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Acylation of natural flavonoids using lipase of candida antarctica as biocatalyst

Melika Ardhaoui [a], Aude Falcimaigne [b], Jean-Marc Engasser [a], Philippe Moussou [b], Gilles Pauly [b], Mohamed Ghoul [a,*]

[a] *Laboratoire Bioprocédés Agroalimentaire, ENSAIA-INPL 2, Av de la Forêt de Haye, 54500 Vandoeuvre-les-Nancy, France*
[b] *Laboratoires Sérobiologiques, Département R&D, division de COGNIS France, 5-7 rue de Seichamps, 54425 Pulnoy, France*

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Abstract

Several flavonoids (quercetin, hesperidin, rutin and esculin) were acylated with fatty acids using an immobilised lipase from *Candida antarctica* in 2-methyl-2-butanol at 60 °C. It appears that esculin with primary OH on the sugar part is the most reactive substrate. With palmitic acid as acyl donor, the conversion yields were of about 80, 71 and 38%, respectively, for esculin, rutin and hesperidin. No reaction was observed with aglycon flavonoid (quercetin). For a given flavonoid (rutin), the conversion yield increased from 42 to 76% when the carbon number of the fatty acids rose from C6 to C12. For fatty acids with higher carbon-chain length, both conversion yield and initial rate dropped slightly. Furthermore, compared to the saturated fatty acid (C18: 0), the unsaturated one (C18: 1) exhibited a lower reactivity. For all molecules studied ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR analyses indicated that only flavonoid monoester was produced.

Keywords: Flavonoids; Fatty acids; Enzymatic; Acylation; *Candida antarctica*

1. Introduction

Flavonoids are a class of secondary phenolic plant metabolites that have recently received keen attention due to their antioxidant, antimicrobial, anticarcinogenic properties [1–5]. Many of these compounds are already used in pharmaceutical, cosmetic and food preparations [1,6]. Unfortunately, the use of some of them is limited by their low solubility and stability in both lipophilic and aqueous media [7]. Therefore, the acylation or the glycosylation of these molecules can be used as a tool to improve their properties. These reactions can be performed either chemically or enzymatically. However, due to the polyhydroxylated nature of these molecules, the enzymatic approach is more selective.

In recent years, the feasibility of the enzymatic acylation of flavonoids has been investigated using both lipase and protease as a biocatalyst [8–10]. Gao et al. [11], using *Candida antarctica* lipase observed that the highest conversion yields were reached when monosaccharidic flavonoids having a primary hydroxyl group were used. However, Danieli et al. [12], using subtilisin protease as a catalyst, reported that the acylation of glycosylated flavonoid with monosaccharide takes place only if the sugar moiety is glucose. No reaction was observed with rhamnose as a sugar residue. These results indicated that both the structure of the flavonoid and the nature of the enzyme have a great effect on the selectivity of these reactions.

In addition to the flavonoid structure, the carbon-chain length of the acyl donors also exerts an influence on the *C. antarctica* lipase reactivity. However, this effect is not yet clear. During sugar ester syntheses, Björkling et al. [13] observed a low specificity of this lipase when fatty acids were varied from C8 to C18. These results are in contradiction with those described by Pedersen et al. [14] and Vaysse et al. [15]. These authors indicated that the conversion

* Corresponding author. Tel.: +33-3-8359-5892; fax: +33-3-8359-5778.
E-mail address: ghoul@ensaia.inpl-nancy.fr (M. Ghoul).

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yield increases with the carbon-chain length (C12–C18). Min Sin et al. [16] showed that *C. antarctica* lipase is more reactive with intermediate chain length fatty acids, i.e. C12–C14. Finally, during the synthesis of flavonoid esters, Kontogianni et al. [17] mentioned that carbon-chain length does not affect the performance of flavonoid acylation. These contradictions between the cited results could be attributed to the change of the lipase selectivity depending on the composition of the reaction medium (solvent, water content, substrate chemistry) or the operating conditions [18–21].

The aim of this paper is to investigate, under a water-controlled environment, the effect of the flavonoid structure (quercetin, hesperidin, rutin and esculin) and the carbon-chain length of the fatty acids (C6 and C18) on the flavonoid acylation performance. These reactions were catalyzed by *C. antarctica* lipase using 2-methyl-2-butanol as a solvent and were carried out in a totally automated synthesis workstation. In order to avoid the effect of the water, all syntheses were performed under water content of less than 300 ppm.

2. Material and methods

2.1. Enzyme and chemicals

Immobilised *C. antarctica* SP 435 lipase (Novozym, EC 3.1.1.3, 7000 PLU/mg: propyl laurate units synthesized per gram of catalyst) was purchased from Novo Industries. The fatty acids from C6 to C18 (purity >97%) were supplied by Fluka (Buchs, Switzerland). Quercetin (Sigma), hesperidin (Sigma), rutin (Sigma), esculin (Fluka) were used as flavonoid substrates. All reactions were performed in 2-methyl-2-butanol (Merck).

2.2. Flavonoid ester synthesis operating conditions

The enzymatic synthesis of flavonoid esters was performed in automated synthesis workstation (ASW1000) equipped with micro-reactors of 27 ml. Before each experiment, 5 g/l of flavonoid were added to 20 ml of solvent previously dried with 4 Å molecular sieves. The acyl donor concentration was adjusted to have a flavonoid/acid molar ratio equals 1. Then, the mixture was solubilized at 60 °C for 12 h under stirring. The direct esterification reaction was started by adding 10 g/l of lipase. The water content of the medium was controlled and maintained at less than 300 ppm with molecular sieves (150 g/l).

2.3. Analytical methods

2.3.1. Karl Fisher analysis

The water content of the reaction medium was determined by a coulometric Karl Fisher apparatus (KF 737II coulometer). The reagent was Hydranal-Coulomat AG-H (Riedel-de-Haën).

2.3.2. High-performance liquid chromatography (HPLC) analysis

The acylation reaction was monitored by HPLC analysis carried out in a system (LaChrom, Merck) composed of a column (Purospher® RP-18^e 5, 250 mm × 4.0 mm, Merck), a column oven (L-7350, Merck), an autoinjector (L-7200, Merck), a light-scattering detector (LSD 31, Eurosep) and an RI detector (Merck). The various compounds were separated using a methanol (A)/acetic acid solution (water/acetic acid (97/3)) (B): 0 min (30/70), 5 min (100/0), 10 min (100/0), 15 min (30/70). The elution was performed at 55 °C with a flow-rate of 1 ml/min.

2.3.3. Nuclear magnetic resonance (NMR) analysis

After purification of the synthesized products by liquid–liquid extraction, the chemical structures of rutin and esculin palmitate were determined by ^{1}H NMR and ^{13}C NMR in DMSO-d_6 using a Brucker AM 400 spectrometer at 400 MHz.

2.3.3.1. Esculin palmitate. ^{1}H NMR (DMSO-d_6): δ 7.85 (1H, d, J: 11.4, H_4), 7.3 (1H, s, H_5), 6.8 (1H, s, H_8), 6.2 (1H, d, J: 11.4, H_3), 5.35 (1H, d, J: 6, $H_{1'}$), 4.85 (1H, d, J: 8, $H_{6'a}$), 4.35 (1H, d, J: 12, $H_{6'b}$), 4.1 (1H, dd, J_1: 8, J_2: 12, $H_{5'}$), 3.65 (1H, dd, J: 8, $H_{3'}$), 3.5–3.1 (2H, $H_{2'}$ and $H_{4'}$), 2.25 (2H, m, $CH_2\alpha$ fatty chain), 1.4 (2H, m, $CH_2\beta$ fatty chain), 1.15 (24H, m, CH_2 fatty chain), 0.8 (3H, t, CH_3 fatty chain) ppm.

^{13}C NMR (DMSO-d_6): δ 173.6 (C=O ester), 161.3 (C2), 152.3 (C6), 151.3 (C9), 145.1 (C4), 143.3 (C7), 114.9 (C5), 112.9 (C3), 111.4 (C10), 104 (C8), 102.3 (C1′), 76.6 (C3′), 74.7 (C5′), 73.9 (C2′), 70.8 (C4′), 64.1(C6′), 34.3 (aliphatic chain), 32 (aliphatic chain), 29.3 (aliphatic chain), 25.3 (aliphatic chain), 23 (aliphatic chain), 15 (CH_3, aliphatic chain) ppm.

2.3.3.2. Rutin palmitate. ^{1}H NMR (DMSO-d6): δ 12.6 (s, OH_5), 7.55 (2H, m, $H_{2'}$ and $H_{6'}$), 6.85 (1H, d, J: 8, $H_{5'}$), 6.35 (1H, d, J: 2, H_8), 6.2 (1H, d, J: 2, H_6), 5.45 (1H, d, J: 8.4, $H_{1''}$), 4.7 (1H, t, J: 9.8, $H_{c\,acylated}$), 4.5 (1H, s, $H_{1'''}$), 3.7–3.1 (9H, H rhamnoglucosyl), 2.15 (2H, m, $CH_2\alpha$ fatty chain), 1.45 (2H, m $CH_2\beta$ fatty chain), 1.2 (24H, m, CH_2 fatty chain), 0.85 (3H, t, J: 14.3, CH_3 fatty chain), 0.75 (3H, d, J: 11.4, CH_3 rhamnosyl) ppm.

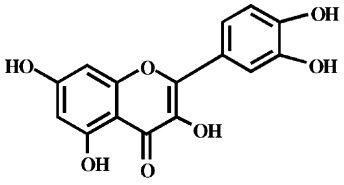
^{13}C NMR (DMSO-d_6): δ 178.2 (C4), 173.5 (C=O ester), 165 (C7), 162.1 (C5), 157.4 (C9 or C2), 157.2 (C2 or C9), 149.2 (C4′), 145.7 (C3′), 133.9 (C3), 122.3 (C6′), 121.9 (C1′), 117 (C5′), 116 (C2′), 104.7 (C10), 101.7 (C1″), 101.3 (C1‴), 99.5 (C6), 94.3 (C8), 77.3 (C3″), 76.3 (C5″), 74.9 (C2″), 74.2 (C4‴), 71.2 (C4″ or C2‴), 70.4 (C2‴ or C4″), 68.9 (C3‴), 67.6 (C6″), 66.6 (C5‴), 34.5 (aliphatic chain), 34.3 (aliphatic chain), 32 (aliphatic chain), 29.7 (aliphatic chain), 25.3 (aliphatic chain), 23 (aliphatic

chain), 17.9 (CH_3 rhamnose), 14.8 (CH_3 aliphatic chain) ppm.

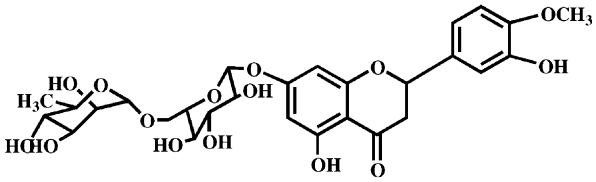
3. Results and discussion

3.1. Alcohol donor structure effect

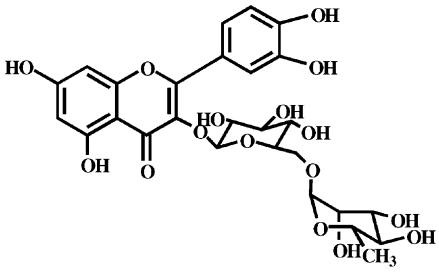
The effect of flavonoid structure on the performance of this reaction was investigated using quercetin, hesperidin, rutin and esculin with palmitic acid as an acyl donor.



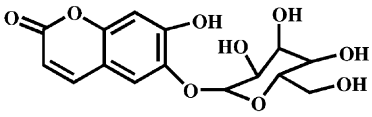
Quercetin



Hesperidin



Rutin



Esculin

Fig. 1 summarises the conversion yields and the initial rates of flavonoids obtained for all substrates studied. These results indicate that esculin was the most reactive substrate. In fact, conversion yields were of about of 80, 71 and 38%, respectively, for esculin, rutin and hesperidin. The same behaviour was observed for the initial conversion rate. For esculin, this was three times higher (8.7×10^{-3} mmol/h) than for rutin and nine times higher than for hesperidin. For an aglycon flavonoid, such as quercetin, no product was observed. The non-reactivity with this flavonoid seemed to be due to our operating conditions, because the enzymatic acylation of this flavonoid has been already reported by Saija et al. [22].

The high conversion yield obtained with esculin can be explained by the presence of the primary OH sugar on this molecule which is more reactive than the secondary OH group of rutin and hesperidin. These results are in accordance with those of Gao et al. [11] and Kontogianni et al. [17]. The high reactivity of the esculin can also be explained, as has been suggested by Gao et al. [11], by the fact that there is only monosaccharide as sugar moiety on this flavonoid.

The difference of the reactivity observed between rutin and hesperidin could be attributed to the low solubility of the hesperidin compared to that of rutin. These solubilities are, respectively, of about 0.2 and 45 mM for hesperidin and rutin.

3.2. Acyl donor structure effect

The effect of the carbon-chain length of the acyl donors on the performance of the flavonoids acylation was demonstrated using fatty acids with carbon number varying from 6 to 18 as acyl donor and rutin as flavonoid. The results obtained (Fig. 2) show that the conversion yields and initial rates of the reaction are affected by the chain length of acyl donors. For fatty acids with a carbon number of less than 12, both conversion yields and initial rates increased progressively to reach, respectively, 76% and 3.5×10^{-3} mmol/h with lauric acid. The lowest conversion yield was obtained with caproic acid (40%). For higher chain length (C12–C18), except for myristic acid, no significant difference was observed whatever fatty acid was used. The conversion yield was almost equal to 70% and the initial rate was about 3.0×10^{-3} mmol/h. For myristic acid, both conversion yield and initial rate were low. Similar behaviour for this acid is already reported by Lee and Parkin [23].

The evolution of conversion yield and initial rate obtained in this work is comparable to that usually observed during sugar ester syntheses [14,15]. However, they are not in accordance with the data of Kontogianni et al. [17]. These authors indicated that the chain length has no effect on flavonoid acylation. The effect of carbon-chain length of fatty acids could be attributed to the specificity of the lipase used, the hydrophobicity of the media [24–26] and the bulkiness of the long fatty acids [27,28].

The influence of the unsaturation was shown by the comparison of results obtained with stearic and oleic acids. It appears that the unsaturation has only a slight effect. The

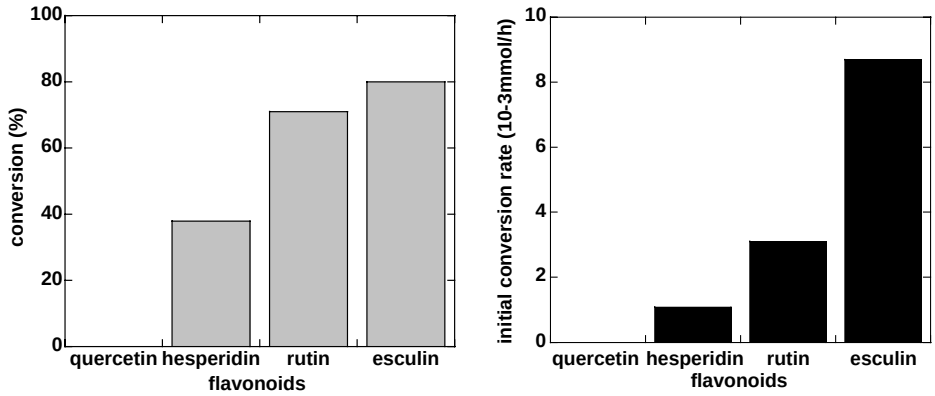


Fig. 1. Flavonoid structure effect on the acylation performance with palmitic acid carried out in 2-methyl-2-butanol using from *Candida antarctica* lipase.

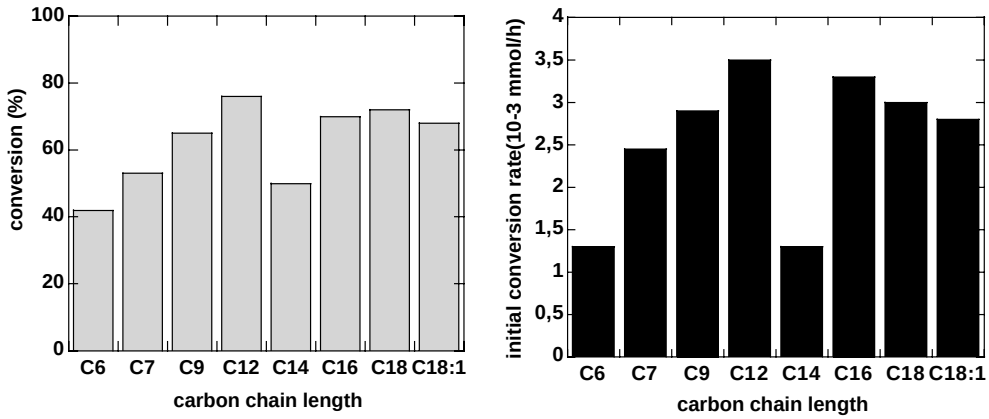


Fig. 2. The effect of carbon-chain length and the unsaturation of the fatty acids on the acylation performance of rutin carried out in 2-methyl-2-butanol using *Candida antarctica* lipase.

conversion yields were of about 72–68%, respectively, with stearic and oleic acids.

3.3. Regioselectivity of esterification

For all acids tested and glycosylated flavonoids, only one product was identified by HPLC analysis. For further investigation, the palmitate esters of esculin and rutin were purified and characterised by ^{1}H and ^{13}C NMR. The ^{1}H NMR data indicated only a monoacylation for the two analysed esters.

For the esculin, with a glucose moiety, the acylation occurred at the 6′-OH of the sugar. The enzymatic acylation of esculin with fatty acids has never been described. For other glucoside flavonoids, similar results have been reported by Gao et al. [11] and Nakajima et al. [29] who showed that the acylation of isoquercitrin with aromatic acid vinyl esters takes place at the primary hydroxyl group of the glucose. However, Danieli et al. [30] indicated for the same flavonoid that the esterification occurs both on the primary 6″-OH and secondary 3″-OH of the glucose.

Rutin has a disaccharide moiety, a 6-*O*- (α-L-rhamnopyranosyl)-D-glucose, which has only secondary hydroxyl groups. The ^{13}C NMR spectrum of rutin palmitate suggested that acylation occurred on the secondary 4‴-OH of the rhamnose moiety. However, Danieli et al. [30] obtained rutin 3″, 4‴-(*O*-diacetate), while Nakajima et al. [29] observed the synthesis of rutin 3″-(*O*-cinnamate). The difference of the regioselectivity of *C. antarctica* lipase could be attributed to the fact that both acyl donors and solvents used in these studies were not similar.

4. Conclusion

The feasibility of the enzymatic esterification of hesperidin, rutin and esculin by *C. antarctica* lipase was demonstrated in this investigation. The performance of the flavonoid ester synthesis depends on both alcohol and acyl donor structures. The study of the alcohol donor effect showed that the substrate with a primary hydroxyl group on the sugar moiety was acylated easily, while glycosylated flavonoids with sugars having secondary OH were found to be poor substrates. Moreover, under our operating conditions, no reactivity was observed with quercetin. The investigation undertaken on the effect of the fatty acid carbon-chain length showed that for carbon numbers lower than C12, the performance depends upon the fatty acids,

whereas for higher carbon-chain length no significant effect was observed. The highest conversion yield and initial rate were obtained with rutin and lauric acid (C12) and equal to 76% and 3.5×10^{-3} mmol/h, respectively.

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