Food Sci. Biotechnol. Vol. 18, No. 3, pp. 803 ~ 807 (2009)

RESEARCH NOTE

Food Science and Biotechnology

Lipase-catalyzed Production of Solid Fat Containing Conjugated Linoleic Acid in Binary Models

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Abstract Solid fats were esterified with solid phase of rice bran oil (S-RBO), palm stearin (PS), and conjugated linoleic acid (CLA) at 2 substrate mole ratios (S-RBO:PS:CLA of 1:1:2 and 1:3:4). The major fatty acids were palmitic, oleic, and CLA in 36 hr products. The solid fat content (SFC) of the 1:1:2 product was 12.8% while the SFC of 1:3:4 product was 45.1% at 20°C. The SFCs after 20°C reduced when the reaction time increased from 1 to 36 hr, suggesting that the change of triacylglycerol species was augmented by extending reaction time.

Keywords: rice bran oil, palm stearin, conjugated linoleic acid, enzymatic esterification, solid fat content

Introduction

Conjugated linoleic acid (CLA) refers to the mixture of positional and geometric isomers of linoleic acid with conjugated double bonds. The nutritional and healthy benefits of CLA have been widely studied. Lee *et al.* (1) investigated the effect of CLA on retardation of atherosclerosis. Besides, Chin *et al.* (2) found the activity of CLA on enhancement of growth, and O'Shea *et al.* (3) proved CLA could increase of immunity. Additionally, effect of reducing body weight of CLA was reported by Park *et al.* (4). Therefore, supplement of CLA into foods attracts considerable attention due to these pharmaceutical and nutritional values.

One feasible approach to increase the CLA amount in foods is transesterification of CLA with triacylglycerols (TAG) by chemical or enzymatic catalysts. Through incorporation of a required acyl group into a specific position of TAG, lipase-catalyzed esterification provides a useful way to improve the nutritional properties of lipids, whereas a random chemical esterification does not have this specificity capability (5,6). In addition, enzyme-catalyzed reaction can occur in non-solvent system at a mild condition (7). Enzymatic esterification is used to modify the physicochemical characteristics of oils and fats because the reaction can be used to produce modified fats and oils by changing the fatty acids profile in the TAG molecules (8). For example, palm stearin (PS) was esterified with vegetable oils to potentially promote the formation of desirable fats. Some researches have been successfully attempted to prepare bakery fats with PS with other vegetable oils through lipase-catalyzed esterification (9-11).

Rice bran oil (RBO) has higher oxidative stability than soybean oil due to its lower level of linoleic and linolenic acid as well as the presence of relatively abundant amount of natural antioxidants such as tocopherol, oryzanol, and tocotrienol (12-14). Also it is known that RBO reduces the harmful low density lipoproteins (LDL) cholesterol (15).

As a useful approach, fractionation could alter the melting point and solidify characteristics by enriching the fat or fatty acids. Fractionation was successfully used by Yu *et al.* (16) to selectively enrich the content of unsaturated fatty acid (UFA) by fractionation with acetone at various temperatures from RBO. Also high saturated fatty acids (SFA) were enriched from RBO by Alim *et al.* (17). In their results, CLA was incorporated to the solid phase of RBO (S-RBO) and PS to prepare nutritional solid fat by enzyme-catalyzed esterification, in which response surface methodology was used to optimize the reaction. However, the rate of incorporation of CLA was relatively low (10.9%). Therefore, in this study, reaction time as the single factor was investigated to raise the incorporation amount of CLA in solid fat products from binary model of substrate molar ratios. Two substrate ratios were selected from our previous studies for having ideal solid fat contents (SFC) in the products, containing CLA.

Materials and Methods

Materials and reagents Rice bran oil (RBO) was obtained by Searim Company (Daejeon, Korea). Palm stearin (PS) was generously supplied by CJ Co. (Seoul, Korea). Conjugated linoleic acid (CLA) mixtures were provided by Livemax Co. (Seongnam, Korea), which contained 94.1% of CLA isomers. Lipozyme *Thermomyces lanuginosus* (TL IM), which is a 1, 3-specific lipase granulated onto silica, was purchased from Novozymes A/S (Bagsvaerd, Denmark). According to the manufacturer specification, the specific activity of Lipozyme TL IM was 175 IUN/g catalytic activity, having 0.54 g/mL bulk density and 0.3-1.0 mm particle diameter. Acetone, hexane, and heptadecanoic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Fractionation of rice bran oil (RBO) The protocol of preparing fractionated S-RBO was similar to our previous

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Received October 16, 2008; Revised January 22, 2009;
Accepted February 2, 2009

reports (17,18). RBO and acetone were mixed at volume ratio of 1:5 in a flask, maintaining at −16°C for 24 hr. The solid phase was collected, and removal of acetone was carried out by nitrogen blowing.

Enzymatic esterification The substrates were prepared by mixing 0.6 g of S-RBO (Mw 861), 0.6 g of PS (Mw 850), and 0.4 g of CLA (Mw 280.4) with the mole ratio of 1:1:2 (S-RBO:PS:CLA) in a screw-capped test tube. Another blend of 1:3:4 was prepared in the same way. After loading lipozyme TL IM (10% weight of total substrate) into each blend, the esterification was conducted in a shaking water bath at 75°C. The effect of time on incorporation of CLA and solid fat contents (SFC) was ascertained by preparing a series of reactions in which 1, 3, 6, 12, 18, 24, 30, and 36 hr reaction. After each reaction time passed, 1 g of reactant was dissolved in 5 mL of hexane with 5 drops of phenolphthalein solution. Then, the mixture was titrated with a 0.5 N KOH in 20% ethanol for deacidification. After titration, moderate hot water (30-40°C) was added and shaken vigorously. The hexane phase was then isolated. After passing through an anhydrous sodium sulfate column the hexane phase was collected, and hexane was removed under nitrogen gas with heating module (60°C) for obtaining each product.

Fatty acid composition Thin layer chromatography (TLC) was used for separation of the reactants on a silica gel 60 F_{254} plate (Merck KGaA, Darmstadt, Germany) developed with hexane/diethyl ether/acetic acid (50/50/1, v/v/v). The triacylglycerol (TAG) bands were located by spraying with 0.2% 2,7-dichlorofluorescein in methanol. Then, TAG bands were scraped and methylated (18). A Hewlett-Packard 6890 Gas Chromatograph (GC, Avondale, PA, USA) equipped with auto-injection and flame-ionization detection was used for fatty acid composition analysis. The condition of GC was the same as previously described (17). The separation of fatty acid methyl esters was carried out by a fused-silica capillary column (SP-WAX; 60 m ×0.25 mm; Supelco, Bellefonte, PA, USA). Besides, heptadecanoic acid (C17:0; 50 μL of 1 mg/mL in hexane) was added as an internal standard.

Triacylglycerol (TAG) analysis The analysis of TAG species of each reacted product was carried out by Yonglin SP930D HPLC (Yonglin, Anyang, Korea), which consisted by Sedex 75 evaporative light-scattering detector (Sedere, Alfortville, France), and Nova-Pak C18 column (150×3.9 mm, Waters, Milford, MA, USA). The separation was obtained by gradient elution consisted of acetonitrile (A) and isopropanol/hexane (2:1, v/v) (B) at a flow rate of 1 mL/min with the following profile: 0-44 min 20% B; 45-50 min, 46% B; 51-58 min, 100% B. The TAG species were identified with various TAG standards.

Solid fat contents (SFC) Melting thermograms of samples were obtained by differential scanning calorimeter (DSC) 2010 (TA Instruments Inc., New Castle, DE, USA). Purge gas was nitrogen and the base line was obtained with an empty aluminum pan. Each sample was weighed within the range of 5.0 to 9.0 mg. For obtaining thermograms, sample was heated at 80°C and held for 10 min. Then,

Table 1. Fatty acid compositions (area%) of different time-reacted products[1)] of 1:1:2 and 1:3:4

	Time (hr)	14:0	16:0	18:0	18:1	18:2	18:3	20:0	CLA	ΣSFA	ΣUFA
RBO		0.3	19.4	1.7	45.9	32.1	0.6	ND	ND	21.4	78.6
S-RBO		0.2	21.7	1.9	45.9	29.8	0.5	ND	ND	23.8	76.2
PS		1.4	63.6	4.9	24.5	5.1	0.2	0.3	ND	70.2	29.8
CLA		ND[2)]	ND	ND	3.9	2.0	ND	ND	94.1	0	100
1:1:2	1	0.9	42.3	3.6	33.2	14.8	0.4	0.4	4.4	47.2	52.8
	3	0.9	40.4	3.4	32.4	14.6	0.4	0.4	7.5	45.1	54.9
	6	0.8	39.2	3.3	31.8	14.3	0.4	0.4	9.8	43.7	56.3
	12	0.8	37.0	3.0	30.4	13.7	0.4	0.4	14.3	41.2	58.8
	18	0.8	36.1	3.0	29.9	13.4	0.4	0.4	16.0	40.3	59.7
	24	0.8	36.0	3.0	29.7	12.9	0.3	0.4	16.9	40.2	59.8
	30	0.7	35.2	3.0	29.1	12.8	0.3	0.4	18.5	39.3	60.7
	36	0.7	35.0	3.0	28.8	12.6	0.3	0.4	19.2	39.1	60.9
1:3:4	1	1.2	51.8	4.2	28.7	9.7	0.3	0.4	3.7	57.6	42.4
	3	1.1	49.7	4.0	28.0	9.5	0.3	0.4	7.0	55.2	44.8
	6	1.1	47.5	3.9	27.4	9.3	0.2	0.4	10.2	52.9	47.1
	12	1.0	44.5	3.6	26.5	9.1	0.2	0.3	14.8	49.4	50.6
	18	1.0	43.1	3.5	26.1	9.0	0.2	0.3	16.8	47.9	52.1
	24	1.0	42.4	3.4	25.7	8.8	0.2	0.3	18.2	47.1	52.9
	30	0.9	41.8	3.3	25.5	8.7	0.2	0.3	19.3	46.3	53.7
	36	0.9	41.7	3.3	25.0	8.5	0.2	0.3	20.1	46.2	53.8

[1)]Enzymatically esterified solid fat with S-RBO:PS:CLA in a mole ratio of 1:1:2 and 1:3:4 catalyzed by lipozyme TL IM (10% weight) at 75°C in a shaking water bath. RBO, rice bran oil; S-RBO, solid phase of fractionated rice bran oil; PS, palm stearin; CLA, conjugated linoleic acid; SFA, total saturated fatty acid; UFA, total unsaturated fatty acid.
[2)]Not detected.

Table 2. Solid fat content (SFC, %) of different time-reacted products[1] of 1:1:2 and 1:3:4

	Time (hr)	−10°C	0°C	5°C	10°C	15°C	20°C	25°C	30°C	35°C	40°C	SMP (°C)
S-RBO		44.6	8.37	0.2	ND[2]	ND	ND	ND	ND	ND	ND	ND
PS		99.6	92.7	86.3	78.4	74.0	73.6	71.7	63.0	53.2	43.3	52
1:1:2	1	75.9	60.3	51.0	47.0	46.2	39.1	30.8	23.7	16.9	9.5	44.5
	3	80.6	62.3	52.5	46.1	45.2	37.4	27.7	19.5	11.7	3.6	38.0
	6	77.0	58.7	49.7	42.0	41.1	32.9	23.3	15.33	7.6	1.1	34.0
	12	75.6	58.7	48.8	38.0	36.5	27.7	19.1	11.6	4.26	0.8	33.5
	18	73.6	57.5	46.7	35.7	33.6	24.6	16.3	9.2	2.1	0.1	29.0
	24	72.0	47.0	30.1	21.2	17.4	13.7	6.4	1.0	0.3	0	27.5
	30	73.3	45.5	29.1	20.5	16.8	13.4	6.3	0.9	0.2	0	26.0
	36	74.7	45.0	27.2	18.8	15.4	12.8	6.2	1.4	0.1	0	26.0
1:3:4	1	89.9	75.9	68.6	62.1	61.6	59.1	49.5	40.5	31.5	22.3	47.0
	3	88.0	72.6	65.3	57.8	56.8	54.5	44.4	35.1	26.0	16.5	42.5
	6	90.6	74.7	67.2	58.6	56.6	53.4	41.3	31.0	21.4	11.4	41.5
	12	88.3	71.9	65.1	55.78	52.9	48.4	36.3	26.1	16.5	6.76	40.0
	18	87.9	71.4	65.4	55.0	52.1	46.3	33.7	23.2	13.3	3.6	37.5
	24	89.9	73.4	67.5	56.2	52.7	46.9	33.9	22.9	12.5	2.5	36.9
	30	87.8	71.7	66.5	54.2	50.7	46.1	33.6	22.8	12.2	2.0	36.0
	36	86.4	70.7	65.7	54.1	50.52	45.1	32.66	22.0	11.8	1.9	36.0

[1]Enzymatically esterified solid fat with S-RBO:PS:CLA (conjugated linoleic acid) in a mole ratio of 1:1:2 and 1:3:4 catalyzed by lipozyme TL IM (10% weight) at 75°C in a shaking water bath. S-RBO, solid phase of fractionated rice bran oil; PS, palm stearin. SMP, slip melting point.
[2]Not detected.

sample was cooled down at a rate of 10°C/min to −60°C. After holding for 10 min, the melting curve was obtained by heating at a rate of 5°C/min to 80°C.

Slip melting point (SMP) The determination of SMP was followed AOCS Official Method (19). All values were obtained by average reading from 2 samples.

Results and Discussion

Fatty acid composition The fatty acid compositions are listed in Table 1. Compared to original RBO, S-RBO contained higher amount of SFA because high melting TAG species were enriched after fractionation (16,20). Similarly, 70.2% of ΣSFA presented in the PS. Thus the content of ΣSFA in blend of 1:3:4 (S-RBO:PS:CLA) were higher than that of 1:1:2 due to high amount of PS. During the reaction of substrate blend with lipase, the incorporation of CLA was gradually observed, resulting in decreasing amount of ΣSFA in the products. In addition, the effect of reaction time on the incorporation of CLA is shown in Table 1. The highest incorporation of CLA was observed after 36 hr reaction, showing that 19.2 and 20.1% of CLA in each 1:1:2 product and 1:3:4 product, respectively. On the contrary, only 4.4 and 3.7% of CLA were respectively found after 1 hr reaction. Therefore, it is plausible that increasing reaction time allows the prolonged contact time between substrates and lipase, resulting in increasing incorporation rate of CLA.

Effect of esterification on SFC The SFC was calculated according to Lopez *et al.* (21) described. The SFC of S-RBO, PS, 1:1:2, and 1:3:4 product are presented in Table 2. For S-RBO, the SFC at −10°C was 44.6% while most phases were liquid at 5°C (0.2% SFC). However, SFC (at −10°C) of PS was 99.6%, indicating that most phases were solid. Even at 40°C, SFC of PS was 43.3%. The reason could be contributed to high content of ΣSFA (70.2%) in PS, while only 23.8% of ΣSFA was found in S-RBO (Table 1). After 36 hr reaction, the SFC (at 20°C) of 1:1:2 product was 12.8% while the SFC of 1:3:4 product was 45.1%. As predicated, high amount of PS increased SFC in the 1:3:4 product. Generally, the SFC at 20°C determines the tendency of oil exudation, and a value of not less than 10% was recommended to prevent oiling off (22).

Effect of esterification on SMP SMP is defined as the temperature at which the fat begins to rise in the capillary tube. Table 2 shows the SMPs from the different time-reacted 1:1:2 and 1:3:4 products, showing 44.5-26.0°C and 47.0-36.0°C, respectively. When the ratio of PS in the substrate blend increased, SMP of the products generally increased. Similar to the SFC results, the SMP also decreased with prolonging the reaction time, resulting from the reduced ΣSFA mainly due to the incorporation of CLA in the products as shown in Table 1.

Effect of esterification on TAG Figure 1 shows the newly produced TAG species and their composition after reaction. Generally, lipase TL IM-catalyzed esterification resulted in the rearrangement of fatty acids at the external (*sn*-1, 3 positions) glycerol backbone of TAG molecules while fatty acid at the internal position (*sn*-2) remained. In Fig. 1, some peak areas of certain TAG species such as PLO and PLP (P, palmitic acid; O, oleic acid; and L, linoleic acid or CLA) increased in relative proportion while

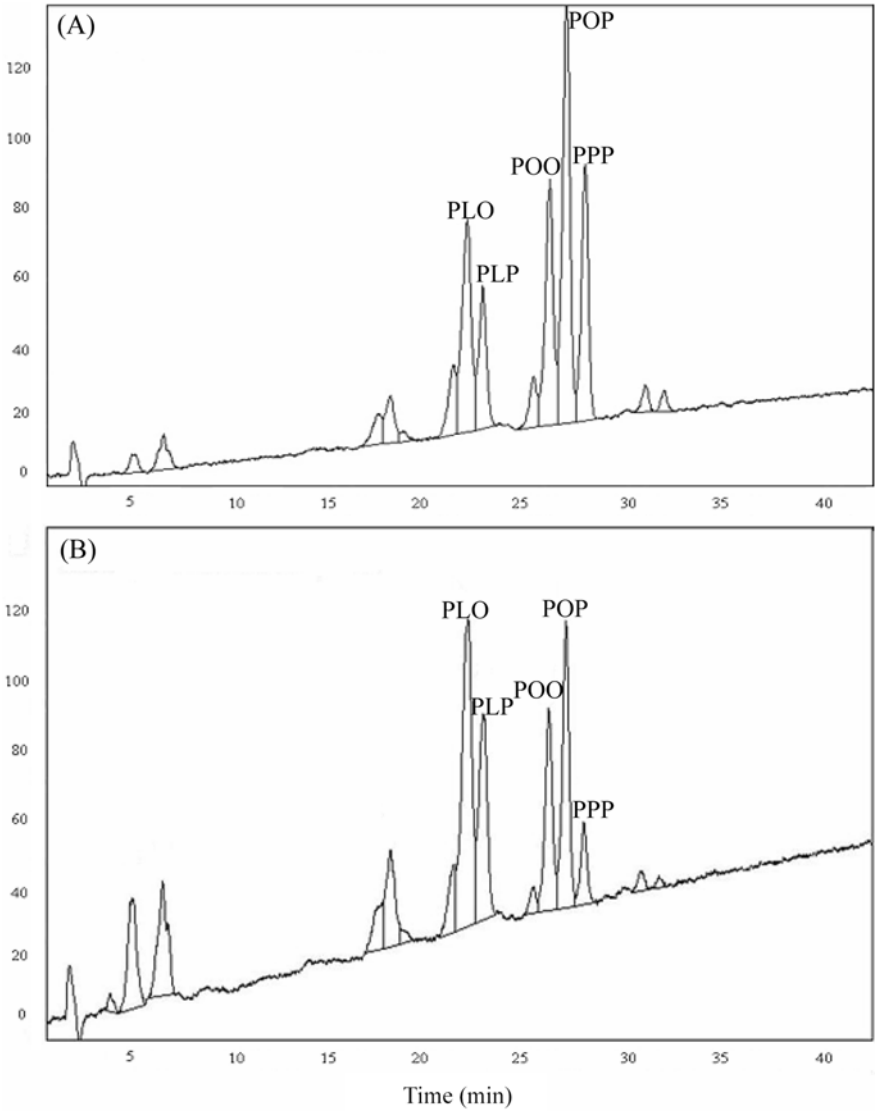

Fig. 1. HPLC chromatography of 1 hr 1:1:2 (S-RBO:PS:CLA; A) and 36 hr 1:1:2 (B) product. P, palmitic; O, oleic; L, linoleic and CLA.

POO, POP, and PPP decreased. In Table 2, changes of SFC were observed during 36 hr reaction. As a whole, the SFC of 1:3:4 product at different reaction times was higher than those of 1:1:2 product due to the high amount of SFA from PS. When the reaction time increased, SFC of both 1:1:2 and 1:3:4 product decreased at any temperature. For example, the SFC (at 20ºC) of 1:1:2 product was 39.1% after 1 hr reaction, which decreased to 12.8% after 36 hr reaction. One reason of decreased SFC by prolonging reaction time is probably the increased incorporation of CLA. As shown in Table 1, only 4.4% of CLA was found in 1:1:2 product after 1 hr reaction, and 19.2% of CLA was in the product after 36 hr reaction as well as high melting (or hard) TAG species such as PPP and POP reduced.

In a conclusion, solid phase of solvent-fractionated RBO was used to prepare the solid fat in which CLA was intentionally incorporated. In this study, maximum content of CLA (19.2% in 1:1:2 product and 20.1% in 1:3:4 product) was obtained after 36 hr reaction, respectively. With prolonging reaction time, the SFC was gradually reduced because of the increased incorporation of CLA in each product. Even though similar CLA content was found in each binary blends (1:1:2 and 1:3:4, S-RBO:PS:CLA) at different reaction times, more solid fat present in 1:3:4 product due to higher amount of PS in the substrate blend.

Acknowledgments

This research was supported by a grant from Korea Food & Drug Administration in 2009.

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