Influence of Water Activity on the Enantioselective Esterification Catalysed by Hydrogel Immobilised Lipase from *Candida Rugosa*

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ABSTRACT

Lipase from Candida rugosa was immobilised onto N-vinyl-2-pyrrolidone–co-styrene (VP-co-ST) hydrogel. The ethylene dimethacrylate (EDMA) and azoisobutyronitrile (AIBN) were used as a cross-linking agent and polymerization promoter respectively. This particular VP-co-ST hydrogel immobilised lipase was used to catalyse the enantioselective esterification of (R,S) -2-(4-chlorophenoxy) propanoic acid with n-tetradecanol. Only VP-co-ST hydrogel immobilised lipase with composition of VP 10% and ST 90% and native lipase (unimmobilised lipase) will be discussed in this study. The reaction was performed at optimum reaction time of 24 hours and at optimum temperature 40°C in organic media. The study revealed that water activity, a_w may influence the enantioselective esterification in which at lower a_w value, the percentage of ester conversion was greater compared with at higher a_w value when using both hydrogel immobilised lipase and native lipase in the reaction. However, when comparing between these two lipases, the hydrogel immobilised lipase exhibited higher percentage of ester conversion rather than native lipase at optimum water activity value of 0.3.

Keywords: *hydrogel, immobilisation, entrapment, water activity and enantioselective esterification*

Introduction

Enzymes are biological catalysts that have been used to bring chemical reactions to its equilibrium position more quickly. The difference between

enzymes and chemical catalyst is that they catalyse the reaction under mild conditions at normal temperature, pressure with extraordinary high substrate specificity and have catalytic powers. Nowadays, the uses of enzymes have expanded globally and are gaining popularity. This is true by looking at the recent development of biochemistry together with the progress in applied microbiology and genetic engineering which has greatly extended the range of enzyme applications (Chibata 1982). With their high substrate specificity properties, enzymes are able to modify specific chemical bonds at specific sites on a substrate molecule successfully. So far, this unique property is not available for any chemical catalysts. Hence, this special characteristic had been targeted to be enormous assets in the application of enzymes to synthesize various compounds such as chiral compounds, chiral drug intermediates, and anti-inflammatory agents (Takahashi et al. 1984). From their effective catalytic powers, enzymes provided reasonable reaction rates under mild condition, whereas for chemical catalysts, they may require high temperature or pressure to satisfy the rate of reaction.

Lipases are the most versatile group of enzymes in which they differ from other enzymes such as proteases and pectinesterase. Lipases have a great tendency to act on a substrate both in aqueous solution and organic media whereby in certain reactions, organic medium is crucial if compared to aqueous solution. The most studied lipases are microbial extracellular enzymes produced by fermentation of yeast, fungi and bacteria (Bagi et al. 1997) where they can be found commercially as *Candida rugosa*, *Chromobacterium viscosum*, *Rhizomucor meihei* and *Pseudomonas flourecens*. All these remarkable lipases are able to catalyse hydrolysis, esterification, interesterification, alcoholysis, acidolysis and aminolysis reactions effectively. It has been reported that lipases from *Rhizomucor meihei*, *Rhizopus delemar*, *Penicillium cyclopium* were used to catalyse the production of esters such as geranyl acetate, isoamyl butyrate which richly used in flavour aroma (Welsh et al. 1990). The synthesis of monoglycerides such as monolaurin, sugar ester and fatty acid amino ester which were used in bread-softening agents used lipase from *Pseudomonas flourecens* to catalyse the synthesis (Servat et al. 1990). Apart from that, this lipase also has been used to catalyse the synthesis of optically active natural products such as manalone, lactone and exo-brevicomin.

However, in most of industrial, analytical, and clinical processes, enzymes are mixed in a solution with substrates and cannot be economically recovered after the exhaustion of the substrates. Thus,

there is an incentive to use enzymes in an immobilised form, so that, they may retain in a biochemical reactor to catalyse further the subsequent feed (Wang 2005). Many entrapment methods are used today for immobilisation in which all are based on the physical occlusion of enzyme molecules within a "caged" gel structure such that the diffusion of enzyme molecules to the surrounding medium is severely limited, if not rendered totally impossible. A highly cross-linked gel has a fine "wire mesh" structure and can more effectively hold smaller enzymes in its cage whereby this "wire" of the cage is the cross-linking of the polymers. It is important to note that the degree of cross-linking depends on condition at which polymerisation is carried out. Recently, hydrogel has become one of the popular gels for immobilisation of enzymes. Interestingly, hydrogel exhibited the ability to swell in water and retained a significant fraction of water within its structure without dissolving it (Peppas and Mikos 1986). It has been reported that entrapping the enzymes within hydrogel did not cause any change in molecular structure of the immobilised enzymes although microenvironment effects may influence its bioactivity (Gombotz & Hoffman 1986).

Water activity may be considered as a measure of water 'preferences' to be in one environment rather than another. Basically, the water molecules in a high water activity 'tend' to move to a medium of lower water activity thus reduces the differences between the water activity values of the media until equilibrium is accomplished. At equilibrium condition, the water activity remained the same but the water concentration is differing. A high water activity (> 0.8) indicates a 'moist' or 'wet' system and low water activity (< 0.7) generally indicates a 'dry' system. Water activity can be measured from the ratio of water vapor pressure over a reaction system divided by the vapor pressure of pure water under the same condition.

$$\text{Water activity, } a_w = P(p, H_2O) / P(p, H_2O, \text{ref})$$
$$= \text{relative humidity} / 100$$

One of the methods to construct a constant water activity is using a saturated salt solution whereby, substrate solution and enzyme preparation were pre-equilibrated separately in a closed container of saturated salt solution. Some of the saturated salt solutions that are frequently used are LiBr ($a_w = 0.10$), CH_3COOK ($a_w = 0.20$), $MgCl_2.6H_2O$ ($a_w = 0.30$), $Mg(NO_3)_2.6H_2O$ ($a_w = 0.49$), NaCl ($a_w = 0.73$), KNO_3 ($a_w = 0.96$). From investigation, water activity affects the rate of lipase catalysed esterification of triolein whereby the rate of reaction was optimum at

water activity value 0.5 (Dudal and Lortie 1995). In esterification reaction, in order to increase the production of ester, the water must be removed during the reaction and, thereby, force the equilibrium position towards ester synthesis. This can be done only by carrying out the reaction at certain controlled water activity to allow the optimization of the initial reaction rate. The influence of water activity towards enantioselectivity of enzyme catalysed reduction of 2-pentanone have been done by Jonsson et al. (1998) who reported that the rate of the reaction increased when water activity was increased from 0.32 to 0.96 and the enantioselectivity reached a plateau at high water activity values.

In this work, the immobilisation of lipase from *Candida rugosa* by entrapment on N-vinyl-2-pyrrolidone-co-styrene hydrogel was carried out. The immobilised lipase and the reaction system were pre-equilibrated in controlled water activity before being used in enantioselective esterification of (*R,S*)-2-(4-chlorophenoxy) propanoic acid with *n*-tetradecanol.

Experimental

Materials

Lipase from *Candida rugosa*, (Type VII), the monomers N-vinyl-2-pyrrolidone (VP) and styrene (ST) the crosslinker, ethylene dimethacrylate (EDMA) were obtained from Sigma Chemical Co. (St. Louis, MO). The initiator, azoisobutyronitrile (AIBN) and the substrate, 1-tetradecanol, were purchased from Fluka Chemical (Buchs, Switzerland). The substrate, (*R,S*)-2-(4-chlorophenoxy)-propanoic acid was from Aldrich Chemical Co. (Milwaukee, WI). All other reagents were analytical grade.

Purification of Monomers

The monomers VP and ST were purified by filtering them through a column containing aluminium oxide, Al_2O_3 (2.5 x 10.0 cm) until colourless yields were obtained.

Preparation of Lipase Solution

Candida rugosa lipase (0.5 g) was dissolved in distilled water (10.0 mL). This lipase solution was agitated on a vortex mixer for a few seconds

and then centrifuged at 13,000 rpm for about 10 minutes. The solid precipitate was removed and the supernatant was collected and used for lipase immobilisation.

Preparation of Hydrogel and Immobilisation of Lipase

In polymerisation tube, the purified monomers VP and ST (10% VP and 90% ST) were mixed together with 1% EDMA. After that the initiator solid AIBN was added to this mixture and was shaken until all AIBN dissolved. The mixtures were then degassed with nitrogen for 15 minutes to discard any present of oxygen. The polymerisation occurred as well as the mixture was incubated in a 55°C-60°C water bath. After 1-4 hours of incubation, the polymer solutions became viscous and were cooled to 45°C-50°C. The lipase solution was added to the mixture and shaken vigorously until a well-mixed solution is obtained. The polymerisation tube was sealed with a rubber stopper and incubating at 50°C for about 5 hrs was continued. After 5 hrs incubation, the polymerisation tube was allowed to cool at room temperature. The solid polymerised rod (hydrogel) was removed from the polymerisation tube. This hydrogel immobilised lipase was cut into pieces and stored at 0°C before use.

Enantioselective Esterification Reaction

The reaction system consisted of 30 mg of native lipase or 0.5 g hydrogel-immobilised lipase, 200 mM (*R,S*)-2-(4-chlorophenoxy) propanoic acid, 200 mM *n*-tetradecanol and 10 mL of carbon tetrachloride. The mixture was incubated at 30°C and shaken in a horizontal water-bath shaker at 120 rpm. Certain amounts of the sample from the mixture were taken from time to time. All the samples will be used for gas chromatography analysis. The reaction system was terminated by separating the mixture from the hydrogel-immobilised lipase by filtration. The filtrate was evaporated with a rotary evaporator (Eyela SB 650 Japan) to remove the solvent and left behind the product containing the ester produced and the remaining acid and alcohol.

Effect of Water Activity (a_w)

The immobilised lipase and the substrates were pre-equilibrated separately in containers containing different salt solutions; LiCl, $MgCl_2.6H_2O$,

$Mg(NO_3)_2.6H_2O$, KI, KCl and KNO_3 with the water activity values 0.12, 0.328, 0.55, 0.689, and 0.960 respectively. The pre-equilibration process was carried out overnight. The enantioselective esterification was carried out as described earlier.

Gas Chromatography Analysis (GC)

The determination of ester formed was carried out by gas chromatography (GC) (Hitachi G3000) with very polar capillary column AT-SILAR, (30 m x 0.32, i.d). The concentration of ester was measured using an internal standard method. The reaction mixture containing 100 *u*L sample and 20 *u*L of internal standard prepared and 0.4 *u*L of this mixture was injected into GC. The internal standard solution was *n*-heptadecane in 10 mL ethanol. The chromatogram obtained is used to calculate the percentage of ester conversion by using internal standard method.

Results and Discussions

The Effect of Water Activity on the Percentage of Ester Conversion in the Enantioselective Esterification

Generally, enzymes with enantiomeric preferences toward the *(R)*-acid would form more *(R)*-ester, thus, leaving a higher concentration of *(S)*-acid in the reaction media. In this cases, both native lipase and hydrogel immobilised lipase are also able to catalyse the enantioselective esterification reaction of *(R,S)*-2-(4-chlorophenoxy) propanoic acid with *n*-tetradecanol to produce the *(R)*-ester, *(R)*-2-(4-chlorophenoxy) propanoate and leaving an excess *(S)*-acid.

Figure 1 shows the effect of water activity values on the percentage of ester conversion in the enantioselective esterification catalysed by both native lipase (NL) and hydrogel immobilised lipase (VP 10%).

In general, the percentage of ester conversion increased as the water activity values increased to 0.5 but gradually decreased at water activity value 0.6. The graph indicated that the optimum water activity value for enantioselective esterification catalysed by hydrogel immobilised lipase was 0.4 and native lipase was 0.5 in which the percentage of the ester conversion was almost 25% and 16% respectively. The percentage of ester conversion decreased to 14% if hydrogel immobilised lipase was used in the reaction without controlled water activity (Harun 2000). This

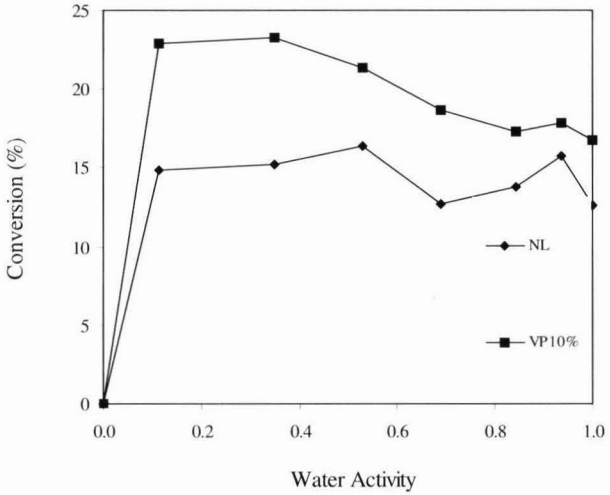

Figure 1: Effects of Water Activity on the Percentage of Ester Conversion for 24 Hrs at 40°C in CCl_4

result revealed that controlled water activity may enhance the percentage of ester conversion. The results also seemed to be in agreement with the results reported by Valivety et al. (1992) whereby lipase showed similar optimum water activity at approximately 0.55. The percentage of ester conversion was lower at lower water activity value may be due to the enzyme was in 'dry' state at lower water activity and, hence, leading to the reduction of enzyme activity. In turn, the percentage of ester conversion also decreased at higher water activity values. This may be due to the higher water level in the reaction media. This situation probably creating the water clusters within the active site of the enzyme and therefore might change the enzyme structure and reduced its activity. Valivety et al. (1994) found those lipases are highly active when only a few molecules of water are associated with the protein molecules. This research result shows percentage of ester conversion decreased at water activities of 0.9 and 1.0 are consistent with the data obtained by Affleck et al. (1992).

Conclusion

The results affirmed that the controlled water activity may enhance the percentage of ester conversion in the enantioselective esterification

catalysed by native lipase and hydrogel immobilised lipase in which the best controlled water activity is the value ranging from 0.4 to 0.5.

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