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C. Lorentz, Amélie Dulac, G. Pencreac'H, F. Ergan, Pascal Richomme, et al.. Lipase-catalyzed synthesis of two new antioxidants: 4– and 3–palmitoyl chlorogenic acids. Biotechnology Letters, 2010, 32 (12), pp.1955-1960. 10.1007/s10529-010-0386-6 . hal-00618196

HAL Id: hal-00618196
https://hal.science/hal-00618196v1

Submitted on 1 Sep 2011

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Section: Microbial and Enzyme Technology

Lipase-catalyzed synthesis of two new antioxidants: 4-*O*- and 3-*O*-palmitoyl chlorogenic acids

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Purpose of work

The goal of our work was to investigate the feasibility of lipase-catalyzed esterification of purified 5-caffeoyl quinic acid (CQA) extracted from *Hydrangea macrophylla*, using palmitic acid as the acyl donor.

Abstract

Chlorogenic acid (5-caffeoyl quinic acid, CQA) extracted from *Hydrangea macrophylla* (44%, w/w) with a 98 % purity, was acylated with palmitic acid by Novozym 435 to yield mono-acylated CQA. Acylation of CQA was achieved in 2-methyl-2-butanol at 60°C, and yielded two mono-acylated products: a major product acylated at the C-4 of the quinic moiety (4-*O*-palmitoyl chlorogenic acid) and a minor product acylated at the C-3 (3-*O*-palmitoyl chlorogenic acid). The bioconversions obtained in 7 days ranged from 14 to 60% and were influenced by the molar ratio of palmitic acid/CQA, which ranged from 10 to 80. The regioselectivity (4-*O*- palmitoyl/3-*O*-palmitoyl ratio) of the reaction was also affected by the molar ratio, and ranged from 90 to 70%. The scavenging activities against 1,1-diphenyl-2-picryl-hydrazyl radicals demonstrated that these palmitoyl CQA derivatives are associated with antioxidant activity (70% versus CQA).

Keywords: Acylation · Chlorogenic acid · Novozym 435 · Palmitic acid · Scavenging activity

Introduction

Antioxidants are important for protecting and prerserving foods, pharmaceuticals and cosmetics. However, antioxidants such as butylated hydroxytoluene (BHT) have been reported to produce undesirable effects (Babich 1982). This has stimulated increased interest in developing alternative natural antioxidants, especially polyphenols of plant origin. However, the antioxidant properties of polyphenols are impaired in oil-based products because of their high hydrophilic/lipophilic balance. Lipophilisation, in which a lipophilic moiety is grafted onto a hydrophilic one, is one way to improve their lipophilicity, and thus their antioxidant activities in oil-based products (Villeneuve 2007). Chlorogenic acids are naturally-occurring polyphenol esters of caffeic acid and quinic acid. 5-caffeoyl quinic acid (CQA) has been associated with numerous biological activities, including antimicrobial (Muthuswamy and Vasantha Rupasinghe 2007), anticarcinogenic and hepatoprotective effects (Xiang and Ning 2008). This has generated considerable interest in synthesising lipophilised CQA derivatives in the hope of enhancing both their biological activity and their antioxidant properties (Lopez-Giraldo et al. 2009). The CQA molecule has several OH groups and one COOH group that can be selectively esterified to produce a variety of lipophilised derivatives by means of acylation or alkylation reactions. The behaviour of CQA towards enzymatic alkylation was first investigated using fatty alcohols as the alkyl donors (Guyot et al. 2000; Lopez-Giraldo et al. 2007). As far as acylation is concerned, only one study reports the results of the lipase-catalyzed acylation of CQA (Stevenson et al. 2006). In this study, acylation was carried out on a fruit extract mixture containing CQA and quercetin glycosides as major polyphenols. Under the experimental conditions used, quercetin glycosides were readily acylated, whereas CQA was not. Moreover, as far as we are aware, hitherto only a chemical approach has been described as leading to the synthesis of lauroyl-CQA (Xiang and Ning 2008).

The goal of our work was therefore to investigate the feasibility of lipase-catalyzed esterification of purified CQA extracted from *Hydrangea macrophylla*, using palmitic acid as the acyl donor. The effects of the molar ratio of the palmitic acid/CQA were studied and discussed in terms of the bioconversion yields and regioselectivity of the reaction. The scavenging activities of the acylated CQA dervatives against 1,1, diphenyl-2-picryl-hydrazyl (DPPH) radicals were also compared, on the one hand, to those of unmodified CQA and an alkylated CQA derivative, namely hexadecyl chlorogenate, and on the other to a synthetic antioxidant, namely butylated hydroxytoluene (BHT).

Materials and Methods

Biological and chemical materials

The lipases used are shown in Supplementary Table 1. All solvents and reagents used were obtained from commercial suppliers, and were either of HPLC or analytical grade. Palmitic acid, silica gel plates and Sephadex LH-20 were purchased from Sigma-Aldrich. Hexadecyl chlorogenate was enzymatically synthesized and purified in our laboratory as previously described (Lopez-Giraldo et al. 2007) with minor modifications.

Extraction of CQA from *Hydrangea macrophylla*

Sepals of *Hydrangea macrophylla* were collected at the Gaston Allard arboretum in Angers (France) and were frozen in liquid N_2, and kept at -80°C before extraction; 550 g of frozen sepals were extracted 3 times under reflux with 3 x 1.5 l methanol/ethanol (50/50, v/v). The resulting extract was concentrated under reduced pressure to obtain 30 g crude extract. Using a commercial standard, the amount of CQA in this extract was estimated to be 238 mg/g using HPLC quantification. 4 g of this extract were then chromatographed over 80 g of Sephadex LH-20, using methanol/water (60/40, v/v) as the eluent. After methanol removal by evaporation under reduced pressure, the precipitate formed in the remaining aqueous phase was filtered and washed twice with water, yielding 424 mg (44%, w/w) of CQA with purity as high as 98 %.

Enzymatic acylation of 5-caffeoyl quinic acid (CQA)

CQA, palmitic acid and enzymes were dried for at least two days over P_2O_5 before use. The water activity (a_w) of the reaction medium was determined using an AqualabLite (Decagon Devices Inc., USA). The initial a_w values of all the reaction media were below 0.2.

Acylation reactions were carried out in Eppendorf tubes in the dark. The reaction medium consisted of molar ratios of palmitic acid/CQA varying from 10 to 80 in 1 ml 2-methyl-2-butanol (2M2B). The initial concentration of CQA was 28 mM or 113 mM. Palmitic acid and CQA were first solubilised for 12 h in 1 ml of 2M2B and stirred at 1000 rpm at 60°C in an Eppendorf Thermomixer. The reaction was initiated by adding 40 mg enzyme. Then 200 mg molecular sieves (3Å), previously dried overnight at 200°C, was added. All assays were carried out in duplicate. The 2M2B had previously been dried on molecular sieves (3Å). Control experiments were conducted without the enzyme.

Thin-layer chromatography

Esterification was qualitatively monitored by TLC using 0.3 mm silica gel plates. TLC analyses were carried out using toluene/ethyl acetate/formic acid (25/20/5, by vol.) as eluent, and spots were located using UV (254 nm) and/or iodine staining. Preparative TLC was performed under the same conditions, using 0.5 mm silica gel plates.

High-performance liquid chromatography

To monitor the reactions, aliquots (20 µl) were withdrawn from the reaction medium and diluted in methanol to 1 ml. Prior to injection, each sample was filtered using a Millex Millipore filter (0.22 µm). Analyses were performed using a Waters system (LC module 1 system, Waters, USA) equipped with an auto-sampler (Waters, USA), an online vacuum degasser (Waters, USA), a UV detector (486 absorbance detector, Waters, USA). The column used was a Lichrospher 100 RP-18 (5 µm, 250x4 mm, Merck). Detection was performed at 327 nm for both CQA and acylated CQA. Elution was performed at 55°C. The chromatogram peaks were integrated using on-line software (Galaxie, Varian, France). The products were separated using a linear gradient of a formic acid solution (0.1%) and acetonitrile ranging from 5/95 to 100/0 (v/v) over 15 min, followed by 100/0 for 10 min, and from 100/0 to 5/95 over 10 min, all at 1 ml/min. The amounts of products were determined using the calibration curves obtained with purified acylated CQA. The method was linear (r^2> 0.98) for acylated CQA over a concentration range of 5-200 mg/l. The bioconversion yield was defined as m mol products/mmol of initial CQA) x 100.

Purification of products

At the end of the reaction, the enzyme and molecular sieves were filtered off (0.22µm), the solvent was evaporated under vacuum using a rotary evaporator, and the products were isolated by Sephadex LH-20 chromatography using chloroform/methanol (70/30, v/v) as the eluent. Final purification was completed by means of preparative TLC.

Determination of the structure of the reaction products

After purification of the acylated CQA by chromatography, each compound was identified by NMR in DMSO-d6, methanol-d4 and/or acetone-d6 using a Brüker Advance at 300 or 500 MHz spectrometer (Germany).

Mass spectrometry analysis was performed using a spectrometer equipped with a quadripole ion trap analyzer, an ESI source, and an integrated syringe pump (Finnigan LCQ Serie MS detector, San Jose, California). Purified compounds were dissolved in methanol at 100 mg/l. Solutions were directly injected into the ESI source (infusion mode) at 5 µl/min. ESI-MS data were acquired in the zoom scan mode for the accurate determination of the parent ion *m*/*z* in MS^n mode. The MS operating conditions (negative mode) were a collision energy of 25%, an ionization voltage of 4.54 kV, and a capillary temperature of 250°C.

Free radical-scavenging activity

This test was performed using the Abdel-Lateff et al. method (2002). It is based on the bleaching of the stable DPPH. In its radical form, DPPH has an absorption band at 520 nm, which disappears when reduced by an antiradical compound. The methanolic solution of the tested compound or standard reference (Trolox) was mixed with DPPH solution. After 30 min at room temperature, the absorbance was recorded at 520 nm. Results are expressed as Trolox equivalent per µmol tested compound (TE/µmol).

Results and discussion

Characterization of reaction products

Of the nine lipases screened (Supplementary Table 1), Novozym 435 was shown to be the best catalyst.

The reaction mixtures were analyzed by TLC after one week. This qualitative approach demonstrated that two products had been synthesised (data not shown). After purification by preparative TLC, products 1 and 2 were obtained as whitish-yellow crystalline powders, and analyzed by MS and NMR. ESI-MS data for products 1 and 2 gave an [M-H] pseudomolecular ion at m/z=591, suggesting that they were in fact regio-isomers. The parent ion fragmentations were also quite similar (data not shown), and NMR analysis was therefore required to distinguish the acyl positions. ^{1}H and ^{13}C chemical shifts for CQA and products 1 and 2 are shown in Supplementary Table 2. The ^{1}H NMR spectra of CQA did not show any substantial change after acylation, except for the two methylenes bearing the OH groups of the quinic acid moiety, and the appearance of new signals at 0.86-1.60 associated with the fatty acid chain (data not shown). For product 1, the ^{13}C-NMR shifts of the two methylenes bearing the OH groups of the quinic acid moiety were unequivocally attributed through a Heteronuclear Multiple Quantum Correlation (HMQC) experiment. This was followed by a Heteronuclear Multiple Bond Correlation (HMBC) experiment that clearly demonstrated correlation between the H4 protons

(4.31 ppm) and the C1'' carbonyl of the fatty chain (174.8 ppm), suggesting that the acylation took place at the C4 position of the quinic acid moiety of CQA. Product 1 was therefore identified as 4-*O*-palmitoyl chlorogenic acid (4-*O*-palmitoyl-CQA, Fig. 1a). Similar observations were made for product 2, which was identified as 3-*O*-palmitoyl chlorogenic acid (3-*O*-palmitoyl-CQA; Fig. 1a).

Fig. 1a

Time course and regioselectivity of CQA acylation

The time course for the CQA acylation with palmitic acid by Novozym 435 in 2M2B is shown in Fig. 2. The equilibrium of the acylation was reached within 20 days, with a bioconversion yield of 35%. This result demonstrated that the enzymatic acylation is feasible with the secondary OH groups of the quinic moiety of CQA unlike results reported by Stevenson et al. (2006). Indeed, these authors did not obtain CQA derivatives when using fruit extracts and palmitic acid as substrates after 7 days and under quite similar experimental conditions (using Novozym 435 and palmitic acid as the acyl donor in 2-methyl-2-propanol at 60°C). This observation is explained as being due to the lipase strong selectivity for acylation of flavonoid glycosides with a primary aliphatic hydroxyl group on the sugar moiety over phenolic hydroxyl groups.

Fig. 2

At the end of the reaction, the two products were quantified separately in order to study the regioselectivity of the reaction. Novozym 435 led preferentially to 4-*O*-palmitoyl-CQA (70%) rather than to 3-*O*-palmitoyl-CQA (30%). In order to check whether the presence of the 3-*O* isomer could be due to an intramolecular acyl migration rather than to an enzymatic reaction, purified 4-*O* isomer was added to the reaction medium without the enzyme. None of the 3-*O* isomer had been formed after incubating for one day, showing that no acyl migration had occurred.

Effect of the palmitic acid/CQA molar ratio on bioconversion and regioselectivity

Acylation reactions were carried out as described above with the palmitic acid/CQA molar ratio ranging from 10 to 80. An improvement in the bioconversion yields was observed with increasing substrate ratios (Fig. 3). Indeed, the best conversion (60%) was obtained with the highest ratios (60-80) in 7 days. These results are comparable to yields reported by Guyot et al. (2000) using alkylation. The yield obtained in organic solvent for CQA alkylation was around 60% after a lengthy reaction time (30 days) using Novozym 435 and fatty alcohols. This reaction was further improved (95% in 96 h) by exploiting an alternative chemo-enzymatic approach based on a two-step reaction: chemical CQA methylation followed by Novozym 435-catalyzed transacylation of the methylchlorogenate in a solvent-free system (Lopez-Giraldo et al. 2007). More recently, the use of supercritical

Fig. 3

CO_2 as solvent for the alkylation of CQA has been described, using pentanol, heptanol and geraniol as acyl donors (Hernandez et al. 2009). The reaction time was reduced to 25 h, and yields of 77, 82 and 85%, respectively, were obtained.

The effect of molar ratio on regioselectivity was also explored. Increasing the amount of palmitic acid reduced the proportion of 4-*O*-acylation from 90% to 68% (Fig. 3). Similar effects of the composition of the reaction medium on the regioselectivity of the acylation reaction have already been reported in the literature. For example, MacManus and Vulfson (1997) showed that regioselectivity of 6-*O*-trityl-β-D-glycopyranoside acetylation was markedly affected by solvent polarity. In our case, increasing the amount of palmitic acid may enhance the hydrophobicity of the medium, and it may be this that alters the regioselectivity.

Free radical-scavenging activity

Fig. 1b

Table 1

The antioxidant activities of acylated CQA derivatives were determined by the widely-used DPPH method, and compared to those of unmodified CQA, hexadecyl chlorogenate (Fig. 1b) and BHT (Table 1). The free radical–scavenging activities of 4-*O*-palmitoyl-CQA, 3-*O*-palmitoyl-CQA and hexadecyl chlorogenate were not significantly different. Indeed, all three displayed activity equivalent to 70-80% of that of CQA. In the literature, lipophilisation has been shown to affect the DPPH-scavenging activities of polyphenols in very different ways. For example, Silva et al. (2000) observed that alkyl esters of caffeic acid displayed enhanced antiradical activity, whereas lipophilisation of dihydrocaffeic acid led to a dramatic decrease in its antiradical activity. Moreover, Sharma and Bhat (2009) recently pointed out that the conditions used for DPPH assays by various authors vary considerably making the results difficult to compare.

Regarding CQA, Lopez-Giraldo et al. (2009) have shown that the hexadecyl chlorogenate exhibits 30% greater DPPH scavenging activity than unmodified CQA. Under our conditions, this ester displays activity equivalent to 80% of that of CQA. These results can probably be explained by differences in the DPPH assay conditions used in these two studies.

Interestingly, under our conditions, the acylated CQA derivatives exhibited significant antioxidant activity which was as much as 5 times greater than that of BHT, which is commonly used to protect lipids against oxidation.

Conclusion

This study shows that a long, saturated fatty chain can be grafted onto the secondary OH group of the quinic moiety of CQA using lipase with palmitic acid as the acyl donor. The synthesised CQA derivatives still display

antioxidant activity. In view of their hydrophobic characteristics, we suggest that these new compounds are interesting candidates for investigating the antioxidant potential, localisation and conformation relationships of antioxidants in emulsified systems. Moreover, so far the structure/activity relationship of polyphenols has remained unclear because of the wide diversity of polyphenol structures. The new compounds proposed here could therefore help to elucidate this structure/activity relationship.

Acknowledgements

This work is part of the AISQAL (Approche Intégrée de la Sécurité et de la Qualité des Aliments) project funded by the region of Pays de la Loire, France. The authors wish to thank Laval Agglomeration and the Conseil Général de la Mayenne for financing a doctoral thesis.

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Table 1: Scavenging effect of CQA, alkylated and acylated CQA derivatives on DPPH radicals

Compound	**TE*/µmol**
CQA	1 ± 0.06
4-*O*-palmitoyl-CQA	0.7± 0.08
3-*O*-palmitoyl-CQA	0.7 ± 0.04
Hexadecyl chlorogenate[a]	0.8 ± 0.08
BHT	0.14 ± 0.004

[a] Hexadecyl chlorogenate was synthesized following Lopez-Giraldo alkylation procedure (2007)

*Trolax equivalent

Figure legends

Fig.1: Chemical structures of CQA derivatives:

(a) 4-*O* palmitoyl-CQA and 3-*O*-palmitoyl-CQA

(b) hexadecyl chlorogenate

Fig. 2. Time course of CQA acylation using palmitic acid and Novozym 435.

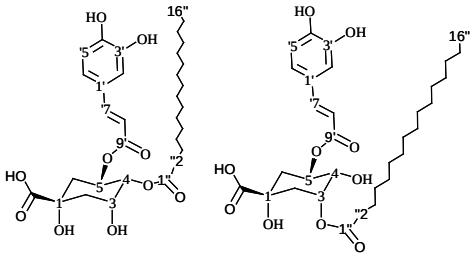
Reaction conditions: 2M2B=1 ml, CQA=113 mM, molar ratio palmitic acid/CQA=10, 60°C, 1000 rpm, Novozym 435 =40 mg, molecular sieves=200 mg.

Fig. 3. Effect of the palmitic acid/CQA molar ratio on bioconversion and on regioselectivity at 7 days.

Reaction conditions : 2M2B= 1 ml, CQA = 28 mM, , 60°C, 1000 rpm, Novozym 435= 40 mg and palmitic acid/CQA molar ratio ranging from 10 to 80.

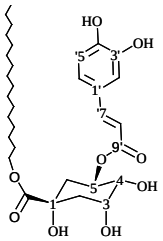
Figure 1

(a)



4-*O*-palmitoyl CQA 3-*O*-palmitoyl CQA

(b)



Hexadecyl chlorogenate

Figure 2

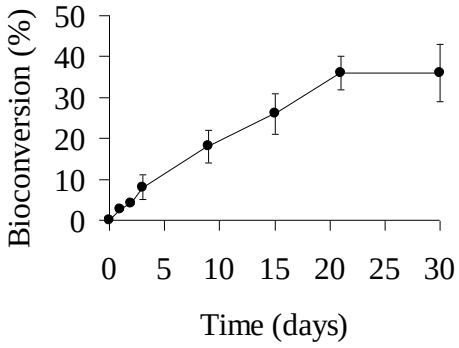
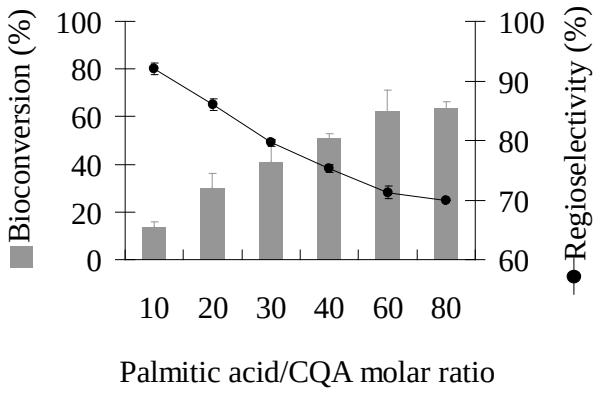


Figure 3


Bioconversion (%)
100
80
60
40
20
0
100
90
80
70
60
Regioselectivity (%)
10
20
30
40
60
80
Palmitic acid/CQA molar ratio

Supplementary Table 1: Lipases screened for CQA acylation with palmitic acid

Lipase source	Commercial name	Enzyme preparation
C. antarctica Lip B	Novozym 435 [a]	immobilised on macroporous acrylic resin
M. miehei	Lipozym RM-IM [a]	immobilised on exchange ion resin
T. lanuginosa	Lipozym TL-IM [a]	immobilised on granulated silica
A. niger	Lipase A [b]	free
M. javanicus	Lipase M [b]	free
R. oryzae	Lipase DF [b]	free
C. rugosa	Lipase AY [b]	free
P. camembertii	Lipase G [b]	free
P. cepacia	Lipase PS [b]	free

[a] purchased from Novozymes (Dk)

[b] purchased from Amano Enzymes (UK)

Supplementary Table 2: ^{1}H and ^{13}C NMR spectroscopic assignments for CQA and acylated CQA

N°	CQA		4-*O*-palmitoyl-CQA		3-*O*-palmitoyl-CQA	
	δH *[(a)]	δC *[(b)]	δH *[(a)]	δC *[(c)]	δH *[(a)]	δC *[(c)]
Quinic acid moiety						
1	-	75.0	-	76.0	-	74.6
2-6	2.1-2.23	36.7-37.9	2.06-2.20	38.2-39.4	2.06-2.36	39.4-39.5
3	4.19	70.2	5.07	69.3	5.23	72.4
4	3.73	72.3	4.31	75.6	3.94	71.9
5	5.34	70.4	5.59	68.6	5.40	70.8
7	-	173.9	-	176.7	-	177.3
Caffeoyl moiety						
1’	-	126.5	-	126.1	-	126.1
2’	7.03	114.7	7.05	113.7	7.07	113.7
3’	-	145.1	-	145.1	-	145.1
4’	-	144.6	-	145.2	-	145.2
5’	6.80	115.2	6.79	113.4	6.80	113.4
6’	6.98	121.4	6.96	127.5	6.99	127.7
7’	7.59	147.6	7.56	146.8	7.59	147.8
8’	6.30	114.0	6.23	114.8	6.28	114.8
9’	-	165.9	-	168.0	-	167.1
Palmitoyl moiety						
1’’	-	-	-	174.8	-	174.0
2’’	-	-	1.58	22.6	1.60	22.6
3’’-15’’	-	-	1.24	24.2-33.6	1.24	24.2-33.6
16’’	-	-	0.91	14.4	0.86	12.8

(*): ppm; Solvent used: (a): DMSO-*d* ; (b): Acetone-*d* ; (c): Methanol-*d*