UNIVERSITI PUTRA MALAYSIA

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

MARIAM BT. TAIB

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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

May 1999
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…
ACKNOWLEDGEMENTS

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

All praise be to Allah the Almighty, for giving me the strength and will to write and at last complete this project.

I am very, very grateful to my supervisor, Professor Dr. Abu Bakar Salleh, for, most of all, believing in me. For all the patience, guidance, advice, ideas, critics, encouragement and talks about life, my deepest gratitude goes to you. To my co-supervisor, Associate Professor Dr. Mahiran Basri, thanks a lot for introducing water activity to me. My deep appreciation goes to the ideas, comments and especially the looks of acknowledgement and appreciation during the weekly meetings, I won’t forget it.

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To mak, ayah, brothers, sisters, nieces and nephews, their love and support keep me going; and to my in-laws who have been very concern about my study all this while - thank you. And last but not least, to my husband Za’ba, for his love, continuous support and understanding – thank you, abang, and I dedicate this success to you, too.
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LIST OF ABBREVIATIONS

- $a_w$ - water activity
- $\text{CO}_2$ - carbon dioxide
- FDA - Food and Drugs Administration
- MW - molecular weight
- SDS - Sodium Dodecyl Sulphate
- $V_{\text{max}}$ - maximum velocity
- $K_m$ - Michaelis constant
- PEG - polyethylene glycol
- PL2000 - PEG2000-lipase
- PL5000 - PEG5000-lipase
- PSL - $Pseudomonas$ sp. lipase
- gm - gram
- ml - milliliter
- ul - microliter
- M - molar
- mmol - millimole
- OD - Optical Density
- nm - nanometer
- rpm - rotation per minute
- w/v - weight/volume
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By

MARIAM BT. TAIB

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, octyldehydro, and dodecyldehydro. 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

MARIAM BT. TAIB

Mei, 1998

Pengerusi : Profesor Abu Bakar Salleh, Ph.D.
Fakulti : Sains dan Pengajian Alam Sekitar

Lipase dari *Candida rugosa* telah diubahsuaian dan keperluan aktiviti air (a_v) 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 propildelheid, oktildehid dan dodesildehid. Dua jenis PEG iaitu PEG2000 dan PEG5000 digunakan untuk kaedah kedua. Dalam kajian kesan a_v ke atas darjah ubahsuaian enzim yang berbeza, a_v 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_v optima. Sebaliknya, a_v 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_v optima bagi enzim terubahsuaian bergantung kepada darjah ubahsuaian, hidrofobisiti dan panjang rantai bahan pengubahsuaian. Tindakbalas relatif untuk kesemua jenis lipase terubahsuaian...
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, $a_w$ optimnya meningkat, dalam pelarut organik yang dikaji. Bagi PL2000, $a_w$ optimnya 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.

<table>
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<th>Classification</th>
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All lypo lytic 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, cholestereosterases, 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