UNIVERSITI PUTRA MALAYSIA

THE EFFECTS OF WATER ACTIVITY AND ENZYME MODIFICATION ON LIPASE ACTIVITY DURING ESTERIFICATION

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By

MARIAM BT. TAIB

Thesis Submitted in Fulfillment of the Requirements for the Degree of Master of Science in the Faculty of Science and Environmental Studies Universiti Putra Malaysia

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DEDICATIONS

To Prof. Bakar,
for his patience and belief in me…

To mak, ayah and family,
for their love and concern…

And to my husband, Za'ba,
for his love, support and understanding…

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In the name of Allah, the most Gracious and the most Merciful

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LIST OF ABBREVIATIONS

a_w	-	water activity
CO_2	-	carbon dioxide
FDA	-	Food and Drugs Administration
MW	-	molecular weight
SDS	-	Sodium Dodecyl Sulphate
V_{max}	-	maximum velocity
K_m	-	Michaelis constant
PEG	-	polyethylene glycol
PL2000	-	PEG2000-lipase
PL5000	-	PEG5000-lipase
PSL	-	*Pseudomonas sp.* lipase
gm	-	gram
ml	-	milliliter
*u*l	-	microliter
M	-	molar
mmol	-	millimole
OD	-	Optical Density
nm	-	nanometer
rpm	-	rotation per minute
w/v	-	weight/volume

Abstract of thesis submitted to the Senate of Universiti Putra Malaysia in fulfillment of the requirements for the degree of Master of Science

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By

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May 1998

Chairman : Professor Abu Bakar Salleh, Ph.D.

Faculty : Science and Environmental Studies

Lipase from *Candida rugosa* was modified and the water activity (a_w) required by the enzyme, in order to be optimally active, was investigated. Two methods of modification were used, which were reductive alkylation and modification with polyethylene glycols. For the first method, three types of aldehydes were studied – propionaldehyde, octyldehyde and dodecyldehyde. Two PEGs were used – PEG2000 and PEG5000, for the second method. On the effect of a_w on different degree of modified-lipases, the optimum a_w for propyl-lipase decreased with increasing degree of modification. As for octyl-lipase, the optimum a_w also decreased with increasing degree of modification, up to 60% modification. Further modification will increase the optimum a_w of the octyl-lipase. The a_w for dodecyl-lipase, on the other hand, increased with increasing degree of modification. A similar trend was also observed with PEG-lipases, up to a certain degree of modification. It was found that the optimum a_w of the enzymes, depended on the

degree of modification, hydrophobicity and also the chain-length of the modifier. The relative activity increased with increasing degree of modification for all modified enzymes tested except for dodecyl-lipase, where the activity decreased as the degree of modification increased. On the effects of solvent on the a_w of the propyl-lipase, there is no significant difference of optimum a_w requirement in the solvents tested, compared to native lipase. The optimum a_w of octyl-lipase generally shifted to a lower value in hydrophobic solvents, while for dodecyl-lipase, the optimum a_w shifted to higher values. In all solvents tested, the optimum a_w of PL2000 are different, while PL5000 required higher a_w , In general, the relative activity of the modified enzymes are better in non-polar solvents, compared to polar solvents.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi syarat untuk mendapatkan Ijazah Master Sains

KESAN AKTIVITI AIR DAN PENGUBAHSUAIAN ENZIM KE ATAS AKTIVITI LIPASE SEMASA ESTERIFIKASI

Oleh

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Lipase dari *Candida rugosa* telah diubahsuai dan keperluan aktiviti air (a_w) untuk aktiviti enzim yang optima bagi lipase tersebut telah dikaji. Dua kaedah ubahsuaian digunakan, iaitu pengalkilan terturun dan ubahsuaian oleh polietilenaglikol (PEG). Untuk kaedah pertama, tiga jenis aldehid digunakan iaitu propildehid, oktildehid dan dodesildehid. Dua jenis PEG iaitu PEG2000 dan PEG5000 digunakan untuk kaedah kedua. Dalam kajian kesan a_w ke atas darjah ubahsuaian enzim yang berbeza, a_w optima bagi propil-lipase menurun dengan peningkatan darjah ubahsuaian. Keputusan yang sama diperolehi bagi oktildehid, sehingga 60% ubahsuaian; pertambahan darjah ubahsuaian akan meningkatkan semula a_w optima. Sebaliknya, a_w optima bagi dodesil-lipase meningkat dengan bertambahnya darjah ubahsuaian. Kesan yang sama seperti dodesil-lipase diperolehi bagi kedua-dua PL2000 dan PL5000. Didapati bahawa a_w optima bagi enzim terubahsuai bergantung kepada darjah ubahsuaian, hidrofobisiti dan panjang rantai bahan pengubahsuaian. Tindakbalas relatif untuk kesemua jenis lipase terubahsuai

meningkat dengan darjah ubahsuaian, kecuali untuk dodesil-lipase di mana aktivitinya menurun dengan peningkatan darjah ubahsuaian. Bagi kajian pelarut organik ke atas a_w optima oleh 60% enzim terubahsuai, tiada perubahan yang signifikan didapati bagi propil-lipase, bila dibandingkan dengan lipase asli. Bagi oktil-lipase, a_w optima secara umumnya menurun, dan sebaliknya bagi dodesil-lipase, a_w optimanya meningkat, dalam pelarut organik yang dikaji. Bagi PL2000, a_w optimanya berbeza-beza; PL5000 memerlukan aktiviti air yang lebih tinggi. Secara umumnya, tindakbalas relatif lipase terubahsuai adalah lebih baik di dalam pelarut organik tidak polar berbanding pelarut organik polar.

CHAPTER I

INTRODUCTION

Fatty acid alkyl esters are used in an extensive range of products and also as synthetic intermediates (Stevenson *et al.*, 1994). Nowadays, 90% of these chemical products are produced from petrochemical feedstocks (natural oil, gas and coal), whereas only 10% are produced from oleochemical feedstocks (vegetable oils and animal fats) (Sibeijn *et al.*, 1994). The use of petrochemicals has several disadvantages. The resources are limited, the use of petrochemicals adds to the greenhouse effect (there is a net production of CO_2) and the biodegradability of petrochemicals is usually not good.

In contrast to petrochemicals, oleochemicals are produced from renewable resources, have no net CO_2 production and usually the biodegradability is excellent. Despite these advantages compared to those of the petrochemicals, the use of oleochemicals is not yet widespread. However, during the last decade, interest in the biotechnology of fats and oils has been growing continuously. The increasing surplus of fats and oils in the more developed countries has supported both fundamental and applied research aimed at the manufacture of alternative lipid-derived products on an industrial scale (Malcata *et al.*, 1990).

Traditional methods of production, such as extraction from plant materials, direct biosynthesis by fermentation or chemical synthesis are high cost and low yield process on the desired components (Rocha *et al.*, 1994). Enzymatic conversions are becoming more and more attractive, not only because highly specific enzymes can be chosen as catalysts, but also because products from enzyme-mediated reactions can be considered as 'natural' accordingly to the FDA requirements, therefore, with a higher economic value.

Among the most promising chemical routes of industrial interest are the hydrolysis, ester synthesis and interesterification reactions of lipids brought about by lipases (Malcata *et al.*, 1990). Lipase-catalysed reactions offer several benefits over chemically catalysed reactions, such as milder operating conditions, cleaner products and reduced waste productions (Yamane, 1987). Lipase have also shown a surprisingly broad substrates specificity. Moreover, those enzymes seem especially well suited to application in organic solvents, and thus, in organic chemistry (Santainello *et al.*, 1993).

True lipases act at an oil-water interface on water-insoluble substrates. The biphasic reaction system pose problems for *in vitro* enzyme assay which can be overcome by using a reaction system containing organic solvents. In such a system, the water content can be reduced, so that the lipase reaction favours esterification instead of hydrolysis (Basri *et al.*, 1991). Zaks and Klibanov (1984) and Nishio *et al.* (1988) have shown that certain lipase can become more thermostable and catalyse transformation in organic media.

The potential of lipase-catalysed reactions in organic solvents can be exploited further using enzymes having new and improved properties following their chemical modification. Covalent coupling of a variety of hydrophobic groups has been used for making enzymes more suitable for catalysis in organic media. Polyethylene glycol (PEG) has been used extensively for this purpose (Inada *et al.* , 1986 ; Veronese *et al.* 1985 and Habeeb, 1966). Reductive alkylation with aldehydes such as acetaldehyde or octaldehyde increased the activity of trypsin (Ampon *et al.*, 1991). Modification of lipase using hydrophobic imidoester was also done by Basri *et al.* (1992).

In general, the solubility of the modified enzyme in organic solvent increases with increasing degree of modification (Adlercreutz, 1996). The hydrolytic activity of *Candida* lipase decreased after modification with hydrophobic imidoesters, but the activity in an esterification reaction increased considerably (Basri *et. al.*, 1992). The esterification activity increased with increasing degree of modification of the lipase and also with increasing hydrophobicity of the modifying reagent.

It is generally accepted that water plays a significant role for biocatalysis in organic media. The correct water level is very important in determining the reaction equilibrium of an enzymic reaction (Ibrahim *et al.*, 1988; Halling, 1992 and Robb *et al.*, 1994). The controlling of the water in the reaction system is also important in order to minimize the hydrolytic reaction, when the esterification reaction is in favour.

There are numerous parameters governing the enzyme activity which are related to the critical water content in the reaction system. Zaks and Klibanov (1988) described that a monolayer of water present around the enzyme molecules is more important than the water present within the system for successful catalytic activity. This monolayer of water which determines the thermodynamic water activity (a_w) of the system will not change although the environment parameters were altered. One of several methods in achieving a low water level in the organic media reaction system is the use of salt hydrate (Halling, 1989). Kvittingen *et al.* (1992) have shown that salt hydrate can be successfully used to buffer the optimum water level during lipase-catalysed synthesis in organic media.

Takahashi *et al.* (1984a) reported that PEG-modified enzymes in organic media bind water . The water activity in the reaction medium greatly influences the catalytic activity of the enzyme. Therefore, similar to that, the objectives of my studies are :

1. To investigate the effects of water activity and reductive alkylation of lipase with different aldehydes, on its activity at different degrees of modification.
2. To investigate the effects of water activity and modification of lipase with different polyethylene glycols, on its activity at different degrees of modification.
3. To investigate the effect of water activity of different modified lipases at fixed degree of modification, in different solvent systems.

CHAPTER II

LITERATURE REVIEW

Enzymes as Biocatalysts

Organic reactions are commonly practiced in industry using acids as catalysts at high temperature and pressure. The chemical route often suffers from poor reaction selectivity, leading to undesirable side reactions and low yields. In recent years, the employment of enzymes as biocatalysts has emerged as a potential route to replace the conventional chemical process (Chand *et al.*, 1997). The use of enzymes in chemical process engineering has been receiving ever increasing attention and new techniques and methodologies for their application are continuously sought (Cremonesi *et al.*, 1975).

Enzymes derived from a number of plant, animal and microbial sources have been recognised as valuable processing aids in a multitude of applications . These biocatalysts are increasingly being used either as whole cells or purified enzymes in organic reactions (Nair and Anilkumar, 1994). Enzymes are remarkably selective catalysts which can discriminate on the basis of chemical functionality, (chemoselectivity), optical activity (enantioselectivity) and molecular position (regioselectivity) (Rich *et al.*, 1995).

Enzymes have three distinguishing characteristic as catalysts:

1. They accelerate the rate of reactions.
2. They are selective : the rate of reaction of a particular substance may be accelerated dramatically, while that of a structurally closely related substance is not.
3. They may be subjected to regulation: that is , catalytic action may be strongly influenced by the concentrations of substrates, products or other species present in solution (Whitesides and Wong, 1985).

Enzymes have certain other characteristics which are important in considering their applications in organic synthesis. Their availability, cost and lifetime in use vary widely. A typical enzyme will contain one active site per 20,000 - 50,000 MW. The economic of enzyme used depend upon a number of factors: the cost of the enzyme, its specific activity and its operation lifetime.

Enzymes are classified and named according to the nature of the chemical reactions they catalyse (Voet and Voet , 1990). There are six major classes of reactions that enzymes catalyse.

Classification	Type of Reaction Catalysed
Oxidoreduction	Oxidation-reduction reaction
Transferases	Transfer to functional groups
Hydrolases	Hydrolysis reaction
Lyases	Group elimination to form double bond
Isomerases	Isomerization
Ligases	Bond formation coupled with ATP hydrolysis

All lypolytic enzymes are hydrolases (Brokerhoff and Jensen, 1974). Among The hydrolases so far investigated, lipase is one of the most advantageous because it is stable, inexpensive and widely used in the development of various applications in the detergents, oils and fats, dairy and pharmaceutical industries. (Stamatis *et al.* 1995).

Lipases

Lipases (E.C.3.1.1.3) or acylglycerol hydrolases are enzymes which catalyse the hydrolysis of long - chain aliphatic acids from acylglycerols at an oil/water interface (Jensen *et al.*,1983). The interface is usually provided by emulsion globules or lipoprotein particles. The element providing the interface has been termed the supersubstrate. These enzymes are serine hydrolases that catalyse reversible ester formation and hydrolyse reaction without cofactor (Derango *et al.*, 1994). Enzymes acting as lipases can in some cases also act as esterases, phospholipases, cholesterolesterases, thioesterase and cutinase (Svendsen,1994).

Sources of Lipases

In general, lipase can be derived from four sources : animals, plants, fungi and bacteria. The role of lipase is the same, that is to monitor the function of lipids in the organisms such as in pancreas, lingual, adipose tissues and other organs. Microbes from the genus of fungi, yeast and bacteria are the main sources of lipase in industry (Macrae, 1983). Most of the microbial lipases are extracellular (Iwai and Tsujisaka, 1984). Purified bacterial lipase can be obtained in large