myristic acid (14:0), which normally exists as less than 1% in cocoa butter (1), ranged from 1.1–4.6% in the isolated phospholipid classes in the Ivory Coast cocoa butter and was greater than 6% in the PG and PE fractions of the seed crystal. The phospholipid classes that contained significantly higher myristic acid in the seed crystals than the original cocoa butter were PG, PE, and PI + LPE. PE and PA had significantly lower stearic acid in the seed crystals and contained significantly less LPC. A comparison of oleic acid in the seven phospholipid classes demonstrated great variability and ranged from 8–26% in the seed crystal fraction. Oleic acid was nearly double in seed crystal PE and three times the concentration in PC of the seed.

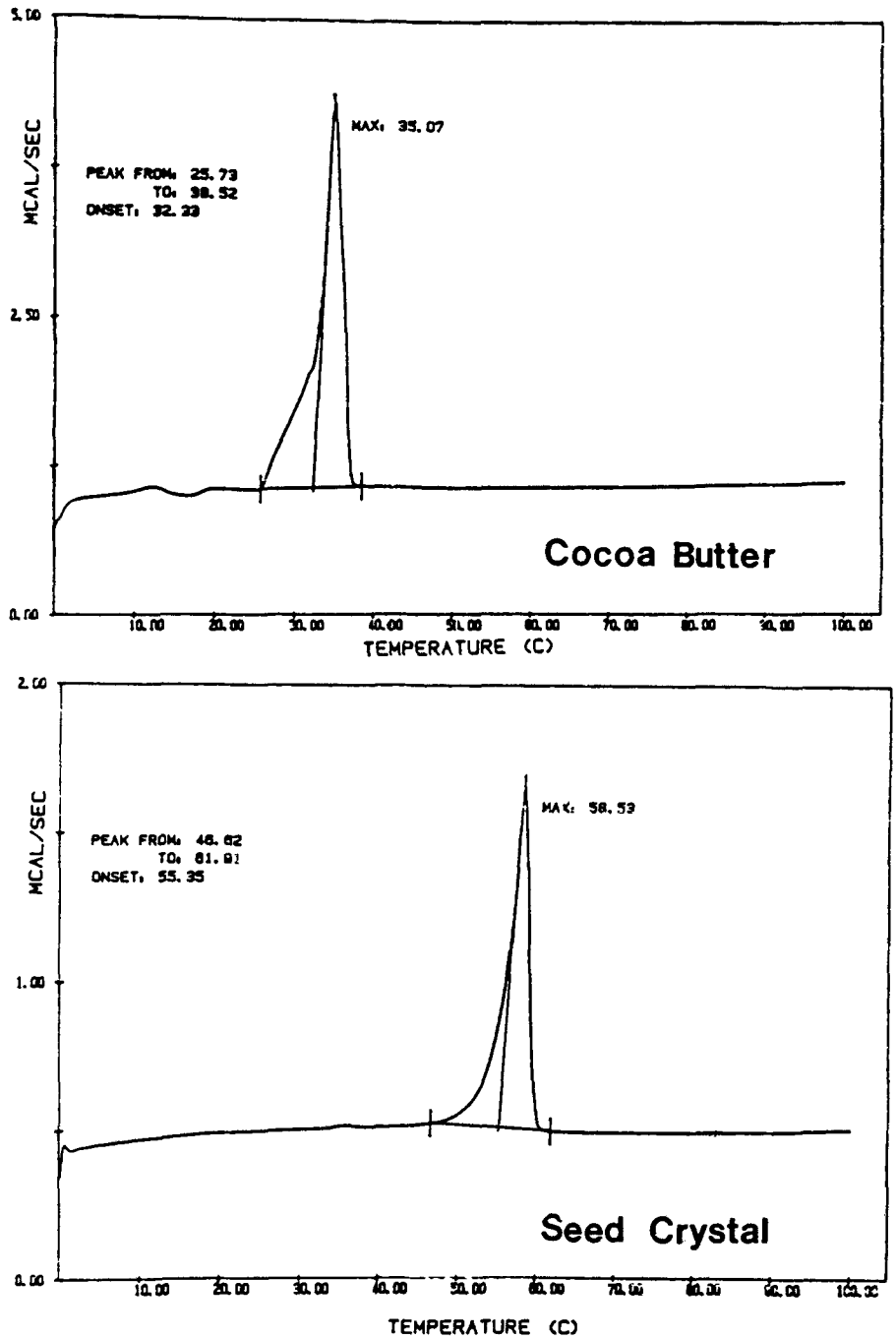

FIG. 1. DSC thermograms (endothermic) of a representative Ivory Coast cocoa butter and its seed crystal sample.

TABLE 1

Distribution of Major Classes of Lipids in Ivory Coast Cocoa Butter and Seed Crystals

Sample	Simple lipids	Phospholipids	Glycolipids	Recovery
		(mean wt% ± S.D.)[a]		
Cocoa butter	99.34 ± 0.05A[b]	0.34 ± 0.07A	0.31 ± 0.01A	101.16 ± 4.25
Seed crystals	87.65 ± 5.80B	3.99 ± 0.44B	5.02 ± 3.13B	96.67 ± 6.23

[a]Mean ± standard deviation (n = 3).

[b]Means within a column with the same upper case letter are not significantly different at $\alpha = 0.05$.

Table 3 presents the calculated percent mass distribution of Ivory Coast cocoa butter and seed crystal phospholipids. Values for cocoa butter phospholipid distribution are generally consistent with previous findings (10). Significant differences between cocoa butter and seed crystal PG, PA, PC and possibly PS are evident. PA and PS quantities were low; therefore, the significance of minor differences in these classes is suspect. Twice the concentration of PE was found in the seed crystal when compared to the original cocoa butter. PG and PC levels were depressed almost equally, 34 and 22% respectively, in the seed sample.

It may be speculated that the charged regions in the PE molecule accommodate water molecules more easily

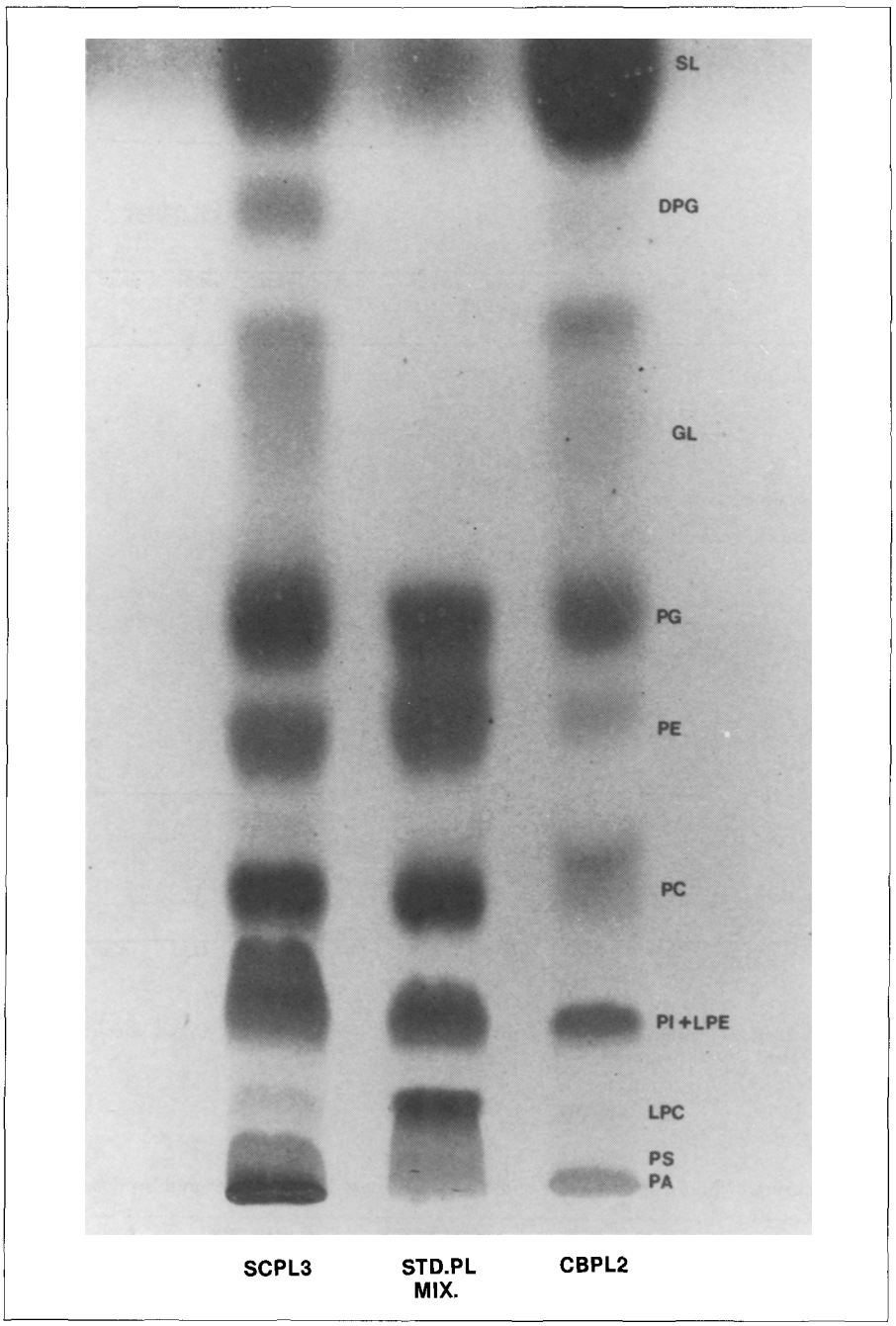

FIG. 2. Thin-layer chromatographic separation of phospholipids from seed crystals (SCPL 3), phospholipid standard mix (STDPL mix) and Ivory Coast cocoa butter sample (CBPL2). SL—simple lipids, DPG—diphosphatidylglycerol, GL—glycolipid, PG—phosphatidylglycerol, PA—phosphatidylethanolamine, PC—phosphatidylcholine, PI + LPE—phosphatidylinositol and lysophosphatidylethanolamine, PS—phosphatidylserine, PA—phosphatidic acid.

TABLE 2

Fatty Acid Composition of Ivory Coast Butter and Seed Crystal Phospholipids

Phospholipid class	Fraction	Fatty Acid Composition (mean wt% ± S.D.)[a]							
		14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0
PG	CB[b]	2.1 ± 0.3A[c]	43.0 ± 1.3A	tr[d]	40.2 ± 3.3A	7.3 ± 0.2A	4.8 ± 0.3A	tr	tr
	SC[e]	6.0 ± 0.7B	38.6 ± 3.0A	5.6 ± 0.6	35.2 ± 2.6A	8.6 ± 0.1A	4.5 ± 2.2A	tr	tr
PE	CB	2.6 ± 0.8A	45.2 ± 3.3A	2.5 ± 0.3A	40.2 ± 3.0A	8.7 ± 0.3A	tr	tr	tr
	SC	6.6 ± 0.8B	39.5 ± 1.8A	3.0 ± 0.7A	29.3 ± 2.1B	19.8 ± 2.0B	2.8 ± 0.2	tr	tr
PC	CB	2.2 ± 0.7	43.8 ± 1.9A	3.4 ± 0.2	43.8 ± 2.1A	7.0 ± 0.4A	tr	tr	—
	SC	tr	39.9 ± 7.7A	tr	32.8 ± 5.6A	25.7 ± 3.5B	tr	tr	—
PI+	CB	2.6 ± 0.1A	41.2 ± 4.2A	tr	35.7 ± 3.2A	19.5 ± 2.5A	—	tr	—
LPE	SC	4.4 ± 0.2B	35.0 ± 2.7A	8.9 ± 0.6	29.7 ± 2.1A	19.7 ± 2.1A	tr	tr	—
LPC	CB	4.6 ± 0.8	34.7 ± 0.6A	13.3 ± 1.5A	27.5 ± 2.8A	20.1 ± 1.5A	tr	tr	tr
	SC	tr	31.6 ± 1.9A	10.7 ± 2.2A	32.1 ± 1.0B	21.3 ± 2.3A	tr	tr	—
PS	CB	1.9 ± 0.2A	22.7 ± 1.4A	3.1 ± 0.2A	39.2 ± 0.5A	21.1 ± 0.7A	4.9 ± 1.0A	6.9 ± 0.2A	tr
	SC	1.0 ± 0.2A	19.2 ± 6.4A	3.7 ± 0.2A	33.1 ± 0.9B	24.4 ± 5.6A	7.4 ± 1.5A	9.2 ± 8.4A	tr
PA	CB	1.1 ± 0.2A	27.3 ± 6.9A	1.5 ± 0.3A	52.8 ± 3.0A	2.7 ± 1.5A	3.9 ± 0.8A	7.5 ± 6.2A	2.7 ± 0.4A
	SC	2.6 ± 0.9A	29.7 ± 2.8A	3.3 ± 0.4A	40.6 ± 3.0B	11.7 ± 7.1A	5.4 ± 1.1A	4.5 ± 2.5A	5.4 ± 4.0A

[a]Mean ± standard deviation (n = 3).

[b]Cocoa butter.

[c]Means within a fraction column with the same uppercase letter are not significantly different at $\alpha = 0.05$.

[d]Trace.

[e]Seed crystal isolated from original cocoa butter.

TABLE 3

Phospholipid Composition Based on Calculated FAMES in Ivory Coast Butter and Seed Crystals

Sample	PG	PE	PC	PI + LPE	LPC	PS	PA
	(mean wt% ± S.D.)[a]						
Cocoa butter	27.3 ± 0.6A[b]	15.6 ± 2.5A	37.7 ± 0.4A	10.9 ± 2.2A	8.0 ± 0.2A	0.3 ± 0.0A	0.2 ± 0.0A
Seed crystals	17.1 ± 1.3B	30.4 ± 2.0B	30.0 ± 0.7B	12.4 ± 0.9A	9.6 ± 0.8A	0.1 ± 0.0B	0.3 ± 0.0B

[a]Mean ± standard deviation (n = 3 of 2 replicates).

[b]Means within a column with the same upper case letter are not significantly different at $\alpha = 0.05$.

than other phospholipids (11). Phospholipids hydrate easily and can exist in different hydration states (11). Maximally dried phospholipids are reported to contain 1-2 molecules of water per molecule of lipid, while 23-34 water molecules per phospholipid have been reported for undried phospholipid systems (12). Ten molecules of water have been found to be directly associated with the polar head group of PC, for example, while 11 molecules were determined to occupy space between PC lamellae in pure PC systems (13). It seems reasonable that approximately 10 molecules of H_2O may be associated with each phospholipid in a mixed lipid system such as cocoa butter. The amphiphilic nature of phospholipids may be the basis of their importance in early crystallization events. Polar lipids, such as phospholipids and glycolipids, are classified as insoluble in water and have the ability to swell into cubic or hexagonal crystalline phases (14). It is evident from this study that these charged molecules preferentially crystallize from the surrounding cocoa butter. Phospholipid polarity may make it energetically favorable for them to crystallize from this non-polar environment. It may be speculated that these insoluble amphiphiles may form inverted hexagonal crystals and that these structures could ultimately result in spiney rod-like crystals in the primary stage of cocoa butter crystallization as seen with scanning election microscopy (15).

During nucleation and in the early stages of crystal growth, conformational shifts within the growing crystals are necessary to accommodate crystallizing triacylglycerols. Mildly hydrated phospholipids may act as slip planes within the crystal, providing an efficient mechanism to relieve conformational stresses within the developing crystal. Given hydrogen bonding possibilities between the nonpolar phospholipid acyl chains and neighboring triacylglycerols, one may speculate on a close association between these two molecules. Phospholipids may anchor triacylglycerols by interdigitation of their nonpolar acyl chains, while polar head groups, with associated water, may act between triacylglycerol layers. Molecular adjustments between triacylglycerol layers would therefore be facilitated during cocoa butter solidification.

It is important to note that the impact of surfactants

on the crystallization of fats has been recognized for some time (16). Lecithins have been utilized to control the viscosity of chocolate by destabilization of the polymorphic forms, thus reducing viscosities and solidification rates (17). It is apparent, therefore, that the high phospholipid concentrations associated with cocoa butter seed crystals may have an opposite effect when compared to the addition of surfactants to chocolate during processing. In order to attempt to understand the effects of the polar fraction (phospholipids and glycolipids) on nucleation and crystal growth rates, additional information is required. This would include knowledge of the concentration of free and bound water, hydrophilic-lipophilic balance, liquid-to-solid ratio and polymorphic form within the chocolate mass.

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