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Contributed Paper

Synthesis of Cocoa Butter Equivalent from Palm Oil by *Carica papaya* Lipase-Catalyzed Interesterification

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ABSTRACT

Cocoa butter equivalent could be synthesized by lipase-catalyzed interesterification of oil. The objective of this research was to investigate the synthesis of cocoa butter equivalent from interesterification of palm oil catalyzed by *Carica papaya* lipase. The study showed that the compositions of cocoa butter equivalent were affected by acyl donor sources, substrate ratio, initial water of enzyme, reaction time, reaction temperature and the amount of enzyme. Among three acyl donors tested (methyl stearate, ethyl stearate and stearic acid), methyl stearate appeared to be the best acyl donor for the synthesis of cocoa butter equivalent from palm oil catalyzed by *C. papaya* lipase. The best reaction conditions were : substrate ratio (palm oil: methyl stearate, mol/mol) at 1 : 4, water activity of enzyme at 0.11, reaction time at 4 h, reaction temperature at 45°C and 18% by weight of the enzyme. The chemical and physical properties of cocoa butter equivalent were 9.75 ± 0.41% free fatty acid, 44.89 ± 0.84 iodine number, 193.19 ± 0.78 sponification value and melting point at 37-39°C. The yield of cocoa butter equivalent was 55% based on palm oil used.

Keywords: cocoa butter equivalent, *Carica papaya* lipase, palm oil.

1. INTRODUCTION

Cocoa butter is the natural fat extracted from the cocoa bean and its color is slightly yellowish [1]. Cocoa butter is an important ingredient in the chocolate and related confectionery industries. It is responsible for the different favorable characteristics such as hardness at room temperature, brightness, fast and complete melting when placed in the mouth [2]. Cocoa butter is composed of three main triacylglycerols (TAGs): 1, 3-dipalmitoyl-2-oleoylglycerol (POP); 1(3)-palmitoyl-3(1)-stearoyl-2-oleoylglycerol (POS) and 1,3-distearoyl-2-oleoylglycerol (SOS), with oleic acid in the *sn*-2 position [3]. The typical fatty acid composition of cocoa butter in mole percentage is : 24.4% palmitic acid, 33.6% stearic acid, 37.0% oleic acid, 3.4% linoleic acid and 1.6% others [4]. Cocoa butter contains stearic acid and palmitic acid in a ratio of 1.3: 1.0 [5]. Due to high cost and fluctuations in the supply and demand of cocoa butter, cocoa butter equivalent (CBE) with a TAGs

composition similar to cocoa butter is used as an alternative source [6].

Cheap commercial oil that have TAGs with oleic acid in the 2-position can be converted to CBE for adding the value of oils. Palm oil is low cost fat and readily available. Therefore, palm oil is a suitable raw material for the production of CBE.

Preparation of CBE through enzymatic-catalyzed interesterification is attractive because lipases offer certain advantages over other chemical catalysts. One of these advantages is that it produces fewer by-products. While chemical catalysts will randomize all of the fatty acids in TAGs mixture, 1, 3-specific lipase can incorporate fatty acids into the *sn*-1,3-positions without changing the fatty acid residues in the *sn*-2-position [6]. Other advantages are lower energy consumption and better product control. Recently, vegetable oils such as Mahua, Kokum and mango fats, palm oil midfraction, teaseed oil, and olive oil have been used to prepare CBE by microbial lipases in batch stirred tank reactor [7-10]. However, procedures of lipase-catalyzed interesterification for CBE production have not been practically applied in industries because the cost of microbial lipase is high.

Over the last century, latex from *Carica papaya* has been well known for containing papain which is a cysteine protease with numerous industrial applications. The particulate of *C. papaya* latex possesses lypolytic activity with 1(3)-regiospecificity [11]. *C. papaya* lipase (CPL) has potential as a biocatalyst in lipid transformations such as milk fat modification [12] and synthesis of low-calorie structured TAGs [13]. This latter observation led us to consider that CPL may be a useful inexpensive biocatalyst for the synthesis of CBE. In this study, CBE was prepared by *C. papaya* lipase-catalyzed interesterification of palm oil. Several factors, such as acyl donor sources, reaction time, temperature, initial water of enzyme, amount of enzyme, and substrate ratio were studied.

2. MATERIALS AND METHODS

2.1 Chemicals and Materials

Palm oil was purchased from Kesorn. Methyl stearate, ethyl stearate, stearic acid, methyl palmitate, ethyl palmitate and palmitic acid were purchased from Sigma. BF_3-methanol and silica gel plate were purchased from Merck. Lithium chloride (LiCl), potassium acetate (CH_3COOK), magnesium chloride ($MgCl_2$) and magnesium nitrate ($Mg(NO_3)_2$) were purchased from Fluka.

2.2 Preparation of *C. papaya* lipase

Papaya latex was obtained by making a longitudinal incision on the unripe fruit (70-100 days) of Thai papaya tree. The latex was then collected and stored at -20°C before defrosting at room temperature and then centrifuged at 9,500×g for 15 min. The insoluble particulate was lyophilized and used as *C. papaya* lipase. The water content and water activity of *C. papaya* lipase were determined by a Karl-Fischer 684 coulometer equipped with a 688 KF oven (Metrohm, Switzerland) and a Thermoconstanter TH200 Novasina (Novasina, Switzerland), respectively. The hydrolysis activity of *C. papaya* lipase was investigated by colorimetric method [14].

2.3 Determination of the Fatty Acid Profiles of Glycerides in Palm Oil

Palm oil (40 mg) was weighted into a small Erlenmeyer flask and then 3 ml of 0.5 M methanolic sodium hydroxide were added. The mixture was heated over a steam bath in hood until a homogeneous solution was obtained. For saponification reaction, BF_3-methanol (5 ml) was added to the reaction mixture and then boiled for 2 to 3 minutes. The solution was cooled and transferred into a separatory funnel containing 25 ml of hexane

and 20 ml of saturated NaCl solution. The solution was gently shaked well and allowed the layers to separate [15]. The hexane layer, containing the fatty acid methyl esters, was dried with about 1 g of anhydrous $MgSO_4$ and filtered into a small vial. The solution was concentrated on the steam bath until the volume was reduced to 0.5 ml. This solution of fatty acid methyl esters was analyzed by gas chromatography-mass spectrometry as described in 2.6. Approximate values of the molecular weights of the oils were calculated using data obtained from the fatty acid profiles [16].

2.4 Optimization of Cocoa Butter Equivalent Preparation

Experiments were conducted to study the factors, such as acyl donor sources, reaction temperature, reaction time, and initial water activity of enzyme, amount of enzyme and mole ratio of plam oil: methyl stearate, affecting the TAGs content of CBE.

First, effect of acyl donor sources on TAGs content of CBE was studied. The reaction mixture consisted of palm oil 2 mmol and each 4 mmol of acyl donor source such as methyl stearate, ethyl stearate and stearic acid. These reactions catalyzed by *C. papaya* lipase (0.5 g) were carried out at 40°C for 24 h and samples (10 mg) were taken and the only modified oil was separated from reaction mixture by Thin-layer chromatography (TLC) as described in 2.5. Effect of reaction temperature (40-55°C) and reaction time (0-24 h) were investigated by using 0.5 g *C. papaya* lipase, 2 mmol palm oil and 4 mmol methyl stearate. For effect of initial water activity of enzyme, before starting of the reaction, the enzyme was pre-equilibrated with the water vapor of saturated salt solutions [17]. Pre-equilibration was done at 25°C for 5 days. The saturated salt solution used were prepared with LiCl (water activity, a_w = 0.11), CH_3COOK (a_w = 0.23), $MgCl_2$ (a_w = 0.33) and $Mg(NO_3)_2$ (a_w = 0.53). A water activity of the bio-catalyst was determined using a Thermoconstanter TH 200 Novasina. The enzyme (0.5 g) with various a_w was added to the reaction mixture consisted of 2 mmol palm oil and 4 mmol methyl stearate. The reaction was carried out at 45°C for 4 h.

To study the effect of amount of enzyme, the enzyme (9-36 wt %) having α_w = 0.11 was added into the same reaction. For effect of mole ratio of substrate, various mole ratio (1:1, 1:2.3, 1:3, 1:4 and 1:5.6) of palm oil: methyl stearate were applied to the reaction that consisted of enzyme 18 wt % and the reaction was carried out at 45°C for 4 h.

2.5 Isolation of Modified Oil by TLC

A single plate of TLC on silica gel plate was applied with a solution of reaction mixture in a row across one side of the silica gel plate, 2 cm from the edge. The plate was developed in hexane-diethyl ether-acetic acid (80:20:1) [15]. After solvent front raised to about 1 cm from the top of the plate, the plate was removed and made a small scratch at the solvent level. The chromatogram was allowed to dry and then placed in an iodine chamber for several minutes. The plate was removed and lightly trace with a pencil around red-brown band. The mobility of each band was calculated relative to the solvent front (R_f). Only band of TAG on the TLC plates was scrapped. The separated TAGs were converted to fatty acid methyl ester with BF_3- methanol for further analysis.

2.6 Analysis of Fatty Acid Profile

The acyl composition was determined by a gas chromatograph (GC 6850, Agilent Technologies) fitted with a capillary column (HP-1MS, 30 m × 0.25 mm, 0.25 μm thickness) and equipped with mass spectrometer (MSD 5973(EI), Agilent Technologies). The

chromatographic conditions were as follow: on temperature of MS Quadrupole and MS Source were 150°C and 230°C, helium as a carrier gas at flow rate 1.0 ml/min, and an injector temperature of 250°C. Separations were made using the following oven temperature profile: initial temperature 140°C, programmed to 240°C at 10°C/min, and final temperature held for 15 min.

2.7 Melting Point Determination

Melting point of CBE was determined by a differential scanning calorimeter (model DSC_2, Perkin-Elmer, Norwalk CT). The data were collected by a Perkin-Elmer data station, model 3000, and analyzed by a standard DSC program.

2.8 Physicochemical Analysis of Cocoa Butter Equivalent

The iodine value and saponification value of CBE were determined by AOAC methods [18]. Acid value was assayed by the IUPAC II.D.19 method [19].

2.9 Yield of Cocoa Butter Equivalent

Cocoa butter equivalent was prepared under optimized conditions: substrate ratio (palm oil: methyl stearate, mol/mol) at 1:4, 18% by weight of the enzyme, a_w of enzyme = 0.11, reaction time at 4 h and temperature of reaction at 45°C. Acetone fractionation [20] was performed to purification of the product. Yield of CBE was calculated with the following equation:

$$\text{Yield of CBE (\%)} = \frac{\text{Mole of CBE} \times 100\%}{\text{Mole of palm oil}}$$

3. RESULTS AND DISCUSSION

3.1 Preparation of *C. papaya* Lipase

Fresh papaya latex was found to contain 80 u of lipase/g of latex. High speed centrifugation (9,500×g) was required to separate the particulate part from the latex. This part of latex possessed all lipase activities which were found to be 200 u of lipase/g of particulate fraction, whereas no activities were found in a clear solution. The optimum temperature and optimum pH of *C. papaya* lipase in hydrolysis activity of palm oil was 45°C and 7, respectively. The lipase activity of dried lyophilized *C. papaya* lipase on palm oil hydrolysis was 725 u of lipase/g of lyophilized particulate. The crude CPL (a_w = 0.396 and 3.60 % water content) was used as biocatalyst in further interesterification reaction of palm oil without purification because of the tight association of lipase with the particulate fraction [21].

3.2 Characterization of the Palm Oil

The fatty acid contents of palm oil were determined as a preliminary step in this characterization (Table 1). Palm oil contained high amount of oleic acid (48%) and palmitic acid (40.8%). However, it contained very little amount of stearic acid. Structure of TAGs in palm oil consisted of 26% POP, 24% LOP, 17% OOP, 4% OOO, 3% POSt and StOO and 23% others [16]. This is indicated that palm oil is suitable for as a starting material because it contained high amounts of oleic acid and the major of this fatty acids was in the 2-position. This is an important prerequisite of starting oil in the production of cocoa butter equivalents since the 2-oleic acid is required for the maintenance of the characteristic sharp melting point of cocoa butter [22].

3.3 Optimization of Cocoa Butter Equivalent Formation by *C. papaya* Lipase

3.3.1 Effect of Acyl Donor

Source of acyl donor plays an important role in the interesterification reaction of oils. In this research, only stearyl donors were incorporated in palm oil because of that the oil already contained palmitic acid at

Table 1. Fatty acid composition of palm oil.

Fatty Acid	%
Myristic acid (C 14:0)	1.0
Palmitic acid (C 16:0)	40.8
Stearic acid (C 18:0)	0.6
Oleic acid (C 18:1)	48.1
Linoleic acid (C 18:2)	9.5

Fatty acid methyl esters were prepared from palm oil by methylation using BF_3-methanol. The methyl esters were analyzed by GC-MS.

concentrations near the desired level. Methyl stearate, ethyl stearate and stearic acid were applied to the interesterification of palm oil which was catalyzed by *C. papaya* lipase. Among the acyl donors used only methyl stearate was an appropriate stearyl donor for interesterification reaction with palm oil catalyzed by *C. papaya* lipase (Table 2) since the product contained stearic and palmitic acid in a ratio of 1.37: 1.0 which similar to cocoa butter [5]. While different lipase such as that from *Rhizopus arrhizus* preferred to catalyzes the interesterification reaction of palm oil with stearic acid or ethyl stearate in CBE preparation [23-25].

Table 2. Effect of acyl donors on fatty acid profiles of cocoa butter equivalent synthesized by *C. papaya* lipase.

Acyl donor source	Fatty acid (%)				Ratio of S:P
	C16:0	C18:0	C18:1	C18:2	
Methyl stearate	26.9	36.8	30.4	6.0	1.3:1
Ethyl stearate	24.5	52.1	23.4	-	2.1:1
Stearic acid	30.2	24.4	37.7	7.7	0.8:1

The lipase (0.5 g) was added to a reaction mixture containing palm oil (2 mmol) and various acyl donors (4 mmol) at 45°C for 4 h. Palm oil composition L : 9.5%; M : 1.0%; O : 48.1%; P : 40.8%; S : 0.6%. L = linoleic acid; M = myristic acid; O = oleic acid; P = palmitic acid; S = stearic acid.

3.3.2 Effect of Reaction Temperature

Temperature also exerts an important influence on enzymatic interesterification. As shown in Table 3, the optimum temperature for interesterification was 45°C. With an increase of reaction temperature, in the range of 50-55°C, the ratio of stearic acid: palmitic acid decreased respectively. There are several possible reasons to explain this phenomenon: (1) substrates, methyl stearate can be well dissolved at the temperature more than 40 °C and lead to a low viscosity reaction mixture

in which interesterification can be carried out quickly [6]; (2) activation energy of the interesterification reation is 30.4 j/mol [26]; (3) high temperatures inactivate the lipase [6], which leads to a strong decrease in reaction rate when the temperature is beyond 45°C. The temperature of 45°C was adopted in further CBE preparation.

Table 3. Effect of reaction temperatures on fatty acid profiles of cocoa butter equivalent synthesized by *C. papaya* lipase.

Reaction temperature (°C)	Fatty acid (%)				Ratio of S:P
	C16:0	C18:0	C18:1	C18:2	
40	32.5	28.9	35.1	3.5	0.9:1
45	26.9	36.8	30.4	6.0	1.3:1
50	31.6	34.8	32.1	1.6	1.1:1
55	31.3	28.2	36.5	4.0	0.9:1

The lipase (0.5 g) was added to a reaction mixture containing 2 mmol palm oil and 4 mmol methyl stearate at various temperatures for 4 h.

3.3.3 Effect of Reaction Time

Reaction time was set from 0 to 24 h to evaluate the time effect on the fatty acid profiles of CBE at 45°C using palm oil and methyl stearate as substrates (Table 4). The content of palmitic acid decreased rapidly from 40.8% in palm oil to desired level (26.9%) at 4 h. Meanwhile, level of stearic acid increased rapidly from 1.58% in palm oil to 36.8%. Incorporation of stearic acid to desired levels was achieved in shorter reaction times of about 4 h. With an increased of reaction time, the content of stearic acid was incorporated over the demand level. Therefore, on the basis of the above finding, reaction time of 4 h appeared to be optimal and was used in the following experiments.

Table 4. Effect of reaction times on fatty acid profiles of cocoa butter equivalent synthesized by *C. papaya* lipase.

Reaction time (h)	Fatty acid (%)				Ratio of S:P
	C16:0	C18:0	C18:1	C18:2	
0	40.8	1.6	48.1	9.5	0.04:1
4	26.9	36.8	30.4	5.9	1.3:1
8	24.4	39.3	31.5	4.9	1.6:1
24	19.4	44.3	30.1	0.2	2.3:1

The lipase (0.5 g) was added to a reaction mixture containing palm oil (2 mmol) and methyl stearate (4 mmol) at 45°C.

3.3.4 Influence of the Initial Water Activity

C. papaya lipase was pre-equilibrated separately at a desired water activity (a_w) using saturated salt solutions (from 0.11 to 0.53). After 5 days, *C. papaya* lipase was mixed with reaction medium and the synthesis of CBE

was followed. Table 5 showed the effect of initial a_w on the composition of CBE. Interesterification of palm oil with methyl stearate performed best when *C. papaya* lipase had initial a_w = 0.11. They decreased when the initial water activity rose. It can be explained by the role of water during synthesis. In lipase-catalyzed interesterification, hydrolysis and esterification take place separately. As a reactant in the hydrolysis step and a product in the esterification step, water shifts the enzymatic interesterification equilibrium [6]. A small amount of water is needed to maintain enzyme activity. However, at high water activity value, the equilibrium of the reaction was shifted towards hydrolysis, leading to higher production of by-products such as diacylglycerols and fatty acids. To avoid producing by-products, *C. papaya* lipase with initial a_w = 0.11 was chosen as biocatalyst in further experiment.

Table 5. Effect of initial water activity on the fatty acid profiles of cocoa butter equivalent.

Initial water activity	Fatty acid (%)				Ratio of S:P
	C16:0	C18:0	C18:1	C18:2	
0.11	26.9	36.8	30.4	6.0	1.3:1
0.23	38.7	29.8	31.5	0	0.8:1
0.33	49.5	20.4	30.1	0	0.4:1
0.53	51.2	16.7	32.1	0	0.3:1

The lipase (0.5 g) with various water activities was added to a reaction mixture containing palm oil (2 mmol) and methyl stearate (4 mmol) at 45°C for 4 h.

3.3.5 Effect of Amount of the *C. papaya* Lipase

The amount of *C. papaya* lipase added was related to the synthesis of CBE (Table 6). Increased enzyme load would improve the incorporation of acyl donors in interesterification under certain conditions. The best amount of CPL was 18% by weight for interesterification of palm oil with methyl stearate. Increasing the amount of lipase above 18 wt% had no significant effect on substrate conversion.

Table 6. Effect of enzyme quantity on the fatty acid profiles of cocoa butter equivalent from interesterification catalyzed by *C. papaya* lipase.

Quantity of enzyme(Wt %)	Fatty acid (%)				Ratio of S:P
	C16:0	C18:0	C18:1	C18:2	
9	44.6	20.3	30.1	5.0	0.5:1
18	26.9	36.8	30.4	6.0	1.3:1
27	28.1	38.5	30.1	3.3	1.3:1
36	28.1	38.5	30.5	2.8	1.3:1

The various amount of lipase with water activity = 0.11 was added to a reaction mixture containing palm oil (2 mmol) and methyl stearate (4 mmol) at 45°C for 4 h.

3.3.6 Effect of Substrate Ratio

To optimize the effect of palm oil: methyl stearate mole ratio on fatty acid content of CBE, various ratios of palm oil: methyl stearate (1:1, 1:2.3, 1:3, 1:4 and 1:5.6) were applied to the reaction and the interesterification reaction was carried out at 45°C for 4 h. Apparently, higher in methyl stearate levels increased the ratio of stearic acid: palmitic acid (Table 7). The fatty acid profiles of CBE similar to that in cocoa butter, when the ratio of palm oil: methyl stearate was 1:4 and 1:5.6. Because of methyl stearate relatively high cost, the mole ratio of palm oil: methyl stearate for cocoa butter equivalent production was optimized to be 1:4.

Table 7. Effect of palm oil: methyl stearate ratio on the synthesis of cocoa butter equivalent.

Ratio of palm oil: methyl stearate (mol: mol)	Fatty acid (%)				Ratio of S:P
	C16:0	C18:0	C18:1	C18:2	
1:1	46.8	23.4	29.8	0	0.5:1
1:2.3	34.2	27.7	31.6	6.5	0.8:1
1:3	29.8	30.2	31.4	8.6	1.0:1
1:4	26.9	36.8	30.4	6.0	1.3:1
1:5.6	28.1	38.5	30.1	3.3	1.3:1

The lipase (18 wt %, a_w = 0.11) was added to a reaction mixture containing various ratios of palm oil: methyl stearate at 45°C for 4 h.

3.4 Physicochemical Properties of Cocoa Butter Equivalent

The result of CBE properties analysis was shown in Table 8. The melting point of CBE was similar to natural cocoa butter. This revealed that CBE had desirable physical properties. Both of iodine value and saponification value of CBE were close to that value of natural cocoa butter. Unfortunately, acid value of CBE was higher than natural cocoa butter. A possible explanation is that palm oil formed hydrolytic rancidity [23].

Table 8. Physicochemical characteristics of cocoa butter equivalent.

Physicochemical properties	Cocoa butter equivalent	Natural cocoa butter [26]
Melting point (°C)	37-39	35-39
Iodine value (g/100g)	44.9±0.8	40.2
Saponification value (mg/g)	193.2±0.8	190.7
Acid value (% as palmitic acid)	9.8±0.4	1.2

3.5 Yield of Cocoa Butter Equivalent

The yield of CBE prepared in our experiment under optimized conditions was 55% based on palm oil used. While the yield of CBE that produced from interesterification of tea seed oil with methyl stearate

and methyl palmitate catalyzed by immobilized lipase from porcine pancreas was 25.6% based on tea seed oil used [6]. It revealed that CPL was an effective catalyst in interesterification reaction for CBE synthesis.

4. CONCLUSION

This study has shown that palm oil can be modified to cocoa butter equivalent by *C. papaya* lipase-catalyzed interesterification. The reaction conditions were optimized to be 45°C for 4 h using palm oil and methyl stearate, at a mole ratio of 1:4, as substrates and 18 wt% of enzyme with initial a_w = 0.11. The chemical and physical properties of cocoa butter equivalent such as iodine value, sponification value and melting point similar to typical cocoa butter. The yield of cocoa butter equivalent under optimized conditions about 55% based on palm oil used.

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