Cocoa Butter Biosynthesis: Effect of Temperature on *Theobroma cacao* Acyltransferases

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Enzymes catalyzing the first and last steps in cocoa butter biosynthesis were studied in microsomal preparations from developing cocoa seeds. Both the acyl-CoA:sn-glycerol-3-phosphate 0-acyltransferase (EC 2.3.1.15) and the acyl-CoA:1,2-diacylglycerol 0-acyltransferase (EC 2.3.1.20) showed broad specificity for three fatty acid donors (palmitoyl-CoA, stearoyl-CoA and oleoyl-CoA), but the relative amounts of incorporation of these fatty acids into lysophosphatidic acid and triacylglycerol were influenced by temperature of incubation. The selectivities of the two acyltransferases for saturated vs unsaturated acyl-CoA's also were investigated over a range of temperatures. The data indicate that, in general, the ratio of saturated to unsaturated fatty acids incorporated into glycerol esters increases with increasing temperature of incubation, consistent with the hypothesis that temperature effects on these enzymes contribute to the phenomenon of "soft" cocoa butter, i.e., that cocoa butter obtained from seeds grown in cooler climates has a lower melting point than that prepared from seeds grown in warmer climates.

Triacylglycerol is the major storage product of cocoa beans, the seeds of the cocoa tree, *Theobroma cacao*. These storage products are a mixture of glycerol esters of palmitic, oleic, stearic and much smaller amounts of other fatty acids. This collection of esters, called cocoa butter, is at $4.50/kg (December 1986, Cocoa Merchants' Association) one of the most expensive edible fats in the world. Cocoa butter is used by both the confectionery and cosmetic industries; its annual world production exceeds two million metric tons (MT). A mature cocoa bean can store up to 700 mg of cocoa butter, and because a tree may produce yearly as many as 2,000 seeds, a single tree could yield up to 1.5 kg of cocoa butter annually.

The physical properties of cocoa butter, most notably its ability to "melt on the palate," explain its value to the chocolate manufacturing industry. Here also is why beans yielding cocoa butter with lower melting points bring correspondingly lower prices for growers. The presence of lower melting cocoa butter, called "soft butter," in a chocolate manufacturing formulation may cause production lines to slow, may require cooler temperatures, and in worst cases may prevent chocolate from hardening at all. Soft butter is a problem of some economic significance to world cocoa growers; those operating at great distances from the equator (1,600 km or more) are most affected because of the positive correlation between cocoa butter melting point and mean daily temperature during cocoa fruit development (1).

Brazilian cocoa, well known for its soft butter, generally brings a lower price ($.06/kg lower, December 1986, Cocoa Merchants' Association). Given annual production approaching 400,000 MT, this amounts to $24 million. Soft butter is due to relatively high unsaturated fatty acid content. Of a variety of factors investigated as possible candidates affecting the degree of fatty acid unsaturation of Brazilian cocoa butter, only temperature variations during seed development were found to have a significant effect (1). Further evidence for the importance of temperature during the growth period in determining the degree of cocoa butter fatty acid unsaturation was obtained in controlled microclimate experiments where it was demonstrated that seeds developing at an artificially elevated temperature produced triacylglycerol containing a lower percentage of oleic and linoleic acids and, consequently, a higher cocoa butter melting point (2). We have designed experiments to test the hypothesis that temperature has an effect on the specificity and selectivity of the acyltransferases catalyzing steps in cocoa butter biosynthesis which can, in part, account for these observations.

MATERIALS AND METHODS

Preparation of microsomes. Cocoa (*Theobroma cacao*,

TABLE 1

Effect of Temperature on Acyl-CoA Specificity of Cocoa Glycerol-3-Phosphate Acyltransferase

	(1-^{14}C) Acyl incorporation into lysophosphatidic acid (nmol 30 min^{-1} mg^{-1} × 10^2)					
Acyl-CoA	15 C	20 C	25 C	30 C	35 C	40 C
palmitoyl	129(0.4)[a]	138(0.4)	210(0.6)	240(0.7)	351(1.2)	300(1.0)
stearoyl	282(0.8)	276(0.8)	273(0.8)	363(1.1)	354(1.2)	426(1.4)
oleoyl	360	354	351	324	300	297

[a]Figures in parentheses express the ratio of palmitate (or stearate) incorporation to oleate incorporation at the indicated temperatures.

L.) seeds were obtained from pods picked four or five days previously at the Hershey Foods Corp. Research Farm in Belize, Central America. Seeds were removed from the pods (aged ca. 120 to 150 days after pollination, the stage of seed development when the rate of triacylglycerol accumulation is at a maximum), and the surrounding pulp and testae were completely removed and discarded. The seeds were placed directly into liquid nitrogen. Between 65 and 70 g of frozen seeds were powdered in liquid nitrogen using a mortar and pestle and then mixed with 150 ml of isolation buffer. The isolation buffer (pH 8.0, 4 C) contained 350 mM mannitol, 40 mM Tris-HCl buffer, 5 mM EDTA, 15 mM β-mercaptoethanol, 1% polyvinylpyrrolidone 10,000, and 100 mM diethyldithiocarbamate. All further procedures were carried out at 4 C. The mixture was dispersed in a Waring Blendor: four 15-sec bursts. The dispersion was strained through four layers of cheese cloth and centrifuged for 15 min at 12,000 × g. The supernatant was recovered and centrifuged for 30 min at 300,000 × g to produce a microsomal pellet. The pellet was recovered and washed in 75 ml of isolation buffer and centrifuged at 12,000 × g for 30 min to obtain a clean pellet. The pellet was suspended in 2 ml of suspension buffer (pH 8.0): 20 mM Tris-HCl buffer, 15 mM β-mercaptoethanol. This microsomal preparation was divided into 50-μl aliquots and stored at −80 C.

The protein concentration of the microsomal preparation was determined using the Pierce Chemical Co. protein assay reagent with bovine serum albumin as the standard.

Assays of glycerol-3-phosphate acyltransferase. The standard assay mixture contained 40 mM Tris-HCl buffer (pH 7.5), 4 mM dithiothreitol, 2 mM $MnCl_2$, 8 mM glycerol-3-phosphate and 95 μg protein of the microsomal preparation in a total reaction volume of 250 μl. Saturating concentrations of the acyl-CoA fatty acid (Sigma Chemical Co., St. Louis, Missouri) donors were used in the specificity experiments: 0.2 mM (1-^{14}C) oleoyl-CoA (1308 cpm/nmol), 0.2 mM (1-^{14}C) palmitoyl-CoA (1307 cpm/nmol), and 0.4 mM (1-^{14}C) stearoyl-CoA (659 cpm/nmol). All radioisotopes were obtained from Amersham. For the selectivity experiments, equimolar concentrations of the two acyl-CoA fatty acid donors to be tested were added to the standard assay mixture. In one-half of the reaction tubes the saturated fatty acid donor was labelled; in the other half the unsaturated fatty acid donor was labelled.

Each reaction tube was incubated 30 min at a designated temperature between 15 and 40 C. The reaction was stopped and the lipids extracted by the addition of 250 μl of ice cold chloroform. The tube was vortexed for 15 sec and centrifuged to separate the aqueous and organic phases. The organic phase was removed and dried under a stream of nitrogen gas. The residue was

TABLE 2

Effect of Temperature on Acyl-CoA Specificity of Cocoa Diacylglycerol Acyltransferase

	(1-^{14}C) Acyl incorporation into triacylglycerol (nmol 30 min^{-1} mg^{-1} × 10^2)					
Acyl-CoA	15 C	20 C	25 C	30 C	35 C	40 C
palmitoyl	1,160(1.5)[a]	1,670(1.3)	2,080(1.1)	1,790(1.3)	2,160(1.7)	2,200(1.9)
stearoyl	640(0.8)	1,070(0.8)	2,060(1.0)	1,660(1.2)	1,690(1.3)	1,600(1.4)
oleoyl	750	1,290	1,960	1,360	1,280	1,180

[a]Figures in parentheses express the ratio of palmitate (or stearate) incorporation to oleate incorporation at the indicated temperatures.

TABLE 3

Effect of Temperature on Acyl-CoA Selectivity of Cocoa Glycerol-3-Phosphate Acyltransferase

	(1-^{14}C) Acyl incorporation into lysophosphatidic acid (nmol 30 min^{-1} mg^{-1} × 10^2)					
Acyl-CoA	15 C	20 C	25 C	30 C	35 C	40 C
palmitoyl	48(0.1)[a]	75(0.2)	96(0.3)	114(0.3)	132(0.2)	213(0.3)
oleoyl	378	414	375	405	615	750
stearoyl	84(0.4)	69(0.2)	75(0.2)	111(0.2)	150(0.3)	159(0.2)
oleoyl	222	315	366	564	471	672

[a]Figures in parentheses express the ratio of saturated to unsaturated fatty acid incorporation at the indicated temperatures.

mixed with a small volume of chloroform and spotted on a precoated silica gel TLC plate for separation and analysis of lipid products.

Assays of diacylglycerol acyltransferase. The standard assay mixture contained 40 mM Tris-HCl buffer (pH 7.5), mM dithiothreitol, 3 mM $MgCl_2$, 5 mM 1-palmitoyl-2-oleoyl glycerol (Serdary) in 0.1% Tween 20, and 200 μg protein of the microsomal preparation in a total reaction volume of 250 μl. Concentrations of 0.2 mM (1-^{14}C) acyl-CoA's were used in the specificity experiments. Selectivity experiments were conducted as described for the glycerol-3-phosphate acyltransferase assay, including incubation and treatment of the reaction mixture.

Identification of reaction products. The radioactive products were co-chromatographed with authentic lipid standard (Supelco Inc., Bellefonte, Pennsylvania) on precoated silica G Uniplates (Analtech). Plates spotted with the products of the glycerol-3-phosphate acyltransferase assay were developed in a polar lipid separation solvent containing chloroform/methanol/acetic acid/water (170:30:20:7, v/v/v/v). The lysophosphatidic acid area was identified by exposure to iodine vapor. This area was removed from the plate, mixed with 5 ml ACS II scintillation fluid, and counted in a Beckman LS-100 liquid scintillation counter.

Plates spotted with the products of the diacylglycerol acyltransferase assay were developed in a neutral lipid separation solvent containing chloroform acetone (96:4, v/v). The triacylglycerol areas were identified and counted as described for lysophosphatidic acid.

RESULTS

Effect of temperature variation on acyl specificity of glycerol-3-phosphate acyltransferase. ^{14}C Labeled palmitoyl, stearoyl and oleoyl-CoA's were offered separately as substrates for the glycerol-3-phosphate acyltransferase reaction at incubation temperatures ranging from 15 to 40 C. All three fatty acid donors were accepted for incorporation into the lysophosphatidic acid product. The data in Table 1 shows that with increasing temperature, the incorporation of palmitate and stearate into lysophosphatidic acid tended to increase, while oleate incorporation decreased. At the lower end of the temperature range (15-25 C), oleate incorporation exceeded that of both saturated fatty acids; at the upper end of the range (35-40 C), the situation was reversed. When palmitate and stearate incorporation are compared to oleate incorporation by the use of simple ratios (Table 1), it is clear that lower temperatures are more favorable for unsaturated fatty acid incorporation than higher temperatures in vitro.

Effect of temperature variation on acyl specificity of diacylglycerol acyltransferase. The three ^{14}C labelled acyl-CoA's were offered separately as substrates for the diacylglycerol acyltransferase reaction. Fatty acid incorporation into triacylglycerol was measured over the range of incubation temperatures. Again, as for the glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase was capable of using all three acyl-CoA fatty acid donors. Palmitate incorporation exceeded oleate incorporation over the entire range of temperatures; however, stearate incorporation was greater than oleate incorporation only at temperatures between 25 and 40 C; at lower temperatures the incorporation of oleate was slightly greater than stearate. Table 2 shows that for increasing temperature of incubation the ratios of stearate to oleate incorporation increase. While the ratios of palmitate to oleate incorporation do not show a steady increase with increasing temperatures, the average ratio between 15 and 25 C (1.3/1) is less than the average ratio between 30 and 40 C (1.6/1). Unsaturated fatty acid incorporation into triacylglycerol via diacylglycerol acyltransferase activity is thus more likely to occur at lower temperatures than at higher temperatures in vitro.

Effect of temperature variation on acyl selectivities of glycerol-3-phosphate acyltransferase and diacylglycerol acyltransferase. Equimolar concentrations of palmitoyl-CoA and oleoyl-CoA were offered together as substrates for the glycerol-3-phosphate acyltransferase reaction and the diacylglycerol acyltransferase reaction at incubation temperatures ranging from 15 to 40 C. The incorporation of each fatty acid into lysophosphatidic acid or triacylglycerol was measured at each temperature. Stearic and oleic acids were likewise offered and their incorporation measured. Tables 3 and 4 show the results of these experiments. The ratios of saturated to unsaturated fatty acid incorporation into lysophosphatidic acid do not vary significantly with temperature of incubation. The ratios of palmitate to oleate incorporation into triacylglycerol show the most variation with temperature, such that at lower temper-

TABLE 4

Effect of Temperature on Acyl-CoA Selectivity of Cocoa Diacylglycerol Acyltransferase

	(1-^{14}C) Acyl incorporation into triacylglycerol (nmol 30 min^{-1} mg^{-1} × 10^2)					
Acyl-CoA	15 C	20 C	25 C	30 C	35 C	40 C
palmitoyl	207(0.6)[a]	225(0.3)	489(0.5)	531(0.7)	888(1.2)	750(0.9)
oleoyl	333	708	1,026	777	768	828
stearoyl	12(0.03)	66(0.1)	102(0.1)	180(0.2)	429(0.4)	282(0.3)
oleoyl	390	612	1,161	1,068	1,176	1,083

[a]Figures in parentheses express the ratio of saturated to unsaturated fatty acid incorporation at the indicated temperatures.

atures (15-25 C) the ratios are smaller than at higher temperatures (30-40 C).

These results suggest that temperature of incubation does not greatly affect the acyl selectivity of glycerol-3-phosphate acyltransferase, but that lower temperatures of incubation may be more favorable for oleate over palmitate incorporation in the diacylglycerol acyltransferase mediated reaction.

DISCUSSION

Temperature dependence of saturated/unsaturated fatty acid ratios is well documented in oilseed plants where it has been observed consistently that plants grown in cooler climates produce seeds which have greater unsaturated fatty acid content (3,4). Concentrations of seed unsaturated fatty acids are controlled by stearoyl-ACP desaturase (5) and by the oleoyl-CoA-phosphatidylcholine exchange reaction (6). Fatty acid composition of seed triacylglycerol is controlled by the acyltransferases catalyzing glycerol ester formation. This control could be regulated in the plant by intracellular substrate concentrations or by intrinsic properties of the enzymes. Ichihara (4,7) studied the properties of safflower diacylglycerol acyltransferase, the final enzyme, and glycerol-3-phosphate acyltransferase, the first enzyme in the Kennedy (8) pathway, and concluded in each case that the composition of the endogenous acyl-CoA pool was the determining factor in triacylglycerol fatty acid composition.

Our experimental design precludes comment on the role of acyl-CoA pools in determining cocoa butter fatty acid composition, but results of the experiments reported herein are clearly consistent with the hypothesis that temperature effects of the cocoa plant acyltransferases contribute to the observed lower melting point of cocoa butter obtained from seeds grown in cooler climates.

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