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Lipase-Catalyzed Esterification of Betulinic Acid Using Phthalic Anhydride in Organic Solvent Media: Study of Reaction Parameters

[1]M. Ghaffari Moghaddam, [1]F.B.H. Ahmad, [1]M. Basri and [1,2]M.B. Abdul Rahman
[1]Department of Chemistry, Faculty of Science, Universiti Putra Malaysia,
43400 UPM Serdang, Selangor, Malaysia
[2]Structural Biology Research Center, Malaysia Genomic Institute,
MTDC-UKM Smart Technology Center, UKM Bangi, 43600 Bangi, Selangor, Malaysia

Abstract: The lipase from *Candida antarctica* immobilized on an acrylic resin (Novozym 435) was employed for the catalytic reaction of betulinic acid and phthalic anhydride. The influence of different reaction parameters, such as effect of single and mixed solvents, substrate molar ratio, reaction time, temperature, amount of enzyme, effect of inorganic bases and effect of substrate support were investigated and optimized. Optimum conditions to produce 3-O-phthalyl- betulinic acid were observed at reaction time; 24 h, temperature; 55°C, amount of enzyme; 176 mg, substrate molar ratio (betulinic acid: phthalic anhydride, 1:1), inorganic base of K_2CO_3, amount of celite; 170 mg in 1:1 mixture of chloroform and n-hexane as solvent. At optimum conditions, it gave 61.8% of 3-O-phthalyl- betulinic acid.

Key words: Betulinic acid, phthalic anhydride, Novozym 435, betulinic acid ester

INTRODUCTION

Betulinic acid is a naturally occurring pentacyclic lupane-type triterpenoid that possess multiple pharmacological activities including inhibition of Human Immunodeficiency Virus (HIV), anti-bacterial, anti-malarial, anti-inflammatory, antioxidant and anticancer properties (Yoggeswari and Sriram, 2005). Betulinic acid was identified as a highly selective growth inhibitor against human melanoma (Pisha *et al*., 1995; Zuco *et al*., 2002), neuroectodermal (Fulda and Debatin, 2000) and malignant (Fulda *et al*., 1999) tumor cells and was reported to induce apoptosis in these cells (Yoggeswari and Sriram, 2005). Nevertheless, the medical use of betulinic acid in pharmaceutical industry is limited due to insoluble of this compound in water which may causes difficulty in absorption by the human body.

Modification of betulinic acid to its derivatives may improve its solubility and enhance its property for drug development. Betulinic acid has three sites, C-3 hydroxyl, C-20 alkene and C-28 carboxylic acid positions, which are highly amenable to derivatization. The introduction of polar groups at the C-3 and C-28 positions of betulinic acid (such as phthalates, amino acids or sugar moieties) was reported in certain cases, increase the hydro-solubility and anticancer activity of betulinic acid derivatives (Gauthier *et al*., 2008; Thibeault *et al*., 2007).

Chemical methods, e.g., esterification of betulinic acid in the presence of acid or alkaline, usually results in a complex mixture and normally occurred at high temperature in relatively toxic solvents which leave traces in the products. Therefore, a difficult and expensive purification method was required. In contrast, the use of enzymes in organic media has enabled new methods for producing many valuable products. The specificity of lipase as a biocatalyst to form an ester- bond allows the control of specific reactions which increase the yield (Yasin *et al*., 2008).

Lipase usually catalyzes hydrolytic reactions. However, when employed in organic solvents, they can perform a reverse reaction, the esterification (Krishna *et al*., 2000). The used of biocatalysts have advantages over chemical catalysts; their specificity, regioselectivity and enantioselectivity allow them to catalyze reactions under mild conditions (i.e., at low temperature and pressure) with lower by products and waste treatments costs (Villeneuve *et al*., 2000).

This study focuses on the reaction parameters that affect lipase (from *Candida antarctica*, Novozym 435) catalyzed esterification of betulinic acid using phthalic anhydride as acyl donor in organic solvent (Fig. 1). The aim of this study was to improve this reaction by optimizing parameters such as reaction time, reaction temperature, solvents, substrate molar ratio, enzyme amount, bases and substrate supported.

Corresponding Author: Faujan Bin H. Ahmad, Department of Chemistry, Faculty of Science, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia Tel: +603-89466784 Fax: +603-89435380

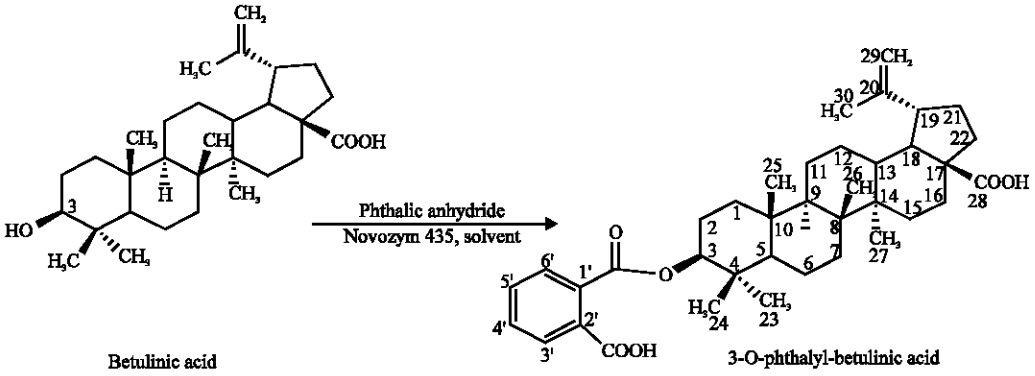

Fig. 1: Enzymatic reaction of betulinic acid and phthalic anhydride

MATERIALS AND METHODS

Enzyme: Immobilized lipase (triacylglycerol hydrolase, EC 3.1.1.3; Novozym®435, 10000 PLU g^{-1}) from *Candida antarctica*, supported on a macroporous acrylic resin with a water content of 3% (w/w) was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark).

Chemicals: All solvents were obtained from Fisher chemicals. Betulinic acid was isolated from Malaysian *Callistemon* sp. as our previous method (Ahmad *et al*., 1999). Phthalic anhydride was purchased from Acros organics, Belgium. Ethyl acetate, Celite®545, Na_2SO_4, K_2CO_3, $NaHCO_3$, Na_2CO_3 and HCl were purchased from Merck, Germany. All chemicals were of analytical reagent grade.

Enzymatic reaction: Ester synthesis was performed in a 50 mL glass stoppered Erlenmeyer flask. Unless otherwise stated, to a solution of betulinic acid (25 mg, 0.0547 mmol), phthalic anhydride (32 mg, 0.218 mmol), chloroform (10 mL) was added Novozym 435(0.088 g). The reaction mixture was magnetically stirred (150 rpm) in a thermostated water bath at 37°C for 24 h. Each reaction was repeated in triplicate and an average value of the product was then calculated. Control experiments were performed in the absence of enzyme. As a result, no chemical acyl transfer reaction was detected.

Analytical methods: Preliminary analysis of the reactions was carried out using Thin Layer Chromatography (TLC) with n-hexane/ethyl acetate (9:1, v/v) as the eluent. The plates were visualized under UV lamp and/or iodine vapor and the product had R_f value of 0.9. Quantitative analysis of samples was performed by column chromatography (Kvasnica *et al*., 2005). At predetermined time intervals, enzyme was removed by filtration and washed twice with chloroform. The filtrate was evaporated to dryness and ethyl acetate was then added and washed twice with aqueous solution of HCl and twice with water. The organic layer was dried (over Na_2SO_4). After solvent evaporation, the residue was chromatographed with gradient on silica gel 60 (n-hexane/ethyl acetate, 9:1-5:1, v/v). Then, the percentage of isolated yield was calculated.

The ^1H and ^{13}C-NMR spectra data of 3-O-phthalyl-betulinic acid were recorded using Varian Unity Inova 500 NMR spectrometer operating at 26°C and matched literature data (Kvasnica *et al*., 2005). ^1H NMR ($CDCl_3$, 500 MHz): δ 0.76, 0.83, 0.94, 0.97, 0.98, 1.69 (each 3H, s, 6×CH_3), 3.00 (1 H, m), 4.40 (1H, dd, J = 4.5, 12.0 Hz), 4.61 and 4.74 (each 1H, br s), 7.55-7.61 (2 H, m), 7.72 (1 H, d, J = 7.0 Hz), 7.92 (1 H, d, J = 8.5 Hz). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 39.50 (C-1), 28.15 (C-2), 80.83 (C-3), 38.56 (C-4), 55.65 (C-5), 18.39 (C-6), 34.47 (C-7), 40.99 (C-8), 50.66 (C-9), 37.34 (C-10), 23.83 (C-11), 25.68 (C-12), 38.19 (C-13), 42.65 (C-14), 30.75 (C-15), 32.31 (C-16), 57.97 (C-17), 47.12 (C-18), 48.42 (C-19), 150.59 (C-20), 29.85 (C-21), 38.10 (C-22), 28.31 (C-23), 16.16 (C-24), 16.72 (C-25), 16.38 (C-26), 14.83 (C-27), 181.11 (C-28, COOH), 109.95 (C-29), 19.52 (C-30), 168.32 (C = O ester), 131.06 (C-1'), 130.57 (C-2'), 169.24 (2'-COOH), 129.18 (C-3'), 133.10 (C-4' and C-5'), 128.76 (C-6').

The betulinic acid ester formation was also confirmed by gas chromatography (G-3000, Hitachi Japan), which was equipped with medium polar DB-17HT capillary column. Helium was used as a carrier gas with flow rate of 1.0 cm^3 min^{-1}. The injector and detector were set at 300°C. The initial temperature of the column was 100°C for 4 min, then increased with flow rates of 11°C min^{-1} up to 300°C.

Optimization study

Effect of single solvent system: To investigate the effect of single solvent system on the enzymatic reaction, the

reaction mixtures were carried out with different solvents (n-hexane, acetone, $CHCl_3$, CH_2Cl_2, and CH_3CN).

Effect of mixed solvent system: To study the influence of mixed solvents system on the enzymatic reaction, the reaction mixtures were performed in n-hexane/chloroform with different composition (90/10, 70/30, 50/50, 30/70 and 10/90, v/v).

Effect of substrate molar ratio: To investigate the effect of substrate molar ratio on the enzymatic reaction, the reaction mixtures which consist of betulinic acid, phthalic anhydride, chloroform (10 mL), n-hexane (10 mL) and Novozym 435 were carried out at different molar ratio of betulinic acid to phthalic anhydride (1:1, 1:2, 1:3 and 1:4).

Effect of reaction time: To investigate the effect of reaction time on the enzymatic reaction, the reaction mixtures which consist of betulinic acid (25 mg, 0.0547 mmol), phthalic anhydride (8 mg, 0.0547 mmol), chloroform (10 mL), n-hexane (10 mL) and Novozym 435 were carried out at various reaction times (1, 3, 5, 8, 12, 24, 36 and 48 h).

Effect of amount of enzyme: To study the influence of amount of enzyme on the enzymatic reaction, the reactions mixtures which consist of betulinic acid (25 mg, 0.0547 mmol), phthalic anhydride (8 mg, 0.0547 mmol), chloroform (10 mL), n-hexane (10 mL) and Novozym 435 were carried out at different amount of enzyme (44, 88, 176, 264 and 308 mg).

Effect of bases: To study the effect of base on the enzymatic reaction, the reaction mixtures which consist of betulinic acid, phthalic anhydride (8 mg, 0.0547 mmol), chloroform (10 mL), n-hexane (10 mL) and Novozym 435 (176 mg) were performed with various bases ($NaHCO_3$, Na_2CO_3 and K_2CO_3).

Effect of celite: To study the influence of celite on the enzymatic reaction, the reaction mixtures which consist of betulinic acid, phthalic anhydride (8 mg, 0.0547 mmol), chloroform (10 mL), n-hexane (10 mL), K_2CO_3 (6 mg) and Novozym 435 (176 mg) were carried out with celite 545 (170 mg).

Effect of reaction temperature: To investigate the effect of temperature on the enzymatic reaction, the reaction mixtures which consist of betulinic acid, phthalic anhydride (8 mg, 0.0547 mmol), chloroform, n-hexane (10 mL), K_2CO_3 (6 mg), celite 545 (170 mg) and Novozym 435 (176 mg) were carried out at different temperatures (30, 37, 45, 50, 55 and 60°C).

RESULTS AND DISCUSSION

Optimization study

Effect of single and mixed solvent system: The enzyme activity in organic solvents was often correlated with solvent hydrophobicity, with the highest activities at high log P values. If the solubility of substrate is considered, hydrophobic solvents are not always good for polar substrates. The effect of single solvent system on the enzymatic synthesis of 3-O-phthalyl- betulinic acid was investigated in various types of solvents (n-hexane, acetone, chloroform, acetonitrile and dichloromethane) with the presence of lipase as biocatalyst. As shown in Fig. 2, chloroform gave the highest yield as compared with other solvents. The substrates are well dissolved in chloroform and produced highest percentage of 3-O-phthalyl-betulinic acid.

It was observed that when chloroform was introduced to the reaction system containing n-hexane; the yield of betulinic acid ester was enhanced since chloroform dissolves the substrate. This phenomenon was also shown by Zhuang *et al.* (1998) and Xu *et al.* (1998). With n-hexane as the bulk solvent, the presence of chloroform as cosolvent was further optimized the yield. The results showed that the system composed of n-hexane (50%, v/v) and chloroform (50%, v/v) gave the best yield (Fig. 3).

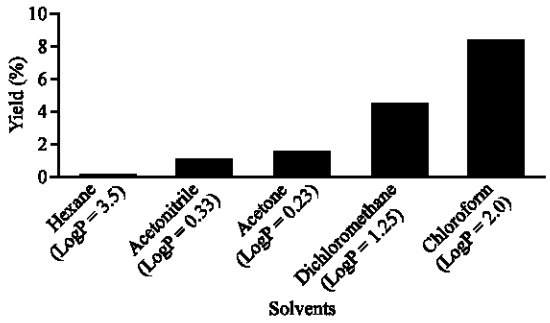

Fig. 2: The effect of single solvent on the enzymatic synthesis of 3-O-phthalyl- betulinic acid

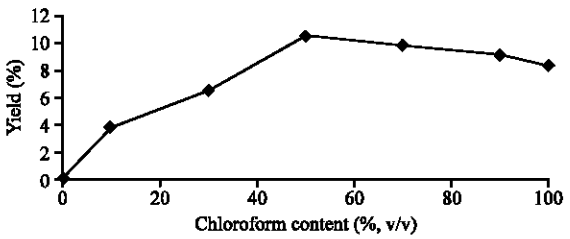

Fig. 3: The effect of mixed solvent on the enzymatic synthesis of 3-O-phthalyl- betulinic acid

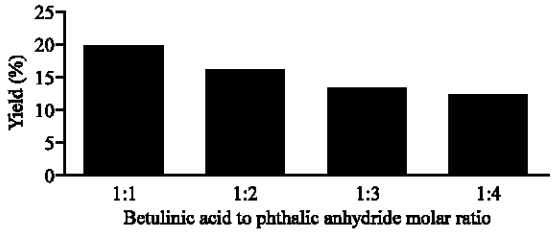

Fig. 4: The effect of molar ratio (betulinic acid: phthalic anhydride) on the enzymatic synthesis of 3-O-phthalyl- betulinic acid

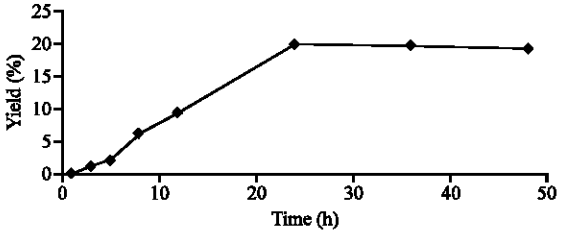

Fig. 5: The effect of time on the enzymatic synthesis of 3-O-phthalyl- betulinic acid

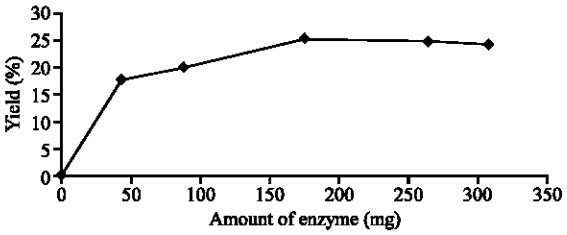

Fig. 6: The effect of amount of enzyme on the enzymatic synthesis of 3-O-phthalyl- betulinic acid

Effect of substrate molar ratio: The effect of mole ratio of substrates was dependent on the types of the enzyme used to catalyze the reaction. High acylation yields can be achieved with high substrate concentration in the reaction media. However, when one substrate is present at very high concentration, viscosity of the liquid phase surrounding the enzyme molecule is increased leading to ineffective mixing of reactants. Thus, the effect of varying the mole ratio of substrate was studied at a constant mole of betulinic acid and varying the mole of phthalic anhydride in a fixed total volume of mixture. The amount of enzyme was held constant in all cases.

As illustrated in Fig. 4, the 1:1 molar ratio of betulinic acid to phthalic anhydride produced the highest percentage (up to about 20% yield) of conversion compared to other molar ratios. Increasing the molar ratio of phthalic anhydride gave decreased the percentage of conversion. The decreased in percentage of yield of 3-O-phthalyl- betulinic acid at higher concentration of phthalic anhydride may be due to the deactivation of enzyme. Phthalic anhydride may perform as a competitive inhibitor and or cause enzyme deactivation by dissolution in the micro-aqueous phase forming phthalic acid, which causes the drop of pH (Krishna *et al*., 2001). The similar phenomenon was also demonstrated by Xu *et al*. (1997).

Effect of reaction time: Time course study is a good indicator of enzyme performance. A good performance of enzymes should have a short time to obtain the highest yields of product. Figure 5 shows the reaction time profile for the enzymatic synthesis of 3-O-phthalyl- betulinic acid in organic solvent composed of 1:1 ratio of hexane and chloroform by used of Novozym 435 as biocatalyst. It was observed that, the rate of reaction and overall conversion increased with increasing reaction time. The results suggested that the reaction reached equilibrium state at about 24 h with highest yield at about 19.8% was recorded. Thereafter, at further longer reaction time the percentage of yield were relatively constant. Apart from the reaction had achieved the equilibrium, as the reaction proceeds, the substrate concentration decreased which led to a fall in the degree of saturation of the enzymes with substrate (Radzi *et al*., 2005a).

Effect of amount of enzyme: From an applied point of reaction, the amount of immobilized enzyme used should be as low as possible to obtain the highest percentages of product. Thus, the effect of amount of enzyme was studied by varying the amount of the enzyme added to the reaction mixture. Figure 6 shows that the percentage yield increased rapidly and reached maximum conversion by the use of 176 mg of enzyme for 0.055 mmole of betulinic acid used. However, in addition of enzyme, the conversions slightly decreased thereafter. This may be due to the limiting effect of the substrate. The findings of Basri *et al*. (2001) have also indicated that, little increased in yield were observed for enzyme loading higher than 100 mg. The excess of enzyme did not contribute to the increase in percentage conversion.

Effect of bases: It was reported that the additions of dissolved organic or suspended inorganic bases significantly improve the yield of enzymatic acylation catalyzed by lipase in organic solvents using anhydrides (Berger *et al*., 1990). Therefore, we were prompted to investigate the effect of a weak base such as sodium bicarbonate ($NaHCO_3$) on the activity of lipase in organic solvent. As shown in Fig. 7, addition of a weak base enhanced the production of 3-O-phthalyl- betulinic acid. The effect of base has been attributed to the formation of

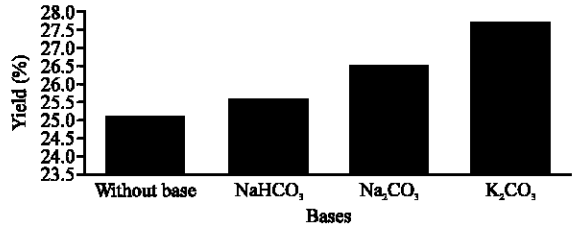

Fig. 7: The effect of addition of bases on the enzymatic synthesis of 3-O-phthalyl- betulinic acid

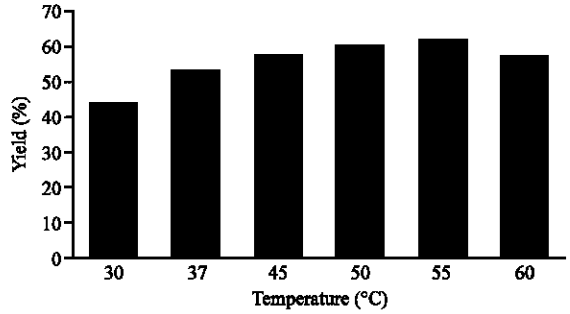

Fig. 8: The effect of temperature on the enzymatic synthesis of 3-O-phthalyl- betulinic acid

an ion pair between the base and any acidic group. The presence of a new acidic group in product may cause inactivation of the enzyme leading to slower reaction rates and thereby the addition of a base might act as a scavenger for the acidic group. Figure 7 also showed that among various inorganic bases used, K_2CO_3 gave the highest yield of 3-O-phthalyl- betulinic acid.

Influence of celite: Castillo *et al.* (1997) reported that addition an inorganic supported such as silica gel in lipase-catalyzed esterification using high polar substrates improved significantly the conversion yields. They reported that the silica gel behaves as a polar substrate reservoir and plays a protective role for the immobilized enzyme avoiding its blockage. To overcome the problems concerning the unfavorable effect on enzymatic process due to blockage of access to the active site of the enzyme, celite as an inorganic supported was directly introduced in the reaction system, which may serve as an alternative surface where substrate could adsorb, thereby prevent excessive adsorption of the substrate on the support of the immobilized lipase. The addition of celite in the present of K_2CO_3 appeared to greatly enhance the formation 3-O-phthalyl- betulinic acid up to 53.4%.

Effect of reaction temperature: The effects of reaction temperature can be apportioned to its effect on substrate solubility as well as its direct influences on the reaction and the enzyme (Facioli and Barrera, 2001). On increasing reaction temperature, substrate solubility is improved by reducing mass transfer limitations and making the substrate more available to the enzyme. Higher reaction temperature also promotes collisions between enzyme and substrate molecules to result in accelerated rates of reaction. In the present study, the influence of temperature was investigated at the temperature range from 30 to 60°C. Figure 8 shows the effect of temperature on the enzymatic synthesis of 3-O-phthalyl- betulinic acid in organic solvent. The percentage of conversion increased with increasing temperature and reached the maximum percentage conversion at 55°C as energy from the heat was used to increase the frequency of interaction of lipase to substrate. The conversion then was reduced when the reaction temperature was increased to 60°C. This is probably because beyond a critical temperature the lipase may have been deactivated (Radzi *et al.*, 2005b). For our reaction using Novozym 435 as catalyst, 61.8% isolated yield was obtained at 55°C. Similar results was reported by most reviewed studies who reported that Novozym 435 was optimally used at 40°C to 60°C (Garros *et al.*, 1998; Lozano *et al.*, 2003).

CONCLUSION

This study was devoted to explore the effect of different operating variables on the synthesis of 3-O-phthalyl- betulinic acid by commercial lipase, Novozym 435 in organic solvent. It was observed that its catalytic activity increased with increasing temperature up to 55°C. It seems that to preserve the high selectivity of the enzyme and avoiding acidification, the addition of a inorganic base was essential when acid anhydride is used as the acylation agent. The maximum percentage of 3-O-phthalyl-betulinic acid produced was 61.8% with substrate molar ratio of 1:1 (for 0.055 mmol of betulinic acid used) and an enzyme amount of 176 mg in 1:1 ratio of hexane: chloroform as the solvent for 24 h at 55°C.

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