SYNTHESIS AND COUNTERCURRENT CHROMATOGRAPHY PURIFICATION OF MYRICITRIN ESTERS

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ABSTRACT

The aim of this study is twofold: firstly, to perform transesterification reactions using nine vinyl esters with Novozyme®435, monitored by Ultra-High Performance Liquid Chromatography coupled with Mass Spectrometry (UHPLC-MS) and High-Performance Liquid Chromatography coupled with Diode Array Detection (HPLC-DAD); and secondly, to isolate the acylated products using High-Speed Counter-Current Chromatography (HSCCC). Initially, the reactions involved 22.5 mg of myricitrin and 1.5 mL of the vinyl ester, mixed with 0.5 mL of either isopropanol or 1-butyl-3-methylimidazolium. After 5 minutes, 4.5 mg of Novozyme 435 enzyme was added, and the reactions were conducted at 150 rpm and 60°C for up to 144 hours. Aliquots of 70 µL were taken every 24 hours, and the reaction kinetics were analyzed using UHPLC-MS in positive mode. Medium and long-chain esters are currently being analyzed using HPLC-DAD. For reactions involving vinyl pivalate and propionate, conversion was observed starting at 24 hours, peaking at 144 hours with the formation of positional isomer monoesters and diesters for the former, and mono-, di-, and triesters for the latter. The purification of myricitrin monopropionate was achieved through countercurrent chromatography, with its structure confirmed by nuclear magnetic resonance.

Keywords: Lipase. Biocatalysis. Myricitrin. Countercurrent cromatography.

1 INTRODUCTION

Biocatalysis is a field within organic synthesis that has made significant progress in recent years. The use of proteins as catalysts for organic reactions in the chemical and pharmaceutical industries has gained prominence, and advancements in the use of enzymes for the partial or total synthesis of certain compounds have been widely reported[1].

Flavonoids constitute one of the largest classes of secondary metabolites and are notable for their pharmacological properties. However, their applicability can be limited due to their moderately hydrophilic nature, especially in the case of glycosylated flavonoids. The use of lipases for the enzymatic acylation of these compounds has resulted in greater stability and solubility in more lipophilic media and has been preferred over chemical acylation, mainly due to its regioselectivity and the mild conditions required for the reaction[2].

Given this, myricitrin is a glycosylated flavonol widely distributed in vegetables, fruits, and various plant species. It stands out for its significant biological potential, exhibiting cardioprotective, anti-inflammatory, and antidiabetic effects, among others. However, its pharmacological application is hindered by its low lipophilicity[3]. Therefore, the objective of this study is to obtain acylated derivatives of myricitrin with biological potential using immobilized lipase from *Pseudozyma antarctica*, and subsequently purify them via countercurrent chromatography.

2 MATERIAL & METHODS

For the reactions, Novozyme® 435 lipase (1531 U/g) (from *Pseudozyma antarctica*) was used along with the flavonoid. The vinyl esters employed included propionate, butyrate, decanoate, laurate, stearate, neodecanoate, benzoate, cinnamate, and vinyl pivalate. Each reaction mixture consisted of 22.5 mg of myricitrin, 4.5 mg of the enzyme (20% relative to the amount of flavonoid), and 1.5 mL of the chosen vinyl ester. Initially, the reactions were carried out using 0.5 mL of isopropanol as the solvent. However, no product formation was observed for medium and long-chain esters. To address this, the ionic liquid 1-butyl-3-methylimidazolium tetrafluoroborate ([C4mim][BF4]) was used instead. Consequently, the following conditions were established for these reactions (table 1), which were performed in duplicate:

Table 1. Reaction conditions for reactions involving each vinyl ester.

Vinyl ester (1.5 mL)	Solvent/Ionic Liquid (0.5 and 7 mL*)	Temperature (°C)	Rotation
Vinyl propionate	Isopropanol	60	150 rpm
Vinyl pivalate	Isopropanol	60	150 rpm

Vinyl butyrate	[C_4mim][BF_4]	60	150 rpm
Vinyl decanoate	[C_4mim][BF_4]	60	150 rpm
Vinyl neodecanoate	[C_4mim][BF_4]	60	150 rpm
Vinyl laurate	[C_4mim][BF_4]	60	150 rpm
Vinyl stearate*	[C_4mim][BF_4] + Isopropanol (5:2; v/v)	60	150 rpm
Vinyl benzoate	[C_4mim][BF_4]	60	150 rpm
Vinyl cinnamate	[C_4mim][BF_4]	60	150 rpm

The reactions proceeded for 144 hours, in a orbital shaker, with 70 µL aliquots withdrawn every 24 hours to determine the respective kinetics. Before adding the enzyme, the ester, compound, and solvent/ionic liquid were mixed together for 5 minutes. The excess ester from these aliquots was removed by washing with hexane (10 washes of 200 µL each). Subsequently, the samples were evaporated and re-dissolved in methanol (350 µL) for further analysis. For the reactions involving ionic liquid, the reaction mixture was extracted with ethyl acetate (10 extractions of 300 µL each) following the hexane wash.

For propionate and vinyl pivalate, analyses of these aliquots were conducted using ultra-high-performance liquid chromatography coupled with mass spectrometry (UHPLC-MS). For the other esters, high-performance liquid chromatography coupled with a diode array detector (HPLC-DAD; λ 250 nm for monitoring) was employed. These analyses were performed using gradient elution (0 min, 10% B; 3 min, 10% B; 10 min, 15% B; 40 min, 95% B; 45 min, 95% B; 45.1 min, 10% B; 50 min, 10% B), with a mobile phase consisting of water (A) and acetonitrile (B), and a Kinetex Polar C18 column (100 x 4.6 mm; 2.6 µm). The UHPLC-MS analyses utilized electrospray ionization with an ion trap analyzer in the positive mode. Additionally, all reactions were monitored by Thin-Layer Chromatography (TLC) using an eluent system of ethyl acetate, methanol, and water (30:2:1; v/v/v), developed with NP PEG.

Once the reaction kinetics with vinyl propionate were established, the scale-up was performed under the standard conditions already established, in triplicate, for 24 hours, aiming to obtain the monoester. The fractions were then combined and the solvent was dried. The excess ester was then removed with hexane (5 x 10 mL).

The monoester purification was carried out using countercurrent chromatography with a biphasic solvent system composed of hexane, ethyl acetate, methanol, and water in a ratio of 2:8:2:8 (*v/v/v/v*). 55 mg of the reaction mixture was used in this process. The chromatography was performed in ascending mode (Vc = 70 mL, 860 rpm, 40°C), collecting 35 fractions during elution and 18 fractions during extrusion. The obtained fractions were analyzed by TLC using an eluent system of ethyl acetate, methanol, and water (30:2:1; v/v/v), developed with NP PEG, and grouped according to their chemical profiles. The fraction corresponding to the purified product had its purity confirmed by nuclear magnetic resonance (500 MHz). The reactions with the remaining esters are in the phase of analysis and establishment of their respective kinetics, and will also be subsequently purified by countercurrent chromatography.

3 RESULTS & DISCUSSION

Currently, the kinetics of reactions involving vinyl propionate and vinyl pivalate are established, while the others are being analyzed using HPLC-DAD. UHPLC-MS analysis of the reactions with these medium-chain and long-chain esters was not pursued due to product fragmentation at the ionization source. As a result, the decision was made to continue the analysis using HPLC-DAD.

Specifically for vinyl propionate reactions, the kinetic profile showed a satisfactory conversion rate within the first 24 hours, achieving more than 50% conversion to monoester during this period. After 144 hours, the highest conversion percentages were observed: 35.77% for the monoester, 32.08% for the diester, and 13.65% for the triester formed.

In the case of vinyl pivalate, there is a modest conversion to positional isomer monoesters observed within the first 24 hours. The highest conversion percentage is observed at 144 hours, with over 70% total conversion into the respective products (29.83, 14.64 and 18.15% for the positional isomer monoesters and 12.52% do the diester). This lower conversion profile compared to propionate is due to the steric hindrance present in the structure of pivalate, which hinders its binding to the enzyme.

The purification of myricitrin monopropionate was successful (tubes 1-7, 15 mg), and its structure was confirmed by 1H-NMR. The acylation position was determined using the HMBC spectrum, revealing acylation at the 4-OH' position of the O-rhamnoside moiety in the myricitrin structure. This highlights the clear effectiveness of countercurrent chromatography in separating two substances with high structural similarity.

Regarding vinyl butyrate, conversion begins after 24 hours, but the percentages remain low, reaching only 10.68% after 144 hours. In contrast, for vinyl neodecanoate, although conversion starts only at 144 hours, it is quite satisfactory, ultimately reaching 50.5%.

While HPLC-DAD analyses for the other esters are still underway, TLC results have already shown that products were formed in all the reactions conducted. Specifically, for vinyl benzoate, these bands appeared starting at 48 hours. For vinyl cinnamate, they

were evident from 72 hours onwards, while stearate displayed them from 96 hours. Finally, decanoate conversion began at 72 hours. These results demonstrate that employing ionic liquids in the reaction medium effectively promoted the conversion to medium and long-chain esters. Furthermore, this approach serves as a greener alternative to traditional solvents for synthesizing acylated derivatives. The use of ionic liquids is notable for their wide liquid-state range and excellent thermal stability. In reactions involving flavonoids, ionic liquids containing the anions TF_2N^-, PF_6^-, and BF_4^- excel as reaction media, generating higher yields compared to other media. This higher performance is likely due to their superior solubilization capacity for flavonoids and their ability to stabilize the enzyme's quaternary structure, which is vital for the catalytic process[4]. These factors also help explain why reactions with medium and long-chain esters, which did not yield any product when isopropanol was used as a solvent, were successful when ionic liquids were employed.

4 CONCLUSION

Thus, the results underscore the feasibility of using immobilized Lipase B from *Pseudozyma antarctica* to produce acylated derivatives of myricitrin with biological potential. The application of ionic liquids in synthesizing medium and long-chain derivatives of myricitrin proved successful. TLC analyses confirmed product formation for all other esters. Currently, these esters are undergoing HPLC-DAD analysis to establish their kinetics. Conversion for vinyl pivalate and propionate begins within 24 hours, peaking after 144 hours. Countercurrent chromatography effectively purified myricitrin monopropionate, with nuclear magnetic resonance indicating acylation at the 4-OH' position of the O-rhamnoside moiety in the myricitrin structure.

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