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Enzymatic hydrolysis of palm olein with mycelium-bound lipase of *Aspergillus flavus* Link

(Hydrolysis minyak olein menggunakan lipase terikat miselium daripada *Aspergillus flavus* Link)

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Key words: *Aspergillus flavus* lipase, high melting glycerides, low melting glycerides, hydrolysis

Abstrak

Hidrolisis minyak olein menggunakan lipase-terikat miselium daripada *Aspergillus flavus* Link telah dikaji. Komposisi asid lemak, profil triasigliserol dan sifat lebur minyak olein sebelum dan selepas 72 jam tindak balas dibandingkan. Kepekatan asid palmitik didapati menurun sedikit diikuti dengan pertambahan asid oleik dan asid linolenik pada minyak tersebut. Kepekatan bandingan bagi triasigliserol tri-tak tepu, minyak olein terubahsuai yang mempunyai takat lebur rendah, seperti trioleoil gliserol, oleoil-dilinoleiol gliserol dan dioleoil oleoil gliserol, didapati meningkat, manakala kepekatan triasigliserol yang mempunyai takat lebur tinggi seperti dipalmitoil-oleoil gliserol dan palmitoil-oleoil steroil gliserol berkurangan kecuali tripalmitoil gliserol. Julat lebur bagi minyak olein terubah suai selepas tindak balas didapati menjadi lebar, iaitu apabila minyak mula lebur (X_1) pada suhu -28 °C dan lebur keseluruhannya (X_2) pada suhu 45 °C.

Abstract

Hydrolysis of palm olein was studied using mycelium-bound lipase of *Aspergillus flavus* Link. The fatty acid composition, triacylglycerol profile and melting properties of the palm olein before and after 72 h hydrolysis were compared. A slight decrease of palmitic acid and increase in oleic acid and linolenic acid concentrations in palm olein was noted. The relative concentration of triunsaturated triacylglycerol, low melting glycerides, such as trioleoyl glycerol, oleoyl-dilinoleoyl glycerol and dioleoyl-linoleoyl glycerol of modified palm olein was increased while the relative concentration of high melting glycerides e.g. dipalmitoyl-oleoyl glycerol and palmitoyl-oleoyl-steroyl glycerol was decreased except for tripalmitoyl glycerol. The melting range of modified palm olein tends to be broad, that is it starts melting (X_1) at -28 °C and totally melted (X_2) at 45 °C.

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Introduction

Aspergillus flavus, isolated from copra meal, was found to have lipase that preferentially hydrolysed saturated long chain fatty acids (FA) than unsaturated long chain FA (Long et al. 1998). This property offers some advantages whereby the lipase can be used in a process to obtain oil enriched with unsaturated FA. Many of the commercially available lipases discriminate against n-3 polyunsaturated FA (Haraldsson 1989). This has enabled their use to concentrate both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) by hydrolysis of fish oils (Lie et al. 1994; Maehr and Lambertsen 1986). Therefore, it is interesting to explore the potential use of *A. flavus* lipase in modifying the palm olein.

Palm olein which contains 46% saturated FA (myristic, palmitic and stearic), 43% monounsaturated FA (oleic) and 11% polyunsaturated FA (linolenic) faces problem such as poor low temperature stability and formation of cloud upon storage (Gunstone 1986). In this study, changes in the FA composition, triacylglycerol (TAG) profile and melting properties of palm olein after being hydrolysed with mycelium-bound lipase of *A. flavus* Link are reported.

Materials and methods

Unless otherwise stated, all chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, England and were of analytical grade. All biochemicals were supplied by Sigma Chemical Ltd., Poole, Dorset, England. Refined, bleached and deodorised (RBD) palm olein was purchased from a local supermarket.

Source of mycelium-bound lipase

The fungus was locally isolated from copra meal (Long et al. 1996a) and was identified as *A. flavus* Link (IMI 361648) by the International Mycological Institute, United Kingdom.

Preparation of defatted mycelia containing lipase

Aspergillus flavus was cultivated in liquid medium as described by Long et al. (1996b). Cultivation was carried out at 30 °C and 150 rev/min in an orbital shaker for 72 h. Mycelia were harvested and washed several times with distilled water, followed by defatting with 100 mL n-hexane (Long et al. 1996b). The defatted mycelia were freeze-dried for 3 h, homogenized in a blender for 90 s, and stored in a desiccator with dehydrated silica gel before use.

Enzymatic reaction of palm olein

The reaction was initiated by adding 0.4 g dried mycelium-bound lipase into a 150 mL flask containing 6.0 g palm olein dissolved in 60 mL n-hexane. The reaction mixture was then agitated in an orbital shaker at 200 rev/min and 40 °C. Each reaction was carried out in duplicate. The FA composition of palm olein was determined using gas chromatography (GC) after free FA were removed from reaction mixture. The TAG profiles of hydrolysed palm olein were analysed using reverse-phase high-performance liquid chromatography (HPLC).

Removals of free fatty acids from reaction mixtures

Free FA were removed from the mixture using the method as described by Foglia et al. (1993) with slight modification by Long et al. (1997).

Analysis of triacylglycerol

The TAG composition before and after the reactions were analysed by nonaqueous reverse-phase HPLC using a Shimadzu Liquid Chromatograph LC-10AD and SLC-10A equipped with an auto-injector and a Shimadzu C-R4AX integrator (Shimadzu Corporation, Kyoto, Japan). A commercially packed RP-18 column (240 mm x 4 mm) with 5 µ particle size (E. Merck, Darmstadt, Germany), was used to separate the TAG. TAG was eluted from the column using

acetone/acetonitrile (63.5:36.5) mixture at a flow rate of 1 mL/min and detected with a refractive-index detector (RID-6A, Shimadzu Corporation, Kyoto, Japan). The injection volume was 10 µL injection. Identification of TAG of palm olein was based on the work done by Ghazali et al. (1995). The total concentration of TAG present in a reaction mixture was calculated by subtracting the concentrations of glycerides (eluted before 12 min) from the total concentration of all glycerides recorded on HPLC chromatogram.

Determination of fatty acid

The FA composition of unreacted and enzyme-reacted oils was determined after the conversion of fatty acid methyl esters (FAME) by sodium methoxide in anhydrous methanol. FAME were analysed by injection of 0.3 µL of a FA-free sample into a gas chromatograph, GC-17AC (Shimadzu Corporation, Kyoto, Japan), equipped with a flame-ionization detector. A polar capillary column BPX 70 (0.32 mm internal diameter, 30 m length and 0.25 µm film thickness: SGE Australia Pty. Ltd., Ringwood, Australia). The flow rate of hydrogen carrier gas was 50 mL/min, and the injector and detector temperatures were maintained at 240 °C while the column temperature was at 180 °C.

Determination of melting profiles

Melting profile of reaction products and control were analysed using a differential scanning calorimeter (DSC 7 Perkin-Elmer Norwalk, CT). The instrument was calibrated with indium and n-decane. Samples, from which FA had been removed, were weighed into aluminium pans (ranging from ca. 9 to 11 mg), and lids were crimped into place. The sample and reference (empty) pan were placed in the calorimeter at room temperature, while the cell block of the DSC was cooled to -55 °C and flushed with nitrogen. Samples were subjected to the following temperature program: -55 °C isotherm for 10 min and heating from -50 °C to 75 °C at the 10 °C/min. The melting points of the palm olein, expressed as X_1 (start melting), X_2 (totally melted) and peak temperature, were recorded based on the means of three replicate runs. The values for X_1 and X_2 were obtained from the intersection of the tangents to the slope against the base line.

Results

The FA composition of modified palm olein (free FA has been removed) is shown in *Table 1* and very little changes in FA composition of palm olein were noted. The relative concentrations of the total saturated FA decreased by 4% and these were mainly due to the reduction of palmitic acid. On the other hand, the relative concentration of unsaturated FA such as oleic acid and linolenic acid increased.

Some changes in the TAG composition of palm olein based on HPLC determination were observed after lipase catalysis and these are shown in *Table 2*. Seven out of twelve TAG, showed an increase in their relative concentration and they were oleoyl-dilinoleoyl glycerol (OLnLn), dioleoyl-linoleoyl glycerol (OOLn), trioleoyl glycerol (OOO), palmitoyl-dilinoleoyl glycerol (PLnLn), myristoyl-linoleoyl-palmitoyl glycerol (MLnP), palmitoyl-oleoyl-linoleoyl glycerol (POLn) and tripalmitoyl glycerol (PPP). These TAG are known as low melting glycerides (LMG) except the PPP,

Table 1. Fatty acid composition of palm olein before and after 72 h reactions

Fatty acid composition	Control (%)	After 72 h (%)
Lauric acid	0.43	0.49
Myristic acid	1.09	1.04
Palmitic acid	37.47	35.91
Stearic acid	3.87	3.81
Oleic acid	46.24	47.49
Linolenic acid	10.90	11.26

The values shown have been corrected to100% and are relative to the total concentration of FA in the reaction mixtures. The above analysis of FA composition was from the monoglyceride, diglycerides and TAG portion of the palm olein.

Table 2. Triacylglycerols (TAG) profiles of the palm olein before and after 72 h reactions

TAG of palm olein	Control (%)	After 72 h (%)
OLnLn	0.64	1.97
PLnLn	3.05	3.09
MLnP	0.67	0.74
OOLn	2.28	4.02
POLn	13.07	14.17
PLnP	10.15	8.47
OOO	4.46	8.25
POO	31.31	27.98
POP	24.7	20.48
PPP	0.53	2.16
SOS	4.10	3.30
POS	5.04	4.35

The values shown have been corrected to 100% and are relative to the total concentration of TAG in the reaction mixtures

which is high melting glyceride (HMG). The highest increase in relative concentration was with PPP (HMG) followed by OLnLn, OOO and OOLn.

In addition, the relative percentage of total TAG remained after 72 h reaction was 80%, indicating that hydrolysis had occurred. The hydrolysis process also increased the concentration of diglycerides *(Figure 1)*. This diglycerides which was identified as 1,3 dipalmitoyl glycerol by Swe et al. 1995, was found to have high melting point.

Most of TAG which contained monosaturated or disaturated TAG, which in their acyl group contains palmitic or stearic acid, showed a decrease in relative concentration e.g. dipalmitoyl-linoleoyl

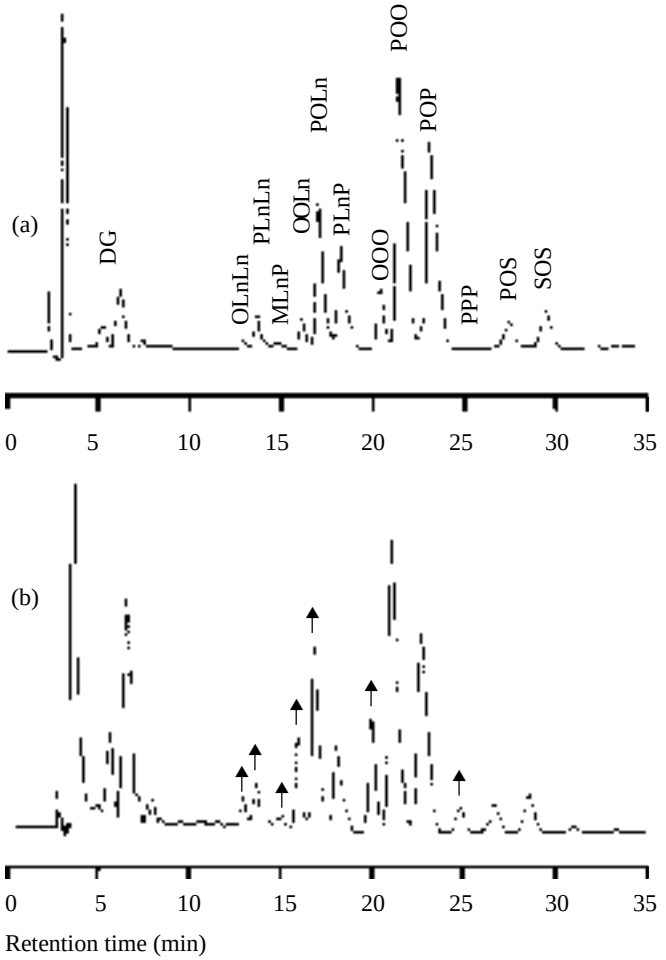

Figure 1. Triacylglycerol (TAG) profiles of palm olein

glycerol (PPLn), dipalmityol-oleyol glycerol (PPO), palmitoyl-oleoyl-steroyl glycerol (POS), dioleoyl-palmitoyl glycerol (OOP), and dioleoyl-steroyl glycerol (OOS). Concomitant with the changes of TAG and FA composition of palm olein after reaction, is the change in melting behaviour. The melting range of palm olein after reaction tends to be broad with the formation of three distinct peaks *(Figure 2)*. The broad melting range in the palm olein after reaction indicates their great degree of heterogeneity. The first peak representing principally glycerides with X_2 at 13 °C and onset value at 6 °C, the second peak representing glycerides with X_2 at 33 °C and onset value at 19 °C, while the third peak representing glycerides with X_2 at 45 °C and onset value at 37 °C.

Discussion

In this study, hydrolysis was initiated with the presence of water from the mycelia (moisture content 6.5% and 7%). During the early state of reaction less hydrolysis occurred. However, as reaction time increased, the amount of water accumulated in the reaction medium increased and the reaction shifted more toward hydrolysis than synthesis. Thus the degree of hydrolysis increased and at the end of reaction (72 h) the remaining TAG was about 80%.

Hydrolysis of palm olein after 72 h reaction lead to the formation of oil enriched with triunsaturated TAG (POL, OOO), polyunsaturated TAG (PLL, LOL, LOO) and trisaturated TAG (PPP) *(Table 3)*. Ghazali et al. (1995) explained the possible mechanisms of the increase in relative

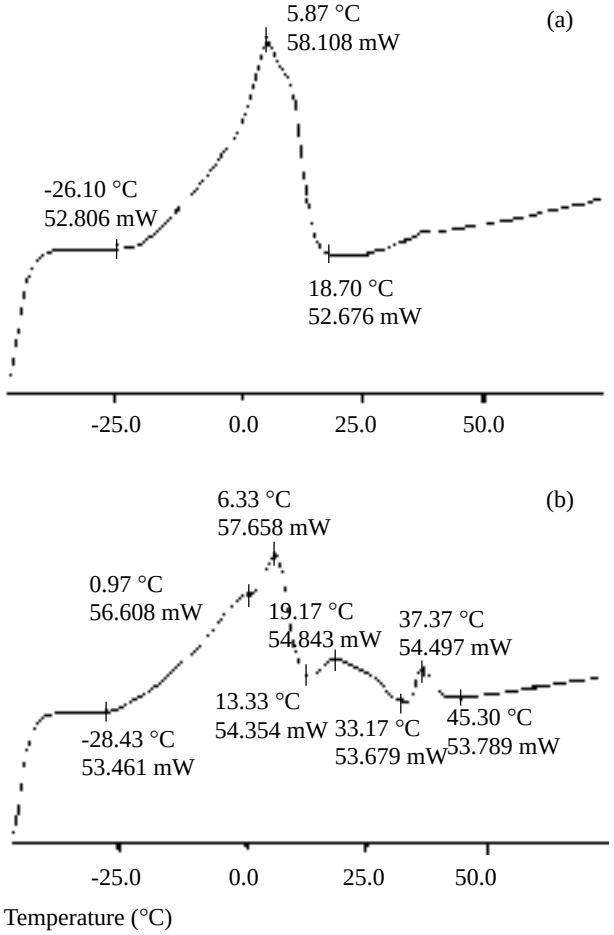

Figure 2. Differential scanning calorimetry heating thermograms of palm olein (a) before reaction (b) after 72 h reactions

Table 3 TAG profiles of the palm olein before and after reaction according to degree of unsaturation

Class	Reaction times	
	Control	72 h
Fully saturated (PPP)	0.53	2.61
Monounsaturated (POP, POS and SOS)	29.74	24.83
Diunsaturated (POO, SOO, PLnP and MLnP)	46.23	40.49
Triunsaturated (POLn, OOO)	17.53	22.42
Polyunsaturated (PLnLn, LnOLn, LnOO)	5.97	10.01

The values shown have been corrected to 100% and are relative to the total concentration of TAG in the reaction mixtures

concentration of PPP of palm olein. One of the possible ways which lead to the formation of PPP could be due to rapid hydrolysis of monounsaturated and diunsaturated TAG *(Table 3)*. Their work showed that the enzymatic reaction of palm olein by *Pseudomonas* sp. (PS-30) (non-specific), R. meihei (IM-60) (1,3-specific) and several other lipases resulted in an increase of OOO, OLL, OOL, SOS and PPP.

As mentioned earlier, the *A. flavus* lipase was found to hydrolyse the saturated FA better than the unsaturated FA (Long et al. 1998). Therefore, as shown in *Table 3*, the lipase will hydrolyse the monounsaturated TAG (POP, POS, SOS) and diunsaturated TAG and leave the oil fraction enriched with the triunsaturated TAG and polyunsaturated TAG. The slight increase in total unsaturated FA in oil fraction *(Table 1)* is expected as previous results have shown that *A. flavus* lipase has less preference towards unsaturated FA and accordingly this FA will be discriminated during hydrolysis and accumulated in the TAG fraction (Long et al. 1998).

The changes in TAG profile affected the melting behaviour of palm olein. The relative increase in triunsaturated (POL, OOO), polyunsaturated (PLL, LOL, LOO), trisaturated (PPP) and dipalmitoyl-glycerol concentration resulted in the change of the heating thermogram of the palm olein. The first peak could possibly consisted of low melting glycerides (6.33 °C), the second peak (19 °C) could be the medium melting glyceride and the third peak could be the HMG (37 °C). According to the work done by Okiy et al. (1978), the triunsaturated and diunsaturated glycerides of palm oil melt at below 20 °C, whereas the trisaturated and disaturated glycerides have melting point above 30 °C.

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