UNIVERSITI PUTRA MALAYSIA

TRANSESTERIFICATION OF PALM OLEIN BY IMMOBILISED MICROBIAL LIPASES

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Thesis Submitted in Fulfilment of the
Requirements for
the Degree of Master of Science in
the Faculty of Food Science and Biotechnology,
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Especially dedicated to my beloved parents.....

Tuan Haji Sidek bin Ludan & Kaimah @ Esah Mohd Nahu

My brother and sister.....

Amid & Siti Hazizah

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of the thesis presented to the Senate of Universiti Pertanian Malaysia in fulfilment of the requirements for the degree of Master of Science.

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January, 1996

Chairman: Associate Professor Hasanah Mohd. Ghazali, Ph.D.

Faculty : Food Science and Biotechnology

Enzymatic transesterification of palm olein in organic solvent was studied. The enzyme was first immobilised to celite, lyophilised for 4 h and then added to a reaction medium composed of water-saturated hexane containing of 10% (w/v) palm olein. The catalytic performance of the enzyme was evaluated by determining changes in the triglycerides (TG) composition and concentration by reverse-phase high performance liquid chromatography (RP-HPLC) and the formation of free fatty acids (FFA) by titration, respectively. For *Candida rugosa* lipase, the optimal water content was controlled by lyophilisation of the lipase preparation for 4 h. The addition of water to the dried immobilised preparation shifted the reaction equilibrium to favour net hydrolysis. Of the commercially available lipases that were investigated, lipases from *Pseudomonas* sp. and the lipase from *Rhizomucor miehei*

resulted in the highest extent of transesterification. Besides palm olein, palm kernel olein and coconut oil showed some changes in the triglyceride composition after transesterification process. Changes in the palm olein concentration in the range 5-100% increased the degree of transesterification of the immobilised *Pseudomonas* sp. and declined with the *R. miehei* lipase. The maximum enzyme activity was reached at an enzyme loading of 0.40% (w/w). The optimum temperature for transesterification by immobilised *Pseudomonas* sp. lipase was 48°C. Hexane, cyclohexane and isooctane were found to be particularly useful organic solvents in the transesterification process. Water-saturated hexane system can be replaced by either dimethysulfoxide- or dimethylformamide-saturated hexane. Methanol was not suitable for the transesterification process. In all cases, the transesterification process resulted in the formation of PPP (tripalmitin), a trisaturated triglyceride initially undetected in the oil, and minor increases in the concentration of OOO, OOL, OLL, SOS, where P, O, L and S are palmitic, oleic, linoleic and stearic acid, respectively thus increasing the slip melting point of the final product.

Abstrak tesis yang dikemukakan kepada Senat Universiti Pertanian Malaysia sebagai memenuhi keperluan syarat untuk Ijazah Master Sains.

TRANSESTERIFIKASI MINYAK OLEIN KELAPA SAWIT OLEH MIKROBIAL LIPASE TERSEKAT-GERAK

Oleh

HAMIDAH SIDEK

Januari, 1996

Pengerusi: Profesor Madya Hasanah Mohd. Ghazali, Ph.D.

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Transesterifikasi secara enzimatik ke atas minyak olein kelapa sawit di dalam sistem pelarut organik telah dijalankan. Enzim disekat-gerak kepada penyokong, celite, kemudian disejuk-keringkan selama 4 jam dan dimasukkan ke dalam medium tindakbalas yang mengandungi 10 mL heksan tepu air yang terdapat di dalamnya 10% (b/b) minyak olein kelapa sawit. Keupayaan pemangkinan oleh enzim ditentukan dengan melihat perubahan kepekatan dan komposisi trigliserida (TG) menggunakan fasa berbalik kromatografi cecair berprestasi tingggi (RP-HPLC) and pembentukan asid lemak bebas (FFA) dengan kaedah titratan. Bagi lipase *Candida rugosa*, kandungan air optimum dikawal dengan menyejuk-kering lipase selama 4 jam. Penambahan air kepada enzim yang kering memindahkan keseimbangan tindakbalas untuk lebih menggemari tindakbalas hidrolisis. Daripada kesemua lipase

komersil yang dikaji lipase dari *Pseudomonas* sp. dan lipase dari *Rhizomucor miehei* menunjukkan darjah tranesterifikasi yang paling tinggi. Selain daripada minyak olein kelapa sawit, minyak olein isirong kelapa sawit dan minyak kelapa juga menunjukkan perubahan minimum komposisi trigliserida selepas tindakbalas transesterifikasi. Perubahan kepekatan minyak olein kelapa sawit dalam julat 5-100% meninggikan darjah transesterifikasi oleh lipase dari *Pseudomonas* sp. tersekat-gerak tetapi tidak dengan *R. miehei*. Aktiviti maksimum enzim dicapai pada muatan enzim 0.40% (b/b). Suhu optimum untuk tindakbalas transesterifikasi oleh enzim *Pseudomonas* sp. tersekat-gerak ialah 48°C. Heksan, sikloheksan dan isooktan adalah pelarut organik yang sesuai untuk tindakbalas transesterifikasi. Sistem heksan tepu air boleh diganti dengan sama ada heksan-tepu dimetilsulfoksida atau heksan-tepu dimetilformida. Metanol tidak sesuai untuk tindakbalas transesterifikasi. Dalam semua kes, tindakbalas transesterifikasi menyebabkan pembentukan PPP (tripalmitin), trigliserida yang pada awalnya tidak dikesan di dalam minyak dan peningkatan kepekatan OOO, OOL, OLL, SOS di mana P, O, L dan S adalah asid palmitik, oleik, linoleik dan stearik masing-masing dan seterusnya meningkatkan takat lebur produk akhir.

CHAPTER 1

INTRODUCTION

The production of fats or oils with desired physical and chemical properties by replacing the fatty acid moieties of triglycerides (TG) with other fatty acid (s) is of great importance and interest from an industrial viewpoint. Although triglycerides can be modified chemically by hydrogenation or chemical interesterification (acidolysis, alcoholysis and transesterification), both reactions occur at random positions. Some of the *cis* unsaturated fatty acids are converted to their *trans* forms in the first reaction which are unfavourable fatty acids in the human diet (Mensink and Katan, 1990). The term `interesterification' is often used to describe reactions that involve the exchange of acyl radicals between an ester and an acid (acidolysis), an ester and an alcohol (alcoholysis) or an ester with other ester (transesterification) (Malcata et al., 1990).

Palm olein is the liquid fraction obtained by fractionation of palm oil after crystallisation at a controlled temperature. The physical characteristics of olein differ significantly from those of palm oil. It is fully liquid in warm climates, has a narrower range of glycerides and blends perfectly with any seed oil (Pantzaris, 1987). Refined, bleached and deodorised (RBD) palm olein, the major form of palm oil consumed and exported by Malaysia, contains 46% saturated fatty acids (myristic, palmitic and stearic), 43% monounsaturated (oleic) and 11%

polyunsaturated acids (linoleic) (Gunstone, 1986). Palm olein is very popular in commercial and industrial establishments where deep frying is a norm because of its high resistance to oxidation (due to low unsaturation) and gumming, low free fatty acid rise and smoking, low rate of foaming, darkening and melting point, and nutritionally good fatty acid composition (no *trans* or iso-acid). One of the very best of its attributes is that it has no unpleasant room odour due to the absence of linolenic acid (Pantzaris, 1987). Idris and Samsuddin (1992) reported that palm olein is suitable as a liquid component of margarine blends and interesterified palm olein was the most suitable material for application both in cream fillings and baking. Although considerable research has been conducted, the benefits of enzymatic modification of edible fats and oils has not yet been realized by industry, especially in such fields as frying oils (Kurashige et al., 1993). Since palm oil will be the most abundant and economical edible oil in the near future, current technological concerns of the edible oil industry is how to expand the multiple usage of palm oil and its fractions. The versatility of palm oil products could be further extended by the interesterification process.

The use of lipases (triacylglycerol acylhydrolase EC 3.1.1.3) to catalyse interesterification reactions has received considerable attention lately because of certain advantages over chemical catalysts. In using lipases, reactions can be done at lower temperatures, the product obtained are cleaner and waste production is reduced (Rattray, 1984; Yamane, 1987 and Mittelbach, 1990). In terms of their substrate selectivity, lipases usually can be classified into three groups (Macrae, 1983). They can be either be 1,3-regiospecific (e.g lipase from *Aspergillus niger, Rhizomucor miehei, Mucor javanicus* and various *Rhizopus* sp.) or non-specific (e.g lipase from *Candida rugosa* (*Candida cylindracea*), *Corynebacterium acnes* and *Staphylococcus aureus*) toward the position of the acyl group of TG during hydrolysis or they can posses selectivity toward particular types of fatty acids (e.g *Geotrichum candidum*). By using a 1,3 specific lipases, the exchange of acyl

moieties is reported to be confined to the 1- and 3- positions giving rise to products with characteristics that cannot be obtained by chemical interesterification (Macrae, 1983). 1,3-positional specificity has been exploited in a number of applications to obtained high-valued speciality fats, such as cocoa butter substitutes of hardened vegetable oils with butter-fat properties (Bloomer et al., 1990; Chang et al., 1990; Sridhar et al., 1991; Chong et al., 1992; Mojovic et al., 1993; Goh et al., 1993). Production of novel triglyceides via enzymatic interesterification and based on vegetable oils to closely mimic the fatty acid distribution in human milk fat also have been developed. This triglyceride can only be made with the use of 1,3-specific lipase by reacting tripalmitin with unsaturated fatty acids. This product is currently under commercial development for use in infant formula under the trade name Betapol (Haumann, 1994). Studies also have been conducted to produce margarine fat without *trans* fatty acids by enzymatic transesterification of cottonseed oil and fully hydrogenated soyabean oil by lipase (Haumann, 1994). The process apparently increase the relative stability of the β'-crystal form indicating possibilities for margarine production without *trans* fatty acids. β' is the preffered crystal form for most margarines and shortening because it produces a smooth texture and an enhanced creaming performance (Marangoni et al., 1993; Haumann, 1994).

Naturally, in aqueous media, lipases catalyse mainly the hydrolysis of triglycerides and other esters. However, in predominantly organic media, they can catalyse a wide range of reactions including interesterification, esterification, aminolysis, acyl exchange, thiotransesterification and oximolysis (Zaks and Klibanov, 1985). It is usually accepted that the positional specificity is retained when lipases are used inorganic solvents (Tsujasaki et al.,1977). Lipase-catalysed transesterification reaction is based on the manipulation of the chemical equilibrium of a thermodynamically reversible reaction and it requires a low water content. During the initial stages of the reaction, a portion of the triglycerides will

be hydrolyzed, consuming water and producing partial (intermediates) glycerides: diglycerides and monoglycerides, and perhaps some glycerol (if the lipase is non-specific) (Reyes and Hill, 1994). Eventually, the concentrations of these glycerides will reach equilibrium values (Goderis et al., 1987; Hansen and Eigtved, 1986; Macrae, 1985). Goderis and coworkers have commented that once this initial degree of hydrolysis is achieved, interesterification can proceed slowly, but smoothly. As the water concentration becomes higher, the activity of lipase usually increases and at the same time, the degree of hydrolysis become higher and deteriorates the quality of modified oils (Kurashige et al., 1993). For industrialization of an enzymatic modification system, the economics and the product quality must be improved. Specifically, it is necessary to improve the modification activity of lipase at minimal water concentration in the reaction system. Hence, the proper choice of solvent that will solubilize the substrate but not affect the enzyme is of great importance (Zaks and Klibanov,1985; Laane et al., 1985; Deetz and Rozzell, 1988). The lipases are mostly immobilised first by means of adsorption in order that the enzyme can be reused in a continuous reaction. The carrier is often celite (Kanasawud, 1992; Macrae, 1983). Immobilisation of lipases on suitable carriers protects the enzyme from the solvent environment and enhances their stability.

At present, there is no report available on lipases suitable for transesterification of palm olein, factors affecting the reaction and the characteristic of transesterified palm olein. Therefore, the objectives of this study are outlined as follows:

1. To select a suitable lipase for transesterification of palm olein.
2. To study factors affecting the transesterification of palm olein by the most suitable lipase.
3. To determine the composition and the concentration of triglycerides in transesterified palm olein and their slip melting point.

CHAPTER 2

LITERATURE REVIEW

Modification of fats and oils

Traditionally, fats and oils processors changed the fatty acid composition either by blending different triglyceride mixtures (combination of natural fats), by chemical modification of the fatty acids (such as hydrogenation), or by rearrangement of the fatty acids on the glyceride backbone (interesterification using alkali catalysts) (Posorske et al., 1988).

According to Sonntag (1982), `interesterification' refers to the reaction in which a fat or other material composed of fatty acid esters is caused to react with fatty acids, alcohols or other esters with the interchange of fatty acid groups to produce a new ester. Thus, the reaction of an ester with an acid is called acidolysis, the reaction of an ester with an alcohol is called alcoholysis and the reaction of one ester with another is termed ester interchange or transesterification.

The interchange of fatty acids between a fat and an alcohol or alcoholysis is analogous to the reaction of acidolysis and is the most important interesterification reaction from the practical point of view because it offers means of preparing fatty esters other than glyceride and is often more suitable and convenient than the alternative method of splitting the fats and reesterifying with the particular alcohol

(Sonntag, 1982). As a consequence, two industrial applications namely, the methanolysis of fat to methyl esters and the glycerolysis of fats to mixed mono- and diglycerides have large scale industrial importance.

Ester interchange reaction, also called transesterification or more precisely, ester-ester interchange, now include a great many intermolecular reactions. Although the ester-ester interchanges of materials intended for non-edible industrial uses are becoming increasingly important, interchanges between triglycerides also have food or food additive applications and command major interest such as the improvement of physical properties of triglycerides product like margarine, shortening and hard butters (synthetic cocoa butter) (Chang et al., 1990; Marangoni et al., 1993)

The main components of fats and oils are the triesters of glycerol and fatty acids (triglycerides) where the physical nature of the oil or fat including melting properties is determined by a) the chain length of the fatty acids in the triglycerides, b) the degree of unsaturation of the fatty acids, and c) the distribution of the fatty acids in the triglyceride molecules (Stevenson et al., 1979; Hammond and Glatz, 1990, Kun et al., 1992). The melting characteristics of fats are of vital importance in many applications. The greater the chain length of a fatty acid, the higher the melting point, and a chain length of ten or twelve carbons marks the transition from liquid saturated fatty acid to those solid at room temperature (Hammond and Glatz, 1990). As natural oils and fats are mixtures of various triglycerides, it has been difficult to study in detail the relationship between the molecular structures and their physical characteristics (Kawahara, 1993). *Cis*-double bonds are more effective in lowering the melting point than *trans* double bonds but the latter seldom occur to any great extent in natural fats and oils. Fats exist in three crystal forms: ∝, ß' and ß. ß' is the preferred crystal form for most margarines and shortenings because it produces a smooth texture and an enhanced creaming performance (Haumann, 1994). The ß

crystal is desirable for fluid or pumpable shortenings for frying and breadmaking (Haumann, 1994).

Changes in physical properties may be achieved through the use of partial or complete hydrogenation, where only the degree of unsaturation of the triglyceride acyl groups is chemically altered, but not the position of the acyl groups on glycerol within the respective triglycerides. Hydrogenated fats have been subjected to criticism on nutritional grounds both because of the decrease in essential fatty acid content, and because of the isomerisation which take place, giving rise to unsaturated *trans*-isomers which behave differently from the *cis*-isomers in some metabolic processes. Recently Mensink and Katan (1990) reported that *trans* fatty acids not only raised the level of blood cholesterol but also decreased the level of good HDL-cholesterol. However, indication is that although *trans* fatty acids have some harmful effects, they are not as harmful as saturated fat (Haumann, 1994).

Unlike hydrogenation, interesterification neither affects the degree of unsaturation nor causes isomerisation of the fatty acid double bond. Thus, it does not change the fatty acid profile of the starting material. Ester-ester interchange of fats and oils may improve physical properties because it changes the original arrangement (distribution) of the acyl group within components of the mixed triglycerides, giving rise to fats with different melting and crystallisation characteristics from those of original fat. Interesterification of oil and fat mixture is very important in food industry, and the products are extremely versatile. Its principle advantage is that it allows the combination of the properties of different oil and fat. Many of these interesterified fat mixture could be used in the formulation for margarine, shortenings and other specialty fats (Chang et al., 1990).

Enzymatic interesterification is now used commercially to produce high value-added products, such as structured triglycerides for confectionery use (Haumann, 1994). The major advantages of enzymatic interesterification over existing chemical technologies lie in the range of specificities available in lipases and the greater degree of control in reactions (Quinlan and Moore, 1993). Performing interesterifications with enzyme catalysts rather than inorganic catalysts eliminates the need to remove and purify the products and by-products of the reaction from the catalysts residues (Mittelbach, 1990), which are concern to consumers. The use of lipases depends on their substrate and position specificities and is very important for food industries (Macrae, 1983; Bloomer et al., 1990). By using a *sn*-1,3-specific lipase, the exchange of acyl moieties is confined to the *sn*-1- and *sn*-3-positions giving rise to products with characteristics that cannot be obtained by chemical interesterification (Macrae, 1983; 1984) eg. production of cocoa butter.

Since the study of Stevenson et al. (1979) many reports concerning enzymatic interesterification have been made. Much of this interest was focused on the production of cocoa butter substitutes using 1,3-specific lipases (Tanaka et al., 1981; Yokozeki et al., 1982 ; Macrae, 1983; Bloomer et al., 1990; Mojovic et al., 1993), reaction between different oils and fat to obtain fat mixture with better melting properties (Eigtved et al., 1986; Forssell et al., 1992; Foglia et al., 1993; Marangoni et al., 1993; Kurashige et al., 1993), enrichment of polyunsaturated fatty acids in fish oils (Yamane et al., 1993; Zuyi and Ward, 1993 a & b), and production of specialty lipids which may have dietetic applications (Schuch and Mukherjee, 1987; Haumann, 1994). Studies by Stevenson et al. (1979) shows that interesterification of glycerol-1-palmitate-2,3-dioleate and palmitic acid with hog pancreatic lipases, which are 1,3-specific in an aqueous system produced palmitate-enriched glyceride. In this system the yield of triglyceride is low due to the large amount of buffer solution used.