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Enantioselective Esterification of (±)-Menthol with Butyric Anhydride in Hexane by modified Lipase from *Candida Rugosa*

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Abstract. Commercial lipase from *Candida rugosa* was chemically modified with the aim to improve their catalytic properties in organic solvents. The chemical modifiers, activated polyethylene glycol derivatives were covalently linked to the lysine residues at the surface of the enzyme. Enantioselective esterification of racemic menthol in hexane using butyric anhydride as acylating agent was achieved with chemically modified lipases. The size of modifier, degree of modification and reaction temperature were examined for the influence on the percent yield and enantioselective formation of (-)-menthyl butyrate. Both enzyme preparations; PEG-2000 and PEG-5000 lipases, the percent yield increased as the degree of modification increased but decreased slightly for the highest degree of modification. The enantioselectivity towards (-)-menthol decreased considerably as the reaction temperature was increased for both derivatives.

Abstrak. Lipase komersial daripada *Candida rugosa* telah diubahsuai secara kimia dengan tujuan meningkatkan ciri kemangkinan didalam pelarut organan. Pengubahsuai kimia, terbitan polietlena glikol teraktif dihubung secara kovalen kepada residue lysin pada permukaan enzim. Pengesteran pemilihanenantio mentol rasemik dalam heksana menggunakan butarik anhidrida sebagai agen pengasilan dicapai menggunakan lipase terubahsuai secara kimia. Saiz pengubahsuai, darjah pengubahsuaian dan suhu tindakbalas dikaji bagi kesan peratus hasilan dan pembentukan pemilihanenantio (-)-mentil butrat. Kedua-dua penyediaan enzim; lipase PEG-2000 dan PEG-5000, peratus hasil meningkat bila darjah pengubahsuaian meningkat tetapi berhurangan sedilkit bagi darjah pengubahsuaian tertinggi. Pemilihanenantio terhadap (-)-mentol berkurangan ketara bila suhu tindakbalas ditingkatan bagi kedua-dua terbitan.

Keywords : lipase, chemical modification, enantioselectivity, esterification, temperature

Introduction

Different approaches have been applied to modify lipases to produce beneficial properties such as to increase activity, stability and solubility. Among the numerous strategies that have been developed to improve the nonaqueous activity include chemical modification with imidoesters [1] and PEG [2], TPI (trapping in the presence of interface) [3], treating with short-chain polar organic solvents [4] and the use of natural fatty acids as amphiphilic modifier [5]. Gonzalez-Navarro and Braco [3] and Chamorro *et. al*. [4] attributed the enhanced activities due to the lipase having attained the open activated conformation.

The cooling and refreshing effects of (-)-menthol made it an important fragrance and flavour compound in candy, beverages, toothpaste and tobacco products. The (±)-menthol cooling effect is not as distinct as that of (-)-menthol, and therefore, it is not highly valued. However, it can be used in medicine and liniments. Here, we report the modification of *Candida rugosa* lipase with *p*-nitrophenyl chloroformate activated polyethylene glycol 2000 and 5000. We studied the influence of different molecular weight of PEG, degree of modification and reaction temperature on the activity and enantioselectivity of modified lipases.

Materials and Method

Enzymes and chemicals.

Commercial lipase from *Candida rugosa* (Type VII) and methoxypoly-ethylene glycol (PEG) were purchased from Sigma Chemical Co. (St. Louis, MO). (±)-Menthol, butyric anhydride, ethyl caproate were from Fluka (Buchs, Switzerland). All the reagents and solvents used in this study were of analytical grade.

Preparation of lyophilized lipase.

Lipase from *C. rugosa* (5.0 g) was dispersed in distilled water (100 mL). The mixture was stirred using magnetic stirrer, centrifuged at 13,000 rpm for

10 min and the supernatant was frozen and lyophilized.

Activation of PEG.

p-nitrophenyl chloroformate (*p*-NPCF) activated PEG: The hydroxyl groups of PEG were modified by the *p*-NPCF, to yield PEG *p*-nitrophenyl carbamate (PEG-pNPCF). 0.6 g of *p*-NPCF was dissolved in 50 mL of acetonitrile. Then, 5 g of PEG and 0.29 g triethyl amine were added. The mixture was stirred for 24 h at room temperature. 500 mL of diethyl ether was then added to precipitate the PEG-pNPCF and was kept at 4 °C overnight. The precipitate was filtered and washed and recrystallized in actonitrile:diethyl ether (1:10 v/v). Finally the PEG-pNPCF was filtered, dried, sealed and stored at –20 °C.

Modification of Lipase.

The chemical modification of lipase was performed at 4 °C for 2 h, with low stirring of native lipase (2.32 g) in borate buffer (0.025 M borate/0.1 M HCl, pH 8.5, 160.0 ml). Different molar excesses of PEG-pNPCF were added to the lipase solution to prepare lipase of different modification. The reaction was terminated by addition of lysine, the pH of the mixture was then adjusted to pH 7. The unbound active PEG was removed by dialysis (Medicell M_w cutoff 12-14,000) against distilled water at 4 °C. Finally, the PEG-lipase solution was frozen and lyophilized using a Labconco freeze dryer (model FreeZone 6 L) at –45 °C.

Protein Determination.

Protein was determined by Bradford Coomasie blue assay procedure [6] using bovine serum albumin as standard. In a typical protein assay, 100 μL of 0.1 g/mL lipase solution was mixed with 5 mL of Bradford reagent. The protein concentration was determined spectrophotometrically at a wavelength of 595 nm using the calibration curve of serum albumin.

Determination of Degree of Modification.

The content of free lysine (Lys) before and after modification was determined using 2,4,6-trinitro benzene sulfonic acid as described by Hazra *et. al*. [7].

Preparation of standard menthyl esters.

Both esters, (+)-menthyl butyrate and (-)-menthyl butyrate were prepared enzymatically by incubating 100 mmole of (+) and (-)-menthol and 100 mmole butyric anhydride separately in hexane for 48 h at 30 °C. The products were separated by column chromatography (Merck silica gel 60, 70-230 mesh), the eluent was hexane/ethyl acetate, 10:1 v/v for ester. The product was monitored by chiral capillary GC to ensure purity and used as standards for the quantification of menthyl esters in the enzymatic reactions.

Enzymatic Esterification Reaction.

The reaction mixture was composed of (±)-menthol (1mmole) and butyric anhydride (1mmole) in hexane (2.0 mL) and was carried out in 20 x 125 mm screw-capped culture tubes in 30 °C shaking water bath (Hotech Shakerbath Model 903) for 48 h. Native lipase (NL)(10.0 mg) and an equivalent amount containing the same protein amount of modified lipases were used as biocatalysts and added immediately before incubation. The modified lipases used were PEG-2000 lipases; 28% (P2-28%), 44% (P2-44%), 64% (P2-64%) and 75% (P2-75%) and PEG-5000 lipases; 20% (P5-20%), 35% (P5-35%), 47% (P5-47%) and 55% (P5-55%). A control (Control) without the presence of lipase was simultaneously incubated. Enzymatic esterification was also carried out at reaction temperatures of 30, 40, 50 and 60 °C.

Analytical procedure.

After esterification, a 50 μL sample of the reaction mixture was withdrawn. The sample was immediately analyzed by gas chromatograph (HP 4890 D) equipped with a flame ionization detector and a Rt-βDEXsm fused-silica chiral capillary column (30 m x 0.25 mm i.d., film thickness 0.25; Restek, Bellfonte, PA) to separate and identify the optically active menthyl butyrate produced by enzymatic reaction. The injector and detector temperatures were 230 °C and 250 °C respectively, carrier gas was helium. The temperature program used to separate the (+) and (-)-menthyl butyrates was initially at 110 °C, elevated to 150 °C at 4 °C/min and then elevated again at the rate of 4 °C/min to 180 °C. The amount of each enantiomer was estimated by peak area recorded and integrated by a 3395 Hewlett-Packard Integrator. Ethyl caproate was used as the internal standard.

The percent yield of menthyl esters was defined as (mmole ester/initial mmole alcohol) present in the system x 100 % and was estimated from the peak areas that were integrated. The enantiomeric excess, % ee, which is equal to the absolute value of percentage excess of one enantiomer over the other; % ee = {[-] – [+]/[-] + [+]} x 100 %.

Results and Discussion

The use of modified lipases improved the overall yield of (-)-menthyl butyrate from 14 % (native lipase) to 83 % for the PEG-2000 lipase and 66 % for the PEG-5000 lipase (Fig. 1 and 2). Koops *et. al*. [8] reported similar trend although they used tryesyl-activated PEG 2000 monomethyl ether and lipase from different sources. The degree of modification affects the esterification activity, the same trend was observed for both enzyme derivatives. High yields were obtained by PEG lipases with moderate degree of modification.

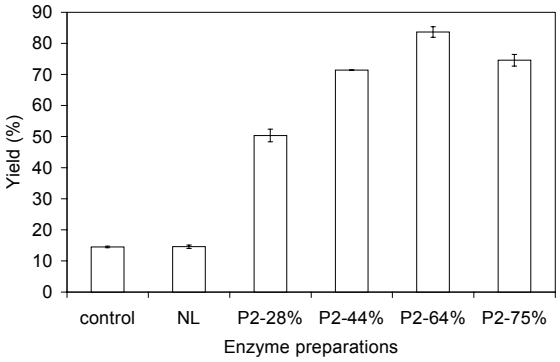

Figure 1 : Effect of degree of modification on the percent yield of (-)-menthyl butyrate by PEG-2000 lipases in hexane at 30 °C. Bars indicate standard deviation.

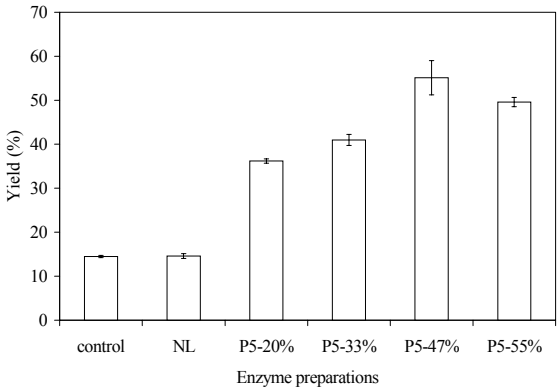

Figure 2 : Effect of degree of modification on the percent yield of (-)-menthyl butyrate by PEG-5000 lipases in hexane at 30 °C

Dramatic increase in % ee when the native lipase was modified for all degree of modification was observed, however, the difference of the % ee for the various degree of modification of the modified lipases was not substantial (Fig 3 and 4). Wu *et. al.* [9] demonstrated that native AY-30 *C. cylindracea* lipase was enantioselective for the same reaction. They used lipase powder directly while we used lyophilized free lipase.

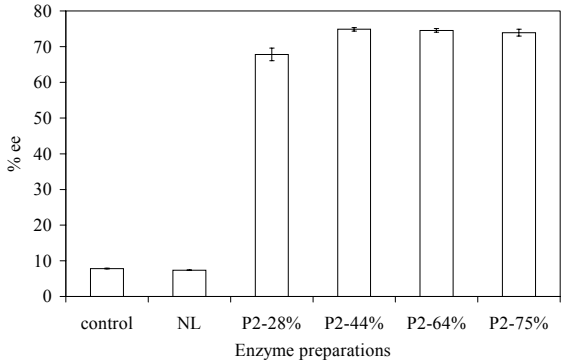

Figure 3 : Effect of degree of modification on enantiomeric excess, % ee by PEG-2000 lipases in hexane at 30 °C.

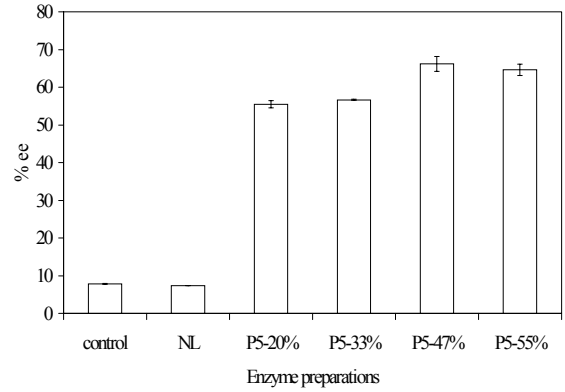

Figure 4 : Effect of degree of modification on enantiomeric ratio, % ee by PEG-5000 lipases in hexane at 30 °C

The molecular size of the modifier seemed to affect the percent yield and enantiomeric excess, % ee. (Fig 5). Lipase modified with lower molecular weight of the modifier produced a higher esterification activity probably due to better accessibility of the substrates towards the active site.

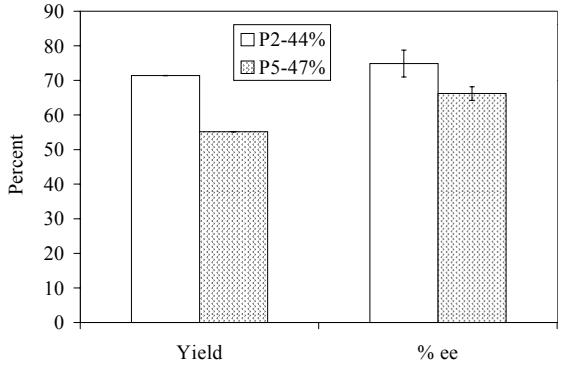

Figure 5 : Effect of size of modifer on percent yield of (-)-menthyl butyrate and enantiomeric excess, % ee.

The esterification activity and enantioselectivity of PEG-lipases are dependent on the temperature. The effect of reaction temperature after 24 h incubation are shown in Fig 6 and 7. Generally, the higher the temperature, the greater the esterification activity, which is shown by the reactions without enzyme and catalyzed by native lipase. The esterification activity, catalyzed by PEG-2000 lipase however was not affected by the reaction temperature, although with PEG-5000 lipase the percent yield was highest at 40 °C. The modified enzyme seemed to maintain the open activated confirmation at high temperature but unable to retain the rigid confirmation as demonstrated by the diminishing enantioselectivity.

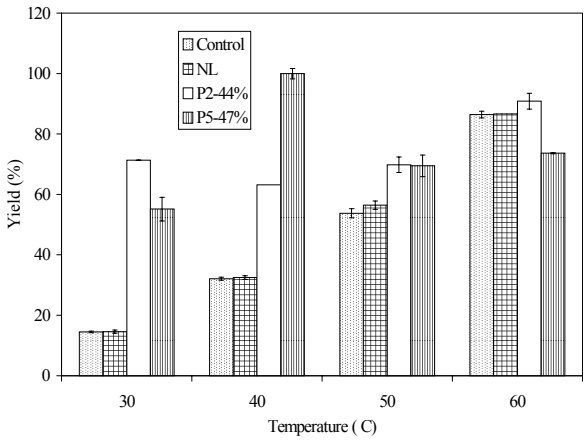

Figure 6 : Effect of reaction temperature on percent yield of (-)-menthyl butyrate by control (C), native lipase (NL), PEG-2000 (P2-44) and PEG-5000 (P5-47) lipases in hexane.

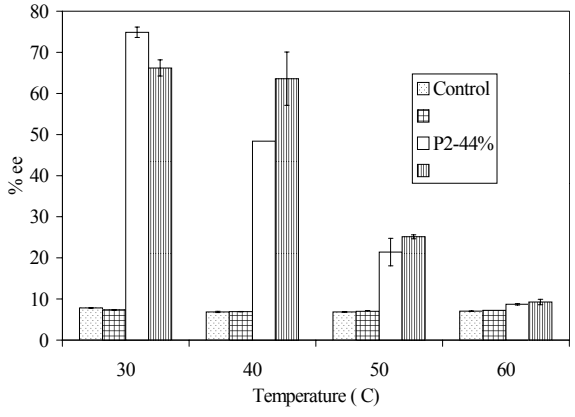

Figure 7 : Effect of reaction temperature on enantiomeric excess (% ee) by control (C), native lipase (NL), PEG-2000 (P2-44) and PEG-5000 (P5-47) lipases in hexane.

The enantioselectivity, on the other hand, decreased as reaction temperature increased. This effect was observed with both of the enzyme derivatives. Temperature affects enantioselectivity according to the equation [10]

$$R\ T_1 \ln E_1 = R\ T_2 \ln E_2$$

Enantioselectivity will decrease when the temperature is increased. Our experimental data are in agreement with this equation.

Conclusion

We have demonstrated an effective means for achieving enantioselective resolution of (±)-menthol with butyric anhydride as acylating agent and modified lipase as biocatalyst. The lower molecular weight of the modifier and moderate degree of modification produced a suitable biocatalyst yielding a high esterification activity and enantioselectivity.

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