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Biocatalytic Synthesis of Betulinic Acid Aromatic Esters

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Abstract: The antitumoral activity of betulinic acid is well known. Therefore, intensive efforts have been made to modify this compound in order to obtain more active derivatives, or derivates with new properties. Enzymatic synthesis of betulinic acid ester using an aromatic acid chloride as acyl donor has been studied, catalyzed by immobilized *Candida antarctica* B lipase (Novozyme 435). The main reaction parameters such as reaction time, molar ratio of betulinic acid/acyl donor, use of molecular sieves and nature of reaction medium were investigated.

Keywords: betulinic acid, lipase, esterification, biocatalysis, acyl chloride

1. Introduction

Triterpenes represent an important category of natural compounds. Among these, pentacyclic lupane-type triterpenes are one of the most significant subclass which has been shown to possess several medicinal properties [1]. Betulinic acid is a naturally occurring compound from this subclass that presents interesting pharmacological activities like: anticancer, anti-bacterial, anti-malarial, and anti-inflammatory properties [2]. It can be isolated from various plants by extraction in suitable solvents. [2-14]. Considerable amounts of betulinic acid (up to 2.5%) are available in the outer bark of a variety of tree species that are valuable for timber purposes. White birch bark, *Betula alba* (which contains betulinic acid) has been used by Native Americans as a folk remedy. They used it in tea and other beverages to treat stomach and intestinal problems such as diarrhea and dysentry. In Russia, it has been reportedly used since 1834. In 1994, scientists at the University of North Carolina reported that chemicals found in white birch bark slowed the growth of human immunodeficiency virus (HIV) [2].

Due to its limited limited solubility in commonly used organic solvents such as methanol, ethanol, hexane, toluene, dimethyl-formamide and ether, researchers turned their attention to the modification of betulinic acid to its derivatives. This approach may result in improved solubility and possible enhancement of the pharmacological activity. Lipases have been already proved to be efficient biocatalysts for synthesis of betulinic acid derivatives [15-17]. Selection of the appropriate enzyme may be difficult, especially when the substrate is non-natural, for example when triterpenes are used as substrate for lipases.

For this study, lipase from *Candida antarctica* B was used as biocatalyst in immobilized form (Novozyme 435). This enzyme was selected following a screening process, with several different lipases, showing the highest activity for this reaction.

Since betulinic acid is not a natural substrate for lipases, carboxylic acid halides were used as acylation reagents, as being more reactive than free acids and other functional derivatives.

2. Experimental

2.1. Materials

The following materials have been employed for the esterification reactions, as purchased: betulinic acid 90% (Sigma Aldrich), benzoyl chloride (Chimreactiv), chloroform (Chimreactiv), tetrahydrofurane 99.8% (Prochem), Novozyme 435 (Novozymes), 4Å molecular sieves (Fluka), ethyl acetate 99% (Chimopar), hexane 98% (Merck), acetonitrile (Merck), 2,5-dihydroxybenzoic acid (Sigma Aldrich), trifluoracetic acid natrium salt (NaTFA, Sigma Aldrich).

2.2. Esterification reactions

Esterification reactions were set up in 4 mL vials, containing betulinic acid and an acyl chloride, at different molar ratios, chloroform or tetrahydrofurane (2 mL), molecular sieves (0,04 g) and 50 mg of immobilized lipase from *Candida antarctica* B. The mixture was incubated using an orbital shaker (MIR-S100, Sanyo, Japan) at 300 strokes/min and 40ºC (ILW 115 STD incubator, Pol-Eko-Aparatura, Poland). Samples were taken at 24, 48 and 72 hrs and assayed by HPLC. The reactions were monitored using TLC.

2.3. Thin Layer Chromatography

Preliminary analysis of the reaction progress was carried out using Thin Layer Chromatography (DC-Kiesegel 60 F_{254} Merck) with n-hexane/ethyl acetate (3:1, v/v) as eluent. The plates were visualized using phosphomolybdic acid, followed by heating.

2.4. High Performance Liquid Chromatography

Quantitative analysis was performed using a Jasco HPLC system equipped with a quaternary pump PU-2089 and UV detector UV-2070 Plus. We used a reversed phase column Synergy 4u Hydro–RP 80A, 250x4.6 mm (Phenomenex). The samples were injected using a Jasco AS-2055 Plus autosampler. The samples were assayed at 210 nm, using a mixture of acetonitrile and water as solvent (86:14, v/v) at 1 mL/min flow.

2.5. Characterization of reaction products

FTIR spectra were recorded on a Jasco FT/IR-430 spectrophotometer on 400-4000 cm^{-1} range, using KBr pellet method.

Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analysis of products was carried out using Bruker BIFLEX III matrix assisted laser desorption/ionization time-of-light mass spectrometry (Bruker Daltonic GmbH, Germany) at an acceleration voltage of 20 kV using 2,5-dihydroxybenzoic acid as matrix. Samples (10 mg/mL), the matrix (20 mg/mL) and the ionization agent NaTFA (5 mg/mL) were individually disolved in organic solvent. 10 µL of sample solution, 5 µL of ionization agent solution were mixed with 50 µL of matrix solution and an aliquot (0.3 µL) was applied on the sample plate. Ions were accelerated with a 25 kV voltage after a delayed extraction time of 300 ns.

3. Results and Discussion

3.1. Influence of molecular sieves on conversion

Esterification is an equilibrium reaction. To move the balance towards synthesis, these processes are carried in non-aqueous media. Influence of water quantity and nature of the organic solvent are parameters have to be considered when optimizing any process. Enzymatic esterification reactions generally occur at temperatures up to 70˚C in the presence of small amounts of water, as water is needed to maintain the biocatalyst's active conformation. A higher water content affects the thermodynamic equilibrium of the reaction, while lowering the concentration of water in the system under a limited value leads to inactivation of the enzyme. Under these circumstances, control of the water amount is an essential element for achieving high values of conversion.

In order to study the effect of water content on conversion, esterification reaction of betulinic acid with benzoyl chloride was carried out with and without addition of molecular sieves. They are used for water retention from the reaction medium, solvent and from the enzyme preparation, in order to minimize the reverse hydrolysis reaction. Although molecular sieves did not allow exact control of water amount in the system, it has been shown that still remains enough water to retain the catalytic activity of the enzyme (this remaining water should not be more than 0.1%, corresponding to a monomolecular layer around the enzyme). The results showed that when molecular sieves were added in the system, betulinic acid conversion increased over time to 52% after 72 hrs of reaction, while in the reaction without molecular sieves was achieved a maximum conversion of 35% after 48 hrs, with no further increase (Fig. 1).

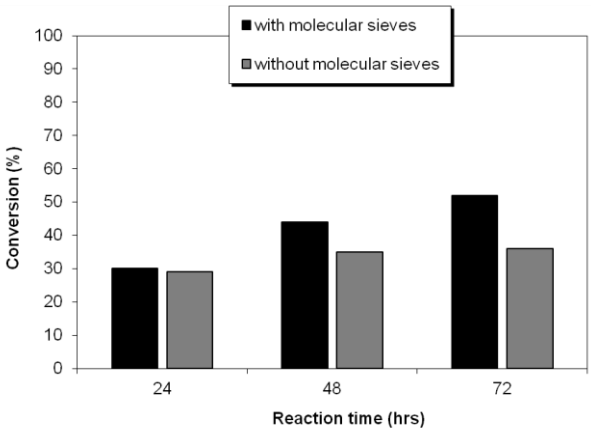

Figure 1. Influence of water content on conversion in the esterification reaction of betulinic acid with benzoyl chloride (molar ratio 1:2) catalyzed by Novozyme 435 at 60˚C in tetrahydrofurane, with and without molecular sieves

3.2. Influence of solvent on conversion

Nature of the organic solvent is an important factor in lipase biocatalysis. Switching from the natural medium of enzymes (water) to a synthetic one (organic solvent) offers many advantages, including improved solubility of substrates. The solvent has an important influence on the activity and stability of the enzyme, and can also modify its specificity. In addition, solvents can interact with substrates and reaction products, thus affecting enzyme activity.

Appropriate choice of the reaction medium is a very important step for enzymatic reaction efficiency. There are literature data on influence of the reaction medium in the enzymatic synthesis of betulinic acid esters with phthalic anhydride. Solvents investigated were n-hexane, acetone, chloroform, acetonitrile and dichloromethane. Chloroform led to higher yields of the reaction [15]. Yasin et al. studied different parameters to optimize the esterification of betulinic acid with benzoyl chloride, catalyzed by Novozyme 435 in chloroform. In optimal reaction conditions, the yield was 48.5% after about 10 hrs [17]. Cheng and colleagues studied the solubility of betulinic acid in a number of organic solvents including

tetrahydrofurane, which proved to be an excellent solvent for the substrate [18].

In this study, we also conducted solubility tests for betulinic acid several organic solvents, but apart from tetrahydrofurane and chloroform, we noticed an acceptable solubility only in ethanol. Ethanol can not be an appropriate environment for this reaction, as it is a competing substrate for lipase. Under these conditions, the reactions were performed in chloroform or tetrahydrofurane, in the presence of molecular sieves at 60˚C. To observe the evolution of reaction, samples were taken after 24, 48 and 72 hrs of reaction.

The substrate was more soluble in tetrahydrofurane, solvent which led to the highest values of conversion (73% after 72 hrs of reaction). Although during the reaction (24 and 48 hrs) tetrahydrofurane proved to be an efficient reaction medium, after 72 hrs conversion values were comparable in the two reaction media (Fig. 2). It is known from other studies that none of these solvents inactivated the lipase; therefore the solubility of raw materials and reaction products is decisive. Even if betulinic acid was not completely soluble in chloroform, if the reaction product is soluble, we can assume that with the evolution of the reaction, the rest of betulinic acid was gradually solubilized and finally (after 72 hrs reaction time) approximately the same conversion as in tetrahydrofurane has been reached.

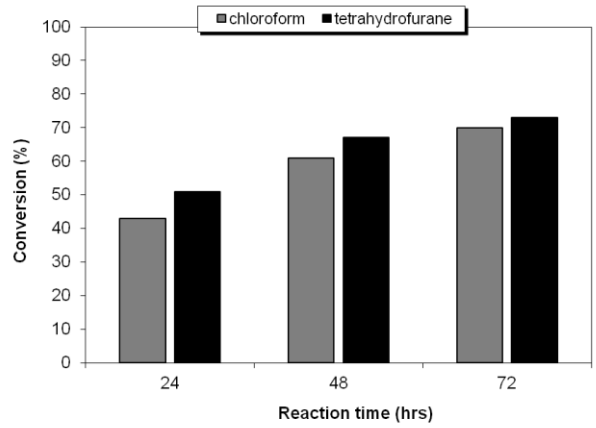

Figure 2. Influence of reaction medium on conversion in the esterification reaction of betulinic acid with benzoyl chloride (molar ratio 1:8.6) catalyzed by Novozyme 435 at 60˚C, with molecular sieves

3.3. Influence of molar ratio of the reactants on conversion

Another important parameter to be taken into account in practical applications is the molar ratio of reactants. Therefore, this study aimed to establish the influence of molar ratio between betulinic acid and benzoyl chloride on conversion. For this, four molar ratios were tested (1:1, 1:2, 1:4 and 1:8.6). Reactions were carried out in tetrahydrofurane medium and samples were taken at 24, 48 and 72 hrs of reaction. It can be seen that the highest values of conversion (70% after 72 hrs) were obtained at a 1:8.6 molar ratio between betulinic acid and benzoyl chloride, meaning that an excess of benzoyl chloride was beneficial (Fig. 3).

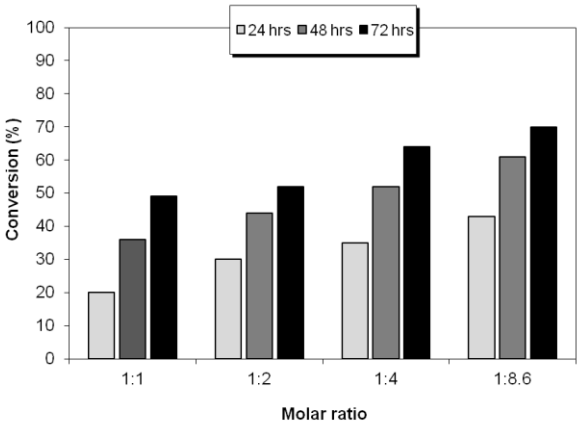

Figure 3. Influence of molar ratio on conversion in the esterification reaction of betulinic acid with benzoyl chloride catalyzed by Novozyme 435 at 60˚C, with molecular sieves

3.4. Characterization of reaction products

The reaction product obtained by enzymatic esterification of betulinic acid with benzoyl chloride was separated using thin layer chromatography, column chromatography, high performance liquid chromatography (HPLC), and characterized using mass spectrometry (MALDI TOF).

MALDI TOF MS analysis confirmed the formation of betulinic acid ester with benzoyl chloride. Pseudomolecular peaks corresponding to sodium adduct with m/z=583 and potassium adducts with m/z=599 for the reaction product were identified in the mass spectrum (Fig. 4).

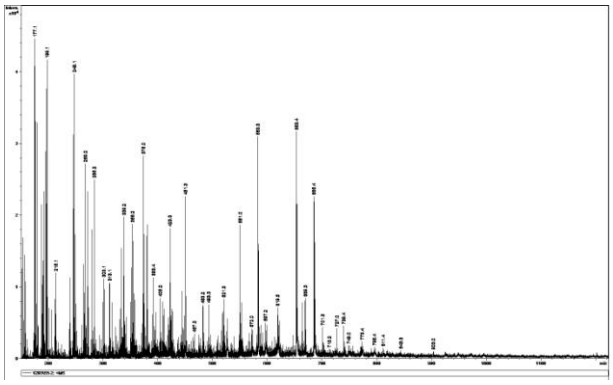

Figure 4. MALDI TOF spectra of the reaction mixture resulted at esterification of betulinic acid with benzoyl chloride, catalyzed by Novozyme 435 in tetrahydrofurane, with molecular sieves

4. Conclusions

Betulinic acid derivatives are compounds of high pharmaceutical interest, mainly due to their cytotoxicity; hence the synthesis of such compounds in a biocatalytic process could be more attractive than the classical chemical synthesis.

High water content affected thermodynamic equilibrium of the reaction, while lowering the concentration of water in the system resulted in inactivation of the enzyme.

The results showed that the addition of molecular sieves increased the conversion to 52% after 72 hrs of reaction, while the reaction without molecular sieves resulted to a maximum conversion of 35% after 48 hrs and did not increase further.

Tetrahydrofurane proved to be the most efficient reaction medium, resulting in highest values of conversion. Also, an excess of benzoyl chloride was beneficial for the conversion values of the studied reaction. MALDI TOF MS analysis confirmed the formation of the reaction products.

The esterification reaction of betulinic acid with terephthaloyl dichloride was also studied in tetrahydrofurane as reaction medium, but it will be subject of a separate communication.

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