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Enzymatic acidolysis of palm olein with PUFA to improve linoleic and α-linolenic acids ratio

(Asidolisis berenzim minyak olein sawit dengan PUFA bagi meningkatkan nisbah asid linoleik dan α-linolenik)

H. Hasnisa* and S. Jumat**

Keywords: PUFA, acidolysis, modified palm olein, RBD palm olein, Lipozyme TLIM

Abstract

An enzymatic acidolysis to produce modified palm olein with high PUFA (linoleic acid, $C_{18:2n-6}$ and α-linolenic acid, $C_{18:3n-3}$) was carried out. An immobilized lipase, Lipozyme TL IM was used as the biocatalyst. The optimum reaction conditions were found at oil:fatty acid mole ratio of 1:2, temperature 50 °C, incubation time 24 h, 1% water in hexane and 10% enzyme. Under these conditions, modified palm olein shows high iodine value of 123.08 mg I/100 g oil due to increasing linoleic acid (24.46%) and α-linolenic acid (3.74%) content. Linoleic and α-linolenic acid ratio of 21:1 in palm olein was improved to 8:1.

Introduction

Linoleic acid (LA; $C_{18:2n-6}$) and α-linolenic acid (ALA; $C_{18:3n-3}$) are essential omega-6 and omega-3 polyunsaturated fatty acids (PUFA) and must be extracted from food because they cannot be synthesized in the human body (Senanayake and Shahidi 2002b). LA is important for healthy skin, helping to keep it smooth and supple, protect it from injury and infections and regulate body temperature and water loss. Individuals with atopic eczema (a skin disorder) are thought to have a deficiency in LA that interferes with the production of other omega-6 PUFA from linolenic acid. LA applied to skin or taken orally has often helped to relieve symptoms of this disorder (Senanayake and Shahidi 2002b). This fatty acid also seems to provide some benefit in premenstrual syndrome, diabetes and old age. It has also been used to treat rheumatoid arthritis, asthma, multiple sclerosis, migraine and cancer (Senanayake and Shahidi 2002b).

The beneficial effects of PUFA have been associated with their ability to lower serum triacylglycerol and cholesterol levels and enhance their excretion, to increase membrane fluidity and to reduce thrombosis (Senanayake and Shahidi 2002a). The omega-3 PUFA are considered essential for normal growth and development and may play an important role in the prevention and treatment of cardiovascular diseases, hypertension, inflammatory and immune disorders, diabetes and cancer.

Modified lipids are less available in nature and scarcely produced by chemical reactions. They can be synthesized by enzymatic methods using *sn*-1,3 specific lipase to change the fatty acid (FA) composition and/or the positional distribution in the glycerol backbone (Cossignani et al. 2004). Modification of

*Food Technology Research Centre, MARDI Headquarters, P.O. Box 12301, 50774 Kuala Lumpur, Malaysia
**School of Chemical Science and Food Technology, Faculty of Science and Technology, National University of Malaysia, 43600 Bangi, Selangor, Malaysia
Authors' full names: Hasnisa Hashim and Jumat Salimon
E-mail: hasnisa@mardi.gov.my

lipids by lipase catalyzed reactions has been reported in a large number of publications in the past few years. The use of lipases is becoming increasingly important in a number of applications. Lipases can be used as biocatalysts for hydrolysis, esterification, interesterification and modification of fats and oils (Gandhi 1997). Lipases may catalyze the incorporation of fatty acids into triacylglycerol (TAG). Application of lipases in the modification of lipids may offer some advantages over conventional chemical catalysts, such as in the synthesis of novel products and incorporation of desirable fatty acids at specific positions of the lipid to improve functionality, absorption, metabolism, nutrition and for clinical use (Akoh 1995). In addition, enzyme-mediated reaction has become the preferred method for small scale modified lipid production because reaction conditions tend to be milder with less side reactions (Senanayake and Shahidi 2002a).

In this study, refined, bleached and deodorized (RBD) palm olein was used as a substrate as it is a widely used edible oil in Malaysia and Asia and it also contains high oleic acid. By using 1,3-specific lipase, linoleic and linolenic acid can be inserted at the *sn*-1,3 position of RBD palm olein, while retaining oleic acid at the *sn*-2 position for more efficient absorption during metabolism (Lai et al. 2005). Palm olein contains low amounts of omega-3 and omega-6 fatty acids compared to corn oil, soybean oil, olive oil and other vegetable oils. The content of essential fatty acids in the RBD palm olein is between 10–13% linoleic acid. Refined, bleached and deodorized (RBD) palm olein needs to be modified to increase its nutritional value by enriching them with essential fatty acids, namely linoleic and α-linolenic acid.

The objective of this study was to synthesize and characterize modified lipid produced from enzymatic acidolysis between palm olein and PUFA extracted from soybean oil using Lipozyme TL IM as a biocatalyst. The effects of mole ratio of reactants, organic solvents, reaction temperatures, incubation times, water content and amount of lipase on PUFA incorporation into RBD palm olein were analysed as major variables.

Materials and methods

Chemicals and materials

Refined, bleached and deodorized (RBD) palm olein was obtained from Golden Jomalina Food Industries Sdn Bhd. Lipozyme TL IM (*sn*-1,3 specific lipase) was donated by Novozymes. PUFA (LA and ALA) were obtained from hydrolysis and urea complexation process of soybean oil. Soybean oil (Sunbeam, Sime Darby Edible Products Limited) was purchased from a local supermarket. All reagents were of analytical grade.

Preparation of PUFA from soybean oil (SBO)

Preparation of free fatty acids from SBO was carried out according to the method of Gamez-Meza et al. (2003). Twenty g of SBO was saponified with 100 ml 1M KOH in 95% ethanol by refluxing for 30 min at boiling temperature of the mixture (60 ± 2 °C) under a blanket of nitrogen. Distilled water (50 ml) was added to the saponified mixture and the unsaponifiable matter was partitioned into hexane (2 x 70 ml) and discarded. The aqueous layer containing saponified matter was acidified (pH = 1.0) with 3N HCl. The mixture was transferred into a separatory funnel and the liberated fatty acids were extracted into 50 ml of *n*-hexane. The hexane layer containing free fatty acids was then dried over anhydrous sodium sulphate and the solvent was removed at 40 °C to recover free fatty acids which was then stored at –20 °C until used in the urea complexation process.

Ten g of free fatty acids were mixed with 30 g urea in 150 ml aqueous ethanol (95%) in a capped bottle and heated at 60 ºC with continuous stirring until the whole mixture turned into a clear homogeneous solution. This solution was then allowed

to crystallize at room temperature and then kept at 4 ºC for 24 h for further crystallization. The formed crystals (urea complexing fraction; UCF) were then separated from the liquid (non urea complexing fraction; NUCF) by vacuum filtration. The filtrate (NUCF) containing PUFA was diluted with an equal volume of distilled water and acidified (pH 4–5) with 6N HCl; an equal volume of hexane was added and the mixture was stirred for an hour. The mixture was transferred into a separatory funnel. The hexane layer containing PUFA was washed with distilled water to remove any remaining urea and then dried over anhydrous sodium sulphate and the solvent was removed at 40 ºC using a rotary evaporator to recover PUFA which was stored at –20 ºC until used in acidolysis (Wanasundara and Shahidi 1999).

Acidolysis of palm olein with PUFA

Acidolysis was performed in screw-capped test tubes in which a mixture of RBD palm olein and PUFA (304 mg RBD palm olein and 196 mg PUFA) were added to n-hexane (2 ml) followed by water (1% by w/w of total substrates) and lipase (10% by w/w of total substrates). The mixture was stirred in an orbital shaking water bath at 250 rpm for 24 h at 50 ºC. All reactions were performed in triplicates.

Effect of substrates mole ratio

The reaction mixture containing lipase (75 mg) was incubated with different mole ratios of substrates (from 1:0.3 to 1:3, RBD palm olein:PUFA). The average molecular weight of RBD palm olein was calculated from its saponification value determined earlier. The mixture was incubated at 45 ºC for 24 h in an orbital shaking water bath at 250 rpm.

Effect of temperature

The reaction mixture containing 304 mg RBD palm olein, 196 mg PUFA (substrates mole ratio of 1:2), 1% (w/w of substrates) added water, 10% (w/w of substrates) Lipozyme TLIM and 2 ml n-hexane was incubated for 24 h at different temperatures (30–60 ºC) in an orbital shaking water bath at 250 rpm.

Effect of enzyme load

The reaction mixture containing 304 mg RBD palm olein, 196 mg PUFA (substrates mole ratio of 1:2), 1% (w/w of substrates) added water, 0–20% (w/w of substrates) of Lipozyme TL IM and 2 ml n-hexane was incubated for 24 h at 50 ºC in an orbital shaking water bath at 250 rpm.

Effect of water content

The reaction mixture containing 304 mg RBD palm olein, 196 mg PUFA (substrates mole ratio of 1:2), 0–3% (w/w of substrates) added water, 10% (w/w of substrates) of Lipozyme TL IM and 2 ml n-hexane was incubated for 24 h at 50 ºC in an orbital shaking water bath at 250 rpm.

Effect of reaction time

The reaction mixture containing 304 mg RBD palm olein, 196 mg PUFA (substrates mole ratio of 1:2), 1% (w/w of substrates) added water, 10% (w/w of substrates) of Lipozyme TLIM and 2 ml n-hexane was incubated for 0–72 h at 50 ºC in an orbital shaking water bath at 250 rpm.

Effect of organic solvents

The effects of various organic solvents in the reaction mixture were investigated. Five organic solvents such as n-hexane, cyclohexane, toluene, acetone and acetonitrile were used in this study. RBD palm olein (304 mg) was mixed with PUFA (196 mg), water (1% by w/w of total substrates) and lipase (10% by w/w of total substrates) were added to the organic solvent (2 ml). A solvent free mixture was used as control. The mixture was stirred in an orbital shaking water bath at 250 rpm for 24 h at 50 ºC.

Separation of acylglycerol after acidolysis
The separation of acylglycerols was carried out according to the method of Senanayake and Shahidi (2002a). The lipase was separated by filtration of the products from the acidolysis reaction. The samples were then placed in 50 ml conical flasks and 12 ml of a mixture of acetone/ethanol (1:1, v/v) was added. The reaction mixture was titrated with 0.5 N NaOH to a phenolphthalein end point (pink). The mixture was transferred to a separatory funnel and thoroughly mixed with 25 ml hexane. The lower layer was separated and discarded. The upper hexane layer containing acylglycerols was passed through a bed of anhydrous sodium sulphate. The acylglycerol fraction was subsequently recovered following hexane removal at 45 ºC using a rotary evaporator.

Fatty acid composition of acylglycerol
Samples were dissolved in 1 ml of hexane in a sample tube. Then 1 ml of sodium methoxide in methanol was added for fatty acid methylation process. The mixture was allowed to stand for 15 min before GC analysis, using Shimadzu GC 17A gas chromatography equipped with a flame ionization detector (FID). Nitrogen was used as the carrier gas and the total gas flow rate was at 0.3 ml/min. BPX 70 capillary column (30m x 0.25 mm x 0.25 μm) was used as the GC column. The injector and detector temperatures were set at 250 ºC and 280 ºC respectively. Fatty acid compositions were calculated based on the percentage peak area of the GC chromatogram. All analyses were performed in triplicates and average values were reported.

Peroxide value
Analysis of peroxide value was carried out according to the American Oil Chemists' Society Official Method Cd 8–53 (AOCS 1989).

Acidity
The acidity of modified palm olein was determined according to the American Oil Chemists' Society Official Method Ca 5a–40 (AOCS 1989).

Iodine value
Iodine value (Wijs method) analysis was carried out according to the American Oil Chemists' Society Official Method Cd 1b–87 (AOCS 1989).

Statistical analysis
All determinations were carried out in triplicates and the mean data ± SD (standard deviation) were reported. Experimental data were statistically analysed by analysis of variance (ANOVA) and the significant differences among means were determined by Duncan Multiple Range Test (DMRT) at a level of $p < 0.05$.

Results and discussion

Effect of substrate mole ratio
The effect of different mole ratio of substrates (palm olein: PUFA) on percentage of PUFA incorporation is shown in *Table 1*. The results indicated that as the mole ratio of substrates increased from 1:0.3 to 1:2, incorporation of PUFA increased accordingly. PUFA incorporation increased significantly ($p < 0.05$) from 3.31% (1:0.3) to 11.47% (1:2) respectively. The results showed that the higher the PUFA mole ratio, the higher the PUFA incorporation in palm olein (Senanayake and Shahidi 2002a). However, at a mole ratio of 1:3, there was a slight decrease of PUFA incorporation (10.89%) into RBD palm olein. This was probably due to inhibition of the lipase activity at high concentration of PUFA in the reaction medium. Desorption of water by fatty acid substrate may also be responsible for the inhibition. At a ratio of 1:2, linoleic and α-linolenic acid incorporations were found highest at 11.47%. Huang and Akoh (1994) also reported that stoichiometric ratio of 1:2 was optimal in modification of lipid via enzymatic esterification of polyunsaturated fatty acid and canola oil (vegetable oil) using *sn*-1,3-specific lipase as catalyst in the presence of n-hexane.

Effect of temperature

The effect of temperature on PUFA incorporation into RBD palm olein is shown in *Table 1*. Results showed that Lipozyme TL IM was more active at higher temperatures. As the reaction temperature increased, the incorporation of PUFA into the products increased (p <0.05). The optimum temperature for incorporation of PUFA into RBD palm olein was 50 ºC. The incorporation of PUFA reached maximum (20.92%) at 50 ºC and started to decline slightly as temperature was further increased. The decline was probably caused by loss of enzyme activity at temperatures above 50 ºC (Senanayake and Shahidi 2002a). Zhao et al. (2007) also suggested that 50–55 ºC were the most suitable temperatures for enzymatic acidolysis. At higher temperatures there is greater migration of enzymes which will lead to higher lipase deactivation rates.

Enzyme load

An increase in enzyme load was expected to have a positive effect on PUFA incorporation into RBD palm olein. As the amount of enzyme increased from 5–20%, differences of PUFA incorporation were significant (p <0.05) (*Table 1*). Highest incorporation of PUFA (27.51%) was obtained at 10% enzyme load. The PUFA incorporation decreased when more than 10% of enzyme was employed. Thus, 10% of enzyme load was sufficient to saturate the reaction system (Senanayake and Shahidi 1999). It is more likely that at higher enzyme load the decrease in PUFA incorporation was due to insufficient amount of water in the reaction mixture. This is because as the amount of lipase in the reaction mixture increased, the amount of added water remained constant. Senanayake and Shahidi (2002a) also reported that inadequate amount of added water may cause the enzyme to be inactive since water was required to maintain their three-dimensional structure.

Water content

An optimum level of water in the reaction medium is required for the enzyme to maintain their activity. However, high amount of water will promote hydrolysis (Senanayake and Shahidi 2002a). The effect of water content on the incorporation of

Table 1. Effect of oil/fatty acid ratio, temperature, enzyme concentrations, water content and reaction time on PUFA incorporation into RBD palm olein

Effect of	PUFA Incorporation (%)
Substrate mole ratio (mole/mole)	
1:0.3	3.31 ± 0.46d
1:0.5	5.76 ± 0.31c
1:1	7.67 ± 0.41b
1:2	11.47 ± 0.43a
1:3	10.89 ± 0.20a
Temperature (°C)	
30	2.59 ± 0.12e
35	3.15 ± 0.64e
40	6.67 ± 0.26d
45	11.42 ± 0.34c
50	20.92 ± 0.38a
55	12.96 ± 0.08b
60	7.25 ± 0.35d
Enzyme load (%)	
0	0 ± 0
5	23.4 ± 0.57b
10	27.51 ± 0.53a
15	22.82 ± 0.53b
20	20.92 ± 0.35c
Water content (%)	
0	23.63 ± 0.11d
0.5	26.30 ± 0.31c
1	28.20 ± 0.57a
2	27.51 ± 0.06b
3	26.88 ± 0.42bc
Reaction time (h)	
0	0 ± 0
12	24.00 ± 0.42d
24	28.20 ± 0.38bc
36	28.58 ± 0.40b
48	29.54 ± 0.12a
60	28.58 ± 0.17b
72	27.98 ± 0.13c

Experimental results are means ± standard deviation (SD) of triplicate determinations. Means with the same letter, for each effect in the column, are not significantly different

PUFA into RBD palm olein is shown in *Table 1*. Lipozyme TL IM performed best at 1% water content for the acidolysis of RBD palm olein with PUFA where the highest incorporation of linoleic and α-linolenic acid (28.2%) was observed. Excessive amount of water however resulted in poor incorporation of PUFA into RBD palm olein. The incorporation of PUFA decreased steadily with increased water content. These results suggested that water content is a critical factor in the production of modified lipid from RBD palm olein and PUFA.

Effect of reaction time

Results showed that the incorporation of PUFA increased as the reaction time increased. *Table 1* shows that after 24 h, the incorporation of PUFA significantly increased as the reaction time increased ($p < 0.05$). The incorporation of PUFA reached a maximum (29.54%) at 48 h, but the differences of PUFA incorporation at 24 h to 48 h is only about 1.3%. After 48 h, PUFA incorporation started to decline slightly due to the inactive enzyme which is affected by the loss of water content in the reaction mixture. Generally, the enzyme requires a certain level of water in the reaction medium to maintain a layer of water molecules around the enzyme and to maintain the enzyme activity for initiating the reaction (Paez et al. 2003). The optimum reaction time considered for the incorporation of PUFA into RBD palm olein was 24 h with the incorporation of 28.2% PUFA.

Effect of solvent type

Table 2 shows the effect of various solvents on PUFA incorporation into palm olein. Organic solvents of various log P values were used in the study: n-hexane, log P = 3.9; cyclohexane, log P = 3.2; toluene, log P = 2.5; acetone, log P = –0.23 and acetonitrile, log P = –0.33. The substrate mole ratio of 1:2 (palm olein to PUFA) was kept constant. Results showed that the incorporation of PUFA increased with the increase in log P values. The incorporation of PUFA in palm olein was highest (28.2%) in n-hexane. In general, log P (logarithm of partition coefficient) was used to describe and predict the polarity of complex mixtures of solvents in a biocatalytic system (Senanayake and Shahidi 2002a). Solvents with log P values in the range of 2 to 4 were suitable for enzymatic applications since they only caused a weak distortion of the essential water in the enzyme molecule (Zhao et al. 2007).

Therefore, n-hexane with a log P value of 3.9 was the most appropriate solvent for the acidolysis reaction as shown by the high catalytic activity in the organic/aqueous two-phase system (Senanayake and Shahidi 2002a). This solvent also increased the solubility of non polar substrates by shifting the reaction towards synthesis rather than hydrolysis (Zhao et al. 2007). The solvent-free system also produced satisfactory incorporation of 22.4% PUFA (linoleic and α-linolenic acids) into palm olein (*Table* 2). Senanayake and Shahidi (2002a) reported that Lipozyme TL IM worked equally well in solvent-free reactions as well as in reactions carried out in inert solvents. Acetone (7.1% PUFA) and acetonitrile (10.0% PUFA); the polar organic solvents with log P value <2 appeared to be poor matrix for enzymatic acidolysis reaction. Solvents with log P value <2 are not suitable in enzymatic reactions as they

Table 2. Effect of selected organic solvents on PUFA incorporation into RBD palm olein

Solvent type	Log P value[a]	PUFA incorporation (%)[b]
Solvent free	–	22.4 ± 0.40
n-hexane	3.9	28.2 ± 0.53
Cyclohexane	3.2	19.0 ± 0.17
Toluene	2.5	18.6 ± 0.26
Acetone	–0.23	7.1 ± 1.29
Acetonitrile	–0.33	10.0 ± 0.30

[a]The logarithm of partition coefficient between water and octanol

[b]Experimental results are means ± standard deviation of triplicate determinations

were strong water distorters and thereby inactivating the enzyme (Zhao et al. 2007). Generally, polar organic solvents compete with the available water which is required for the three-dimensional structure of the enzyme and may disrupt the enzyme activity (Senanayake and Shahidi 2002a).

Fatty acid composition

RBD palm olein contains six major fatty acids, namely, myristic, palmitic, stearic, oleic, linoleic and linolenic acids, which comprise 95% of the total fatty acids in the oil (*Table 3*). After the optimum reaction condition, a total of 24.46% linoleic acid and 3.74% α-linolenic acid were incorporated into the modified oil (*Table 3*). The PUFA incorporation caused the decline of palmitic (37.05–22.67%), oleic (46.36–34.41%) and stearic (4.05–2.52%) acids content. These results showed that the amount of medium chain fatty acids in RBD palm olein were greatly reduced while linoleic and α-linolenic acids were successfully incorporated into RBD modified palm olein. This has been expected due to the application of the specific lipase *sn*-1,3 as catalyst in the acidolysis process.

Generally, the major positions of palmitic, oleic and stearic acids are on the *sn*-1 and *sn*-3 on the glycerol molecule.

Table 3. Fatty acid compositions of RBD palm olein and modified RBD palm olein after acidolysis

Fatty acids	Palm olein (%)	Modified RBD palm olein (%)
Myristic 14:0	0.84 ± 0.05	0.51 ± 0.01
Palmitic 16:0	37.05 ± 0.05	22.67 ± 0.15
Stearic 18:0	4.05 ± 0.10	2.52 ± 0.14
Oleic 18:1n9	46.36 ± 0.05	34.41 ± 0.13
Linoleic 18:2n-6	11.16 ± 0.10	35.62 ± 0.40
Linolenic 18:3n-3	0.53 ± 0.06	4.27 ± 0.66

The major TAG in RBD palm olein are palmitic-palmitic-oleic (PPO), palmitic-oleic-oleic (POO), palmitic-oleic-palmitic (POP), palmitic-linoleic-oleic (PLO) and palmitic-palmitic-linoleic (PPL) while minor TAG are palmitic-oleic-stearic (POS), oleic-oleic-oleic (OOO), palmitic-linoleic-linoleic (PLL), myristic-oleic-linoleic (MOL) and others (Lai et al. 2005). The decline of palmitic, oleic and stearic acids can be explained by the action of specific lipase *sn*-1,3 in removing them from the TAG and allows the PUFA to incorporate into the TAG (Fajardo et al. 2003).

There was also an improvement of linoleic and α-linolenic fatty acids ratio in the modified oil. PUFA n-6 and n-3 ratio of 21:1 in unmodified palm olein were improved to 8:1 in the modified oil (*Table 3*). The FAO/WHO has recommended dietary intakes for omega-6 and omega-3 fatty acids ratio of 10:1 for adults for optimal brain and cardiovascular health and function (Simopoulos et al. 2000).

Chemical properties

Table 4 shows the acidity (AV), peroxide (PV) and iodine (IV) values of the modified palm olein. Lipase-catalyzed acidolysis increased the IV of RBD palm olein from 56 to 123. The increase in IV can be explained by the successful incorporation of PUFA into palm olein to form the modified oil. Linoleic ($C_{18:2}$) and α-linolenic ($C_{18:3}$) acids are polyunsaturated fatty acids and therefore their incorporation resulted in an increase in IV as the degree of unsaturation in modified oil increased. The peroxide (PV) value of modified oil also increased. This is probably due to the oxidation process of the PUFA composition in the modified oil.

Table 4. Characterization of modified RBD palm olein

	Palm olein	Modified RBD palm olein
Acid value (mg/g)	0.17 ± 0.03	0.17 ± 0.03
Peroxide value (meq/kg)	1.19 ± 0.06	19.60 ± 0.03
Iodine value (mg I_2/100 g oil)	56.09 ± 0.10	123.08 ± 0.06

Conclusion

PUFA (linoleic and α-linolenic acids) from soybean oil was successfully incorporated into RBD palm olein. Optimum conditions for the incorporation of PUFA into RBD palm olein were found to be: substrate mole ratio of 1:2, incubation time 24 h, temperature 50 ºC, 1% (w/w) water content and an enzyme load of 10% (w/w) in n-hexane. Acidolysis reactions effectively increased linoleic and α-linolenic acids from 11.16 to 35.62% and 0.53 to 4.27% respectively, in modified palm olein. Linoleic and α-linolenic acids ratio in modified palm olein also improved from 21:1 to 8:1.

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Abstrak

Asidolisis berenzim untuk menghasilkan minyak olein sawit terubah suai yang mengandungi asid lemak politaktepu (PUFA) yang tinggi (asid linoleik, $C_{18:2n-6}$ dan asid α-linolenik, $C_{18:3n-3}$) telah dijalankan. Lipozyme TL IM digunakan sebagai pemangkin. Keadaan optimum tindak balas asidolisis adalah pada nisbah mol minyak:asid lemak 1:2, suhu 50 ºC, tempoh pengeraman 24 jam, 1% air dalam heksana dan 10% enzim. Pada keadaan ini, minyak olein sawit terubah suai menunjukkan nilai iodin yang tinggi iaitu 123.08 mg I/100 g disebabkan oleh peningkatan kandungan asid linoleik (24.46%) dan asid α-linolenik (3.74%). Nisbah asid linoleik dan α-linolenik 21:1 di dalam minyak olein sawit meningkat kepada 8:1.

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