UNIVERSITI PUTRA MALAYSIA

IMMOBILIZATION OF *CANDIDA RUGOSA* LIPASE ONTO N-VINYL-2-PYYROLIDONE-CO-STYRENE HYDROGEL FOR USE IN ENANTIOSELECTIVE ESTERIFICATION

AIZA BT HARUN

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By

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Thesis Submitted in Fulfilment of the Requirement for the Degree of Master of Science in the Faculty of Science and Environmental Studies. Universiti Putra Malaysia

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DEDICATIONS

To Assoc. Prof. Dr. Mahiran,
for his patience, guidance and belief in me....

To umi, ayah and family,
for their love and concern.....

To my husband, Azhan and my childrens,
for their love, support and understanding....

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Chairperson : Associate Professor Mahiran Basri, Ph. D

Faculty : Science and Environmental Studies

Lipase from *Candida rugosa* was immobilized onto N-vinyl-2-pyyrolidone-co-styrene hydrogel (VP-co-ST) The crosslinker and the initiator used were EDMA and AIBN respectively Three different compositions of monomer were used, namely, (VP.ST)% , 10 90, 50 50 and 70 30 The VP-co ST hydrogel immobilized lipases were used in the enantioselective esterification of (R,S)-2-(4-chlorophenoxy) propanoic acid with *n*-tetradecanol

The optimum reaction time was 24h for native and all immobilized lipases Studies on the effect of percentages composition of monomers of the VP-co-ST hydrogel immobilized lipases showed that lipase immobilized onto hydrogel (VP ST)% , 10 90 exhibited the highest percentages of enantiomeric excess at approximately 24%

Generally, increasing the temperature of the enantioselective esterification increases the percentage of ester conversion for all VP-co-ST hydrogel immobilized lipases However, the percentages of enantiomeric excess increases up to 40°C and subsequently decreases At 40°C, the highest percentage of enantiomeric excess was shown by VP-co-ST hydrogel with composition of monomer (VP ST)% , 10 90 at 45%

In the solvent effect studies, the percentage of ester conversion was higher in relative more polar organic solvents with log P value ranging from 2 0 to 3 0 for all immobilized lipases and native lipase The organic solvents such as toluene, chloroform and carbon tetrachloride showed higher percentage of ester conversion as compared with other solvents Immobilized lipases exhibited higher percentage of enantiomeric excess compared to the native lipase Higher percentage of enantiomeric excess were obtained when using chloroform and carbon tetrachloride at approximately 50% and 46% for VP-co-ST (VP ST)% , 50 50 and (VP ST)% , 10 90 respectively

In the water activity studies, at optimum a_w all immobilized lipases showed higher percentage of ester conversion compared to the native lipase at approximately 23%, 16% and 22% for VP-co-ST hydrogel with composition of monomer (VP ST)% , 10 90, 50 50 and 70 30 respectively At optimum a_w, VP-co-ST hydrogel immobilized lipase with composition of monomer (VP ST)% , 10 90, 50 50 and 70 30 exhibited higher percentage of enantiomeric excess at approximately 40%, 21% and 28% respectively compared to the native lipase

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
bagi memenuhi keperluan Ijazah Master Sains

IMOBILISASI LIPASE DARI *CANDIDA RUGOSA* KE ATAS HIDROGEL N-VINYL-2-PYYROLIDONE-CO-STYRENE UNTUK KEGUNAAN DI DALAM TINDAKBALAS ESTERIFIKASI ENANTIOPILIHAN

Oleh

AIZA BT HARUN

Disember 1999

Pengerusi : Profesor Madya Mahiran Basri, Ph. D

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Lipase dari jenis *Candida rugosa* telah diimobilisasikan secara pemerangkapan ke atas polimer hidrogel Polimer hidrogel ini telah disintesiskan secara pempolimeran dari monomer N-vinil-2-pirolidon dan stirena dengan menggunakan EDMA sebagai agen rangkai silang dan AIBN sebagai agen pemula Lipase yang telah diimobilisasikan pada tiga jenis polimer hydrogel yang mempunyai peratus komposisi monomer yang berbeza iaitu (VP ST)%, 10 90, 50 50 dan 70 30 telah digunakan di dalam tindakbalas esterifikasi enantiopilihan mengunakan asid rasemik (R,S) klorofenoksi propanoik dan *n*-tetradekanol

Didapati lipase asli dan lipase terimobilisasi menunjukkan peratus pertukaran ester yang paling tinggi pada masa tindakbalas selama 24jam dan lipase yang terimobilisasi pada hidrogel dengan komposisi monomer (VP ST)% , 10 90 mempunyai peratus kelebihan enantiomer yang paling tinggi iaitu 24%

Umumnya, peratus pertukaran ester bertambah dengan pertambahan suhu tindakbalas bagi semua lipase terimobilisasi Peratus kelebihan enantiomer pula bertambah hingga ke suhu 40°C dan selepas itu nilainya semakin berkurang Pada suhu 40°C, lipase terimobilisasi pada hidrogel (VP ST)% , 10 90 menunjukkan nilai peratus kelebihan enantiomer yang paling tinggi iaitu 45%

Kajian kesan pelarut menunjukkan peratus pertukaran ester bagi lipase asli dan lipase terimobilisasi meningkat di dalam pelarut yang agak polar seperti toluena, klorofom dan karbon tetraklorida Lipase terimobilisasi menunjukkan nilai peratus kelebihan enantiomer yang lebih tinggi berbanding dengan lipase asli Di dalam pelarut klorofom dan karbon tetraklorida, lipase terimobilisasi pada hidrogel (VP ST)% , 10 90 dan 50 50, masing-masing menunjukkan nilai peratus kelebihan enantiomer sebanyak 46% dan 50%

Kajian aktiviti air terhadap peratus pertukaran ester menunjukkan lipase terimobilisasi mempunyai nilai yang lebih tinggi berbanding dengan lipase asli pada aktiviti air yang optima Lipase terimobilisasi pada (VP ST) , 10 90, 50 0 dan 70 30 masing-masing memberikan nilai 23%, 16% dan 22% Lipase terimobilisasi juga mempunyai peratus kelebihan enantiomer yang lebih tinggi dari lipase asli Ini ditunjukkan oleh lipase terimobilisasi pada hidrogel (VP ST)% , 10 90, 50 50 dan 70 30 masing-masing dengan nilai 40%, 21% dan 28%

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LIST OF ABREVIATIONS

VP	N-vinyl-2-pyyrolidone
ST	Styrene
EDMA	Ethyleneglykol dimethacrylate
a_w	Water activity
Log P	Polarity constant
GC	Gas chromatography
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
CCl_4	Carbon tetrachloride
$CHCl_3$	Chloroform
CPPA	Chlorophenoxy propanoic acid
TLC	Thin layer chromatography
AIBN	Azobisisobutyronitrile
HEMA	Hydroxyethylmethacrylate
MMA	Metylmethachrylate
DVB	Divinylbenzene

CHAPTER I

INTRODUCTION

Enzymes are biological catalysts that allow chemical reactions to occur in living organisms at ambient condition. Enzymes bring the reaction to its equilibrium position more quickly and have the ability to catalyze the reaction under mild conditions in neutral aqueous solution at normal temperature, pressure and with high substrate specificity. Generally, the use of enzymes has gradually been extended into variety of fields, such as brewing, food production, textile industry and medicine. Further, the recent development of biochemistry together with the progress in applied microbiology and genetic engineering, has greatly extended the range of enzyme applications (Chibata, 1982). Although enzymes are efficient and effective catalysts, they are not always ideal for practical applications. This is because some of the advantages of enzymes may even be disadvantageous in practical use as catalysts.

Enzymes are generally unstable and cannot be used in organic solvents or at elevated temperatures. Because of that, immobilization is one of the approaches to obtain a better enzyme that could withstand organic solvents and high

temperature. It is a technique that makes enzyme use in industries more attractive. This is because enzymes offer certain processing advantages over free enzymes such as ease of separation from the product, localization within reactor, improved stability or activity retention, continuous operation and the possibility of obtaining superactivity (Bailey and Ollis, 1986).

Entrapment is one of the popular methods of enzyme immobilization and is achieved either by the inclusion of enzymes within polymeric matrices or by separation from the bulk phase by a semi-permeable microcapsules. An important feature of these methods is that the enzyme is not actually attached to anything. Therefore none of the stearic problems associated with covalently or electrostatically binding an enzyme on to a polymer, for example binding the enzyme in such a way that its active site is obstructed by a portion of the polymer matrix. The three-dimensional gels such as polyacrylamide gels, acrylamide and methylene-bis-acrylamide are almost used to entrap the enzymes whereby the porous matrix totally surrounds the enzymes and it is the barrier through which the solution must pass (Trevan, 1980). The hydrophilic polymers are normally selected to form an open crosslinked hydrogel because the solvents can move freely throughout it. This characteristic enables the substrate to interact very easily with the enzymes. By entrapment method, the enzyme can be protected from environmental stresses such as pH, temperature, solvents, salts, self-destruction, inhibitors and poisons.

The most attractive characteristic that makes enzyme far superior to conventional catalyst is their high enantioselectivity. The enzyme often exhibits a high selectivity for a target substrate, thus it can be used as a biocatalyst for the resolution of optically active materials. Lipases are the most popular enzymes which have been used extensively in the preparation of chiral compound such as anti-inflammatory agents like (S)-ibuprofens, chiral drug intermediates like anticancer drug and hypertensive drug. In aqueous media, lipase catalyzes the hydrolysis reaction of ester compound, whereas in non-aqueous media, it enables the reverse reaction, that is esterification (Goto *et al.*, 1996). This characteristic can be utilized for the preparation of chiral compounds through the resolution of their racemic mixture by esterification or hydrolysis using lipase.

With the recent development of enzyme technology, the immobilized enzymes also can be utilized to produce the optically active materials by the enantioselective reaction. The gel was choosen as a support material because the uniform size can be formed by a mild and simple immobilization procedure (Santoyo *et al.*, 1996).

Objectives

As there is no study reported on enantioselective esterification by using hydrogel immobilized enzyme, thus we embarked on this project with the following objectives:

1. To immobilize lipase from *Candida rugosa* onto hydrogel by entrapment.
2. To determine the enantioselectivity of immobilized lipase with respect to:

    i. Temperature of the reaction.

    ii. Different type of solvents in the reaction mixture.

    iii. Controlled water activity of reaction mixture.

CHAPTER II

LITERATURE REVIEW

Enzyme as Biocatalyst

Enzymes are among the most remarkable biomolecules known because of their extraordinary specificity and catalytic powers, far greater than that of chemical catalysts. Generally, enzymes are distinguished from other catalysts because of their high substrate specificity, mild reaction and reduced waste production. As for specificity, enzymes work to modify specific chemical bonds usually at specific sites on a molecule. These characteristics are great asset when one wants to apply the catalysts in the synthesis of various compounds (Takahashi *et al.*, 1984). As effective catalysts, enzymes provided reasonable reaction rates under mild condition whereas for chemical reactions, they may require high temperature or pressure to satisfy the rate of the reaction.

The most versatile groups of enzymes are lipases. Lipases differ from carboxylesterase, such as proteases and pectinesterase, which act on substrate in aqueous solution whereby lipases can act both in aqueous solution and organic solvents. Lipases normally catalyzed the hydrolysis of fatty acid ester linkages in triglycerides into glycerol and fatty acids. They are active in hydrophobic

organic solvents with limited water content where the chemical equilibrium is shifted towards ester synthesis (Zaks and Klibanov, 1985). The most widely studied lipases are microbial extracellular enzymes produced by the fermentation of yeasts, fungi and bacteria (Bagi *et al.*, 1997). The most promisingly commercially available microbial lipases are from *Candida rugosa*, *Chromobacterium viscosum*, *Rhizomucor meihei* and *Pseudomonas flourecens*. These lipases are able to catalyze the reactions such as hydrolysis and synthesis. For synthesis, it can be further separated into esterification, interesterification, alcoholysis, acidolysis and aminolysis.

Generally, a variety of products were formed when lipase catalyzed these reactions. For example lipases from *Rhizomucor meihei*, *Rhizopus delemar*, *Penicillium cyclopium* were used to catalyze the production of esters such as geranyl acetate, isoamyl butyrate which were used in flavour aroma (Welsh *et al.*, 1990). Servat *et al.* (1990) reported that the lipase from *Pseudomonas fluorecens* was used to catalyze the synthesis of monoglycerides such as monolaurin, sugar ester and fatty acyl amino ester, which was used in bread-softening agents. This lipase was also used to catalyze the synthesis of optically active natural products such as manalone, lactone and exo-brevicomin for the preparation of chiral building block 1,3-syn-diol. Macrea (1983) had reported the transesterification of racemic α-alkyl substituted primary alcohols to obtain separate optical isomers for use in manufacture of antiimflammatory agents.

Recently, the industrial applications of lipases have grown rapidly and are likely to expand further in the coming years. Andree *et al.* (1980) have studied the

use of lipase in detergent industry, whereby its function is to remove the stains from the fabrics. The most promising lipases that were used for this purpose include those from *Candida rugosa* (Nishioka *et al.*, 1990) and *Chromobacterium viscosum* (Minoguchi and Muneyuki, 1989). El Sayed *et al.* (1990) reported that the use of surfactant system containing lipase was very suitable to remove the olive oil from the cotton fabrics. As laundering is generally carried out in alkaline media, the lipase which are active under such conditions are preferred such as *Aspergillus oryzae* – derived lipase (Gerhartz, 1990). Apart from detergents for cleaning fabrics, lipase also can be applied in dishwashing. The interaction of lipase in this process was investigated by Fukano and Abe (1990) and Van Dijk (1989). They found that the lipase component causes an increase in detergency and prevents scaling.

The use of lipases in flavour production in dairy products is well established such as in cheese, butter and margarine. The aroma and the texture of these milk products are produced as a result of fat, protein and lactose metabolism in milk. In that case, the lipases are frequently used for accelerating the maturation of cheese and for the production of typical flavours. Apart from milk products, lipases are also used to improve flavours of rice wine and other alcoholic beverages, such as apple wine (Shay *et al.*, 1990). This process was carried out with continuous fermentation of *C. utilis* in the presence of beef extract or butteroil and the lipase. Lipases are also being used in chocolate industry to obtain flavours of milk chocolate, caramels, tofees and butter creams.

Another important industry with the enormous potential for lipase application is in the food industry. Seitz (1974) reported that lipases have been used in fat removal during fish processing. Meanwhile Johnson and Welch (1964) reported that lipase catalyzed the partial hydrolysis of triglycerides to increase the monoglycerides content in the bread dough. As in the use in bread dough, the formation of monoglycerides is important for the improvement of egg-white whipping properties (Wiseman, 1975)

Immobilization

Many researchers have found that enzymes are generally unstable and cannot be used in organic solvents or at elevated temperature (Chibata, 1982). Thus, to obtain a superior catalyst for practical applications, researchers have investigated two approaches. One is the synthetic approach, which is using recently developed techniques of organic synthesis and polymer chemistry to synthesize catalysts having enzyme-like activities. Another technique is immobilization of the enzyme.

Immobilization is a technique to confine or to localize an enzyme in certain defined region or space with the retention of their catalytic activities, which then can be used repeatedly and continuously. Trevan (1980) has defined immobilization as a separation of biocatalyst in a distinct phase that allow exchange with, but is separated from the bulk phase. Once the immobilization takes place some changes such as changes in substrate specificity and activities of

the immobilized enzymes may occur. The stability may also changes after immobilization. Stability refers to an ability to resist alteration. For example, resistance to loss of activity during storage, loss of activity due to process operations, digestion by proteases and disruption by chemicals (Bickerstaff, 1987). Apart from stability, the optimum pH of the enzyme may also shifted by immobilization due to the changes in electro-arrangement in the enzyme protein and the electric charge on the carrier. Immobilization may also enhanced the heat stability of the enzymes, for example the optimum temperature of immobilized enzyme become higher than that of the native enzymes. The kinetic constant may also change after immobilization which may be due to the electrostatic interaction between an enzyme and the substrate (Chibata, 1982).

Types of Immobilization Technique

Generally there are three main categories of immobilization technique. They are:

i. Carrier Binding

ii. Crosslinking

iii. Entrapment

Carrier Binding

The carrier binding method refers to the binding of enzymes to water-insoluble carrier. Adsorption is one of the carriers binding method, which was performed as the simplest method of obtaining an immobilized enzyme.