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Journal of Molecular Catalysis B: Enzymatic 27 (2004) 1–6
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Enzymatic synthesis of new aromatic esters of phloridzin

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Received 16 June 2003; received in revised form 12 August 2003; accepted 12 August 2003

Abstract

Lipase B from *Candida antarctica* immobilized on a macroporous acrylic resin (Novozym 435®) was used to catalyze the enzymatic synthesis of acylated derivatives of phloridzin a flavonoid from the dihydrochalcone family. Reactions were achieved under reduced pressure, in the presence of a large excess of acyl donor which also acted as a solvent for the acyl acceptor, phloridzin. The acylation of phloridzin by ethyl cinnamate was shown to be total and perfectly regioselective in favor of phloridzin-6″-*O*-cinnamate. The kinetic of the reaction was significantly improved by the temperature of the reaction medium best results being obtained at 80 °C. An amount of immobilized enzyme of 20 g l^{-1} corresponding to 1.2 g l^{-1} of proteins was chosen in order to obtain the total conversion of phloridzin after only few hours. Increasing phloridzin concentration allowed a maximal production of 119 g l^{-1} phloridzin cinnamate after 80 h. Other esters like phloridzin-6″-*O*-benzoate and phloridzin-6″-*O*-salicylate were also prepared in similar conditions. However, only traces of ester were obtained when phenolic acyl donors like methyl *p*-hydroxyphenylacetate and methyl *p*-hydroxyphenylpropionate were used. All esters were purified and characterized.
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Keywords: *Candida antarctica* lipase B; Enzymatic acylation; Flavonoid glycoside; Phloridzin

1. Introduction

Phenolic compounds are efficient antioxidants acting as free radical terminators or metal chelators [1]. Among them, flavonoids which are derivatives of benzo-γ-pyrone, are particularly interesting as natural antioxidants for foods and cosmetics. Flavonoids generally occur as glycosylated derivatives in plants where they contribute to the brilliant shades of leaves, flowers and fruits. Besides their antioxidant activity, some of them have a wide variety of physiological effects in both animals and plants as enzyme activators or inhibitors, transcription regulators and phytohormones [2]. However, their use is strongly limited by their weak solubility either in hydrophilic or hydrophobic formulations. An interesting way to enhance the solubility of flavonoid glycosides, and then to exploit their properties as well as possible, is their functionalisation with appropriate hydrophilic or hydrophobic radicals. Moreover functionalisation of flavonoids could be a way to enforce their antioxidant activity with phenolic radicals or to bring additional properties with other active radicals.

A few studies are reported describing flavonoids functionalisation and especially acylation reactions which can be made either by a chemical way or an enzymatic way. Chemical acylation of flavonoids by various fatty acids was patented [3], but this process is not regioselective and leads to unwanted functionalisation of phenolic hydroxyl groups which are responsible for antioxidant activity of flavonoids [4].

Enzymatic acylation of flavonoids by fatty acids is also described in a few studies. Flavonoid disaccharides like rutin, hesperidin or naringin were acylated by butanoic acid in the presence of subtilisin [5]. Conversion yields were comprised between 33 and 65% and acylation was not regioselective, leading to mixtures of products. Danieli et al. [6] described the effective acylation (conversion yields around 80%) of flavonoid glycosides by vinyl acetate using *Candida antarctica* lipase as biocatalyst. More recently, Kontogianni et al. [7] presented the acylation of naringin and rutin by C8–C12 acids. Maximal conversion yield was about 65% after 10 days of reaction.

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doi:10.1016/j.molcatb.2003.08.002

The regioselective acylation of flavonoids with phenolic acids which may enhance not only their solubility in various media but also their stability [8] and their antioxidant activity [9] is an interesting way too. A two steps process could be applied where the subtilisin is firstly used to catalyze the acylation of flavonoid substrate with malonate [10]. This transformation was not total (65% conversion yield) and not regioselective which means intermediate purification steps. Secondly, a chemical Knoevenagel type condensation is applied leading to the desired arylaliphatic ester. A single step enzymatic process was also described in which Novozym 435® catalyzes alcoholysis reaction between flavonoid substrate and aromatic vinyl esters but yields and reaction rates remained low (maximal conversion yield was 70% after 5 days) [11,12].

One way to move esterification equilibrium towards synthesis is to work with a large excess of one substrate which also acts as a solvent for the second substrate (melting media). Such conditions were applied for the esterification of cinnamic acid esters with aliphatic alcohol [13]. High conversion yields up to 98% were obtained when using the acyl acceptor (primary alcohol C4–C12) as excess substrate. Acylation of flavonoids like naringin and rutin by carboxylic acids in excess was attempted too but was much less effective in melting medium comparing with solvent medium (conversion yields around 13% after 10 days) [7].

This work describes firstly the enzymatic acylation of phloridzin by cinnamic acid ethyl ester as excess substrate catalyzed by the Novozym 435® as a feasibility study. Phloridzin is a flavonoid glucoside of dihydrochalcone family which is found in *Malus* species (up to 10% of dry weight in young apple leaves and twigs) [14]. This flavonoid presents interesting biological activities such as inhibitor of glucose adsorption by cell [15]. Cinnamate radical is expected to bring additional anti-UV properties while increasing phloridzin solubility in lipidic phases. Secondly a few parameters such as temperature, enzyme and substrates concentrations were studied in the aim of improving phloridzin cinnamate production while reducing reaction time. Then the process was applied to other aromatic acyl donors like methyl benzoate and methyl salicylate which show anti-UV and/or antibacterial activities. Finally acylation of phloridzin by phenolic substrates like *p*-hydroxyphenylacetate and *p*-hydroxyphenylpropionate was attempted. All these acyl donors are liquid above 60 °C and are susceptible to make phloridzin soluble.

2. Materials and methods

2.1. Materials

2.1.1. Enzyme

Lipase B from *C. antarctica* immobilized on a macroporous acrylic resin (Novozym 435®, Novo Industry A/S, protein content between 2 and 10%) was used to catalyze acylation reactions. Maximum activity of the lipase is 7 propyl laurate Units/mg (the enzyme activity refers to the synthesis of propyl laurate at 60°C). Enzyme was dried for 16 h over P_2O_5 before use.

2.1.2. Chemicals

Phloridzin was supplied by Extrasynthese (France). Ethyl cinnamate (mp 7 °C), methyl benzoate (mp −12.4 °C), methyl *p*-hydroxyphenylacetate (mp 58 °C) of purity over 99% and methyl *p*-hydroxyphenylpropionate (mp 40 °C) of purity over 97% were purchased from Aldrich. Methyl salicylate (mp 19.4 °C) and methyl cinnamate (mp 36 °C) of purity over 99% were supplied by Fluka and Merck, respectively.

Methanol (BDH), hexane (Carlo Erba) and trifluoroacetic acid: TFA (Sigma) were of analytical grade.

2.2. Synthesis

Acylation reaction was carried out in the 25 ml evaporation flasks of a rotary evaporator (R-144, Büchi) under reduced pressure (200 mbar). Stirring was provided by a 40 rpm rotation. Temperature of the reaction medium is specified as needed.

Reaction mixture was prepared with 23 mg (50 μmol) phloridzin dissolved in 5 ml acyl donor (30 mmol). Reaction was started by adding 100 mg Novozym 435® which corresponds to about 6 mg of proteins.

Fifty microliters samples were taken from the reaction mixture at relevant time intervals for analytical monitoring of the reaction. Acyl donor excess was extracted twice with 0.5 ml hexane. Then precipitated phloridzin and derivatives were diluted in 0.5 ml methanol (0.1%TFA). Seventy-five microliters of this solution were diluted with 1.425 ml of a mixture MeOH/H_2O/TFA (50/50/0.1) for HPLC analysis.

2.3. HPLC analysis

Quantitative analysis of samples was made by high performance liquid chromatography (HPLC) on a 100 RP 18 Lichrosphere column (250 mm × 4 mm, 5 μm). Elution system was: A = methanol/water/TFA (50/50/0.01 (v/v/v)) and B = methanol/TFA (100/0.01 (v/v)). A two steps gradient starting from 20% B up to 70% within 20 min followed by an isocratic step at 70% B for 10 min was applied. Elution flow rate was 0.6 ml min^{-1}. Detection was achieved at 280 nm on a Merck Hitachi L-3000 UV detector. Calibration curves for phloridzin and acyl donors were obtained using standards in methanol. Calibration curves for phloridzin cinnamate, phloridzin salicylate and phloridzin benzoate were obtained using purified samples.

2.4. Conversion yields determination

Conversion yields were expressed by the molar ratio of ester formed to flavonoid introduced at the beginning of the reaction.

2.5. Water activity measurement

Flavonoid and enzyme were dried for 16 h over P_2O_5 before use, whereas acyl donors were used straight from the bottle.

Water activity (a_w) of the reaction medium was determined using a Thermoconstanter TH200 Novasina (20 °C). Initial a_w of all reaction media was under 0.1.

2.6. Purification of phloridzin esters

Purification of reaction mixtures was achieved in a quite simple procedure which led to pure phloridzin esters: at the end of the reaction, hexane was added to the reaction medium (5/1 (v/v)). A filtration on a Duran sintered disc filter funnel (no. 3) removed hexane phase containing ethyl cinnamate while retaining precipitated flavonoid and enzyme particles. Phloridzin esters were dissolved with 20 ml methanol. Methanol phase was extracted with 3 ml × 20 ml hexane in order to remove residual acyl donor traces. Esters were recovered after evaporation of methanol.

2.7. Chemical structure determination

Chemical structure of phloridzin cinnamate, phloridzin benzoate and phloridzin salicylate were determined by ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) in dimethylsulfoxide-d_6 by using tetramethylsilane as the internal reference on an Avance Bruker 300 MHz spectrometer.

^{1}H chemical shifts for phloridzin-6″-*O*-cinnamate were: δ(H-β) = 2.76, δ(H-2, H-6) = 7.03, δ(H-3, H-5) = 6.64, δ(H-3′) = 6.18, δ(H-5′) = 5.91, δ(H-1″) = 5.09, δ(H-6″a) = 4.20, δ(H-6″b) = 4.47, δ(H-*x*‴) = 7.63, δ(H-*y*‴) = 6.59, δ(H-3‴, H-7‴) = 7.65, δ(H-4‴, H-5‴, H-6‴) = 7.41.

^{13}C chemical shifts were: δ(C=O) = 204.3, δ(C-α) = 45.2, δ(C-β) = 29.5, δ(C-1) = 131.9, δ(C-2, C-6) = 129.5, δ(C-3, C-5) = 115.3, δ(C-4) = 155.7, δ(C-1′) = 104.8, δ(C-2′) = 161.0, δ(C-3′) = 97.6, δ(C-4′) = 165.8, δ(C-5′) = 95.5, δ(C-1″) = 101.1, δ(C-2″) = 74.3, δ(C-3″) = 76.9, δ(C-4″) = 73.6, δ(C-5″) = 70.0, δ(C-6″) = 63.9, δ(C-1‴) = 166.5, δ(C-*x*‴) = 118.2, δ(C-*y*‴) = 145.0, δ(C-2‴) = 134.3, δ(C-3‴, C-7‴) = 128.7, δ(C-4‴, C-6‴) = 129.3, δ(C-5‴) = 130.8.

^{1}H chemical shifts for phloridzin-6″-*O*-benzoate were: δ(H-β) = 2.76, δ(H-2, H-6) = 7.00, δ(H-3, H-5) = 6.30, δ(H-3′) = 6.22, δ(H-5′) = 5.86, δ(H-1″) = 5.08, δ(H-6″a) = 4.23, δ(H-6″b) = 4.62, δ(H-3‴, H-7‴) = 7.96, δ(H-4‴, H-6‴) = 7.47, δ(H-5‴) = 7.67.

^{13}C chemical shifts were: δ(C=O) = 205.0, δ(C-α) = 45.3, δ(C-β) = 29.4, δ(C-1) = 131.9, δ(C-2, C-6) = 129.7, δ(C-3, C-5) = 115.5, δ(C-4) = 155.6, δ(C-1′) = 105.5, δ(C-2′) = 160.9, δ(C-3′) = 97.2, δ(C-4′) = 166.0, δ(C-5′) = 94.8, δ(C-6′) = 164.8, δ(C-1″) = 100.7, δ(C-2″) = 74.3, δ(C-3″) = 76.8, δ(C-4″) = 73.5, δ(C-5″) = 70.3, δ(C-6″) = 64.6, δ(C-1‴) = 165.7, δ(C-2‴) = 129.9, δ(C-3‴, C-7‴) = 129.5, δ(C-4‴, C-6‴) = 129.0, δ(C-5‴) = 133.7.

^{1}H chemical shifts for phloridzin-6″-*O*-salicylate were: δ(H-β) = 2.76, δ(H-2, H-6) = 7.03, δ(H-3, H-5) = 6.63, δ(H-3′) = 6.27, δ(H-5′) = 5.89, δ(H-1″) = 5.03, δ(H-6″a) = 4.24, δ(H-6″b) = 4.68, δ(H-4‴) = 6.92, δ(H-5‴) = 7.46, δ(H-6‴) = 6.83, δ(H-7‴) = 7.80.

^{13}C chemical shifts were: δ(C=O) = 204.4, δ(C-α) = 45.2, δ(C-β) = 29.5, δ(C-1) = 131.9, δ(C-2, C-6) = 129.5, δ(C-3, C-5) = 115.3, δ(C-4) = 155.6, δ(C-1′) = 104.9, δ(C-2′) = 161.0, δ(C-3′) = 97.5, δ(C-4′) = 166.7, δ(C-5′) = 95.4, δ(C-6′) = 169.1, δ(C-1″) = 100.9, δ(C-2″) = 74.2, δ(C-3″) = 76.9, δ(C-4″) = 73.5, δ(C-5″) = 70.3, δ(C-6″) = 64.8, δ(C-1‴) = 165.9, δ(C-2‴) = 113.7, δ(C-3‴) = 161.1, δ(C-4‴) = 118.0, δ(C-5‴) = 135.8, δ(C-6‴) = 119.1, δ(C-7‴) = 130.8.

3. Results and discussion

3.1. Synthesis of phloridzin-6″-O-cinnamate

3.1.1. Feasibility study

Enzymatic acylation of phloridzin was achieved by an alcoholysis reaction in a large excess of ethyl cinnamate, at 60 °C, under reduced pressure (200 mbar). Such conditions should allow the elimination of the by-product of the reaction, ethanol, in order to move thermodynamic equilibrium of the reaction towards synthesis. Initial water activity of the reaction medium was less than 0.1 which is also in favor of ester synthesis [16].

HPLC analysis of the reaction medium (Fig. 1) showed the formation of a single new product while phloridzin disappeared. This product had a middle polarity between phloridzin and ethyl cinnamate and was expected to be the phloridzin-6″-*O*-cinnamate.

NMR analysis of the purified product showed that acylation did actually take place on the 6″-OH of phloridzin glycoside moiety (Fig. 2) which agrees with the selectivity of the enzyme towards primary alcohols [11].

Time course of the enzymatic acylation of phloridzin by ethyl cinnamate is reported in Fig. 3.

Phloridzin was totally converted after 15 h of reaction leading to a production of phloridzin cinnamate of 5.6 g l^{-1} (10 mM). This result shows that enzymatic acylation of phloridzin by an aromatic substrate like ethyl cinnamate is possible. High efficiency of the process is explained by the large excess of acyl donor and the good elimination of ethanol which moves the equilibrium of the reaction in favor of ester synthesis, and also by the low a_w level (<0.1) of the reaction medium which limits possible hydrolysis phenomena.

3.1.2. Effect of the temperature

In order to diminish time necessary for the total conversion of phloridzin the effect of temperature on the kinetic of the reaction was studied (Fig. 4).

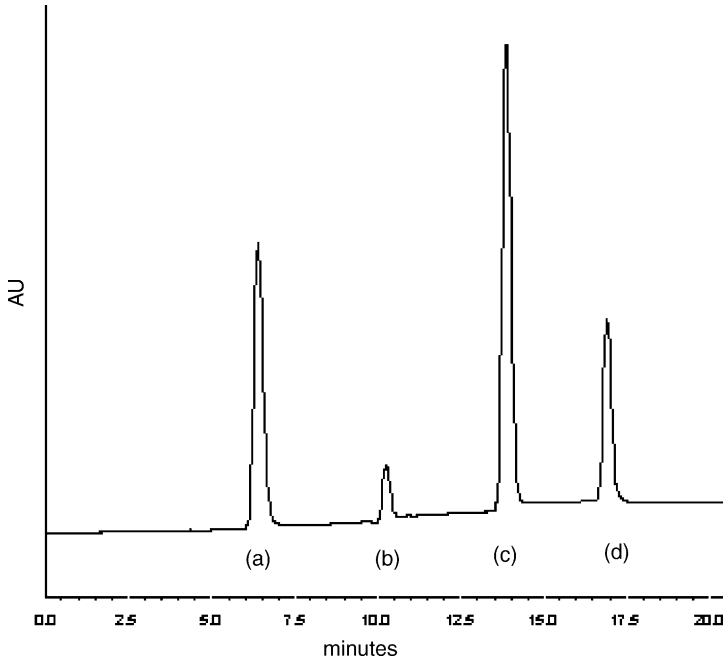

Fig. 1. Chromatogram from HPLC analysis of reaction medium, after 5 h reaction at 80 °C, under 200 mbar pressure. Reaction medium was composed of phloridzin (50 μmol) and ethyl cinnamate (30 mmol): (a) phloridzin; (b) cinnamic acid; (c) phloridzin cinnamate; (d) ethyl cinnamate.

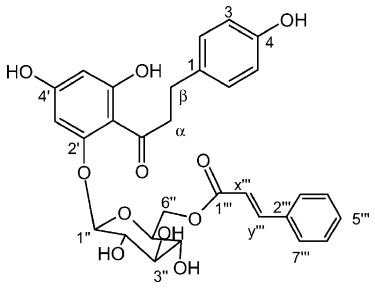

Fig. 2. Chemical structure of phloridzin-6″-*O*-cinnamate.

As shown in Fig. 4 phloridzin was totally converted into phloridzin cinnamate after 15, 4 and 2 h at 60, 80 and 100°C, respectively. Such result shows the higher the temperature was the shorter the reaction time. Possible explanations are the thermal activation of the lipase and also the more efficient elimination of ethanol. An other explanation consists in the better diffusion of phloridzin in the reaction medium at high temperatures. Moreover this study shows the perfect stability of phloridzin and phloridzin cinnamate in ethyl cinnamate whatever the temperature was.

Next experiments were conducted at 80 °C which appears as the best temperature for the rapid and total conversion of phloridzin while preventing enzyme from thermal inactivation.

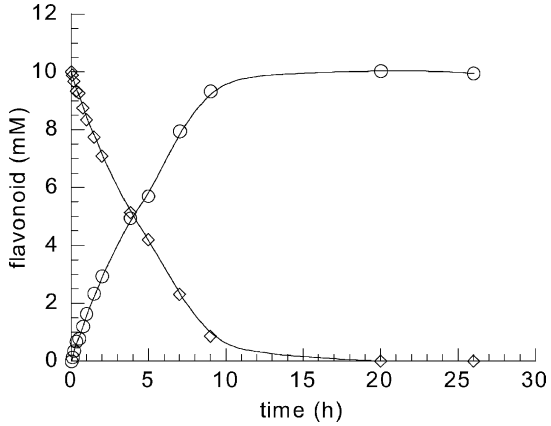

Fig. 3. Time course of the enzymatic acylation of phloridzin (50 μmol) by ethyl cinnamate (30 mmol) catalyzed by the Novozym 435®, at 60 °C, under 200 mbar: (◇) phloridzin; (○) phloridzin cinnamate.

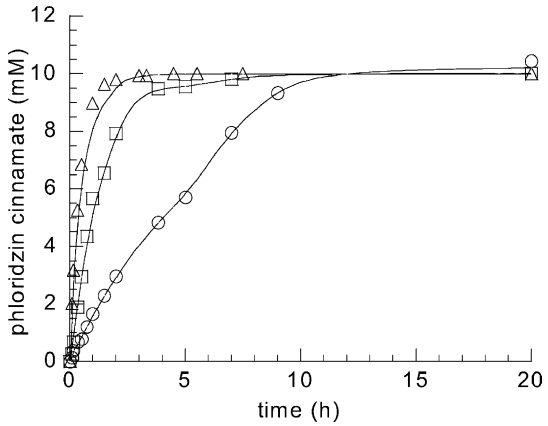

Fig. 4. Effect of temperature on time course of the enzymatic acylation of phloridzin (50 μmol) by ethyl cinnamate (30 mmol) catalyzed by the Novozym 435®, under 200 mbar, at 60 °C (○), 80 °C (□) and 100 °C (△).

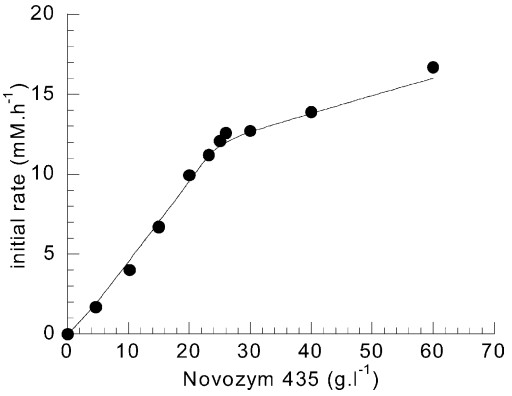

Fig. 5. Effect of the amount of Novozym 435® on the initial rate of phloridzin (50 μmol) acylation by ethyl cinnamate (30 mmol), at 80 °C, under 200 mbar.

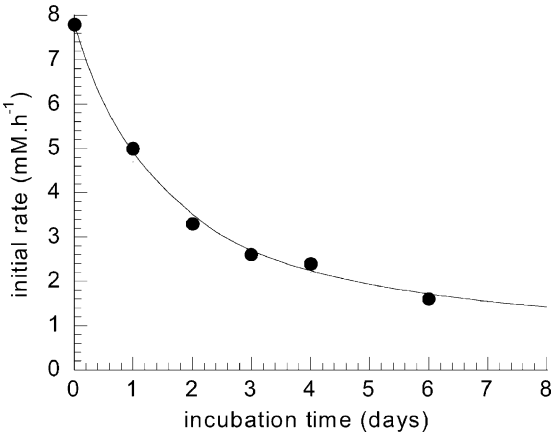

Fig. 6. Initial rate of the enzymatic acylation of phloridzin by ethyl cinnamate after variable incubation times of the Novozym 435®, at 80 °C.

3.1.3. Effect of enzyme amount

In order to minimize the amount of enzyme while maintaining the effective acylation of phloridzin, reactions were achieved in the presence of various amounts of Novozym 435® (Fig. 5).

As expected, phloridzin conversion yield was not affected by the amount of enzyme and was maintained to 100%.

The initial reaction rate was proportional to the concentration of Novozym 435® until 20 g l^{-1} which corresponds to about 1.2 g l^{-1} of proteins. As previously shown before, this concentration of enzyme led to the total conversion of phloridzin after 4 h at 80 °C. A weaker effect was observed for higher concentrations which could be explained by diffusion limitations. Similar result was obtained when methyl benzoate was used as acyl donor (results not shown). Next experiments were all conducted with 100 mg of Novozym 435® per 5 ml ethyl cinnamate.

3.1.4. Stability of the Novozym 435® in ethyl cinnamate at 80 °C

In order to consider the further development of this process the stability of the enzyme in such reactional conditions has to be evaluated. Hundred milligrams of Novozym 435® were incubated in 4 ml ethyl cinnamate, at 80 °C, under atmospheric pressure, for 1–4 and 6 days. Then 1 ml of a 50 mM solution of phloridzin in ethyl cinnamate was added while reducing pressure up to 200 mbar. Residual enzymatic activity was evaluated through initial reaction rate measurements (Fig. 6).

As shown in Fig. 6, a rapid loss of the catalytic activity of the Novozym 435® was observed in ethyl cinnamate, at 80 °C. For instance, 30% of the activity was lost after 1 day of incubation. This unstability was explained by the thermal denaturation of the lipase and also by possible interactions between enzymatic protein and ethyl cinnamate. Such a result should not limit the eventual development of the process as phloridzin was totally converted into phloridzin cinnamate after only 4 h at 80 °C.

3.1.5. Effect of phloridzin concentration

In order to enhance the production of phloridzin cinnamate, initial concentration of phloridzin was increased from 10 to 210 mM (Table 1).

Phloridzin was totally converted whatever the initial concentration was, leading to a maximum of 118.9 g l^{-1} of phloridzin cinnamate after 80 h. This result represents a significant amelioration comparing with the literature, showing the high efficiency and the originality of the process. Actually, Nakajima et al. [12] and Gao et al. [11] both used 20 g l^{-1} *C. antarctica* lipase B and vinyl cinnamate as acyl donor to synthesize flavonoid glycoside esters. At best, a 70% conversion yield of flavonoid was obtained when starting from 44 mM (+) catechin 7-*O*-α-D-glucopyranoside [11] or 100 mM isoquercitrin [12]. In the present process, up to 210 mM phloridzin was involved. Conversion of the flavonoid was complete and a quite simple purification procedure (described Section 2.6) allowed recovering of all the ester formed which corresponds to 100% isolation yield.

Table 1
Phloridzin conversion yields and final reaction times for various initial concentrations of phloridzin in ethyl cinnamate

	Initial concentration of phloridzin (mM)						
	10	20	40	65	85	125	210
Phloridzin conversion yield (%)	100	100	100	100	100	100	100
Final phloridzin cinnamate concentration (g l^{-1})	5.7	11.3	22.6	36.8	48.1	70.8	118.9
Final reaction time (h)	5	10	25	28	40	60	80

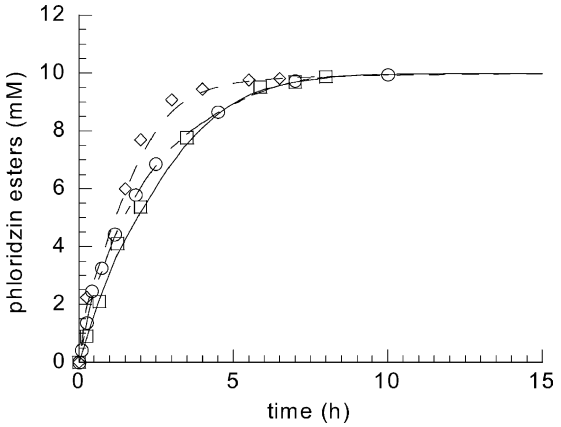

Fig. 7. Time course of the enzymatic acylation of phloridzin by methyl cinnamate (○), methyl benzoate (□) and methyl salicylate (◇) catalyzed by the Novozym 435®, at 80 °C, under 200 mbar.

The more the concentration of phloridzin was the higher the final reaction time. This result could be naturally explained by the growing amount of substrate to be transformed. Another explanation consists in mass transfer limitations due to the increasing viscosity of the reaction mixture.

3.2. Effect of the acyl donor

In order to study the influence of the acyl donor and to extent the process to the production of other original derivatives of phloridzin several available aromatic acid methyl esters were tested as acylating agents (Fig. 7).

Phloridzin was totally converted into a single product after 6 h when methyl cinnamate, methyl benzoate or methyl salicylate were used as acyl donors. NMR analysis of purified products confirmed the obtention of phloridzin-6″-*O*-cinnamate, phloridzin-6″-*O*-benzoate and phloridzin-6″-*O*-salicylate. All these transformations were slightly slower than acylation by ethyl cinnamate, which could result from the lower affinity of the enzyme for these substrates. Another explanation could be the higher viscosity of reaction mixtures.

The acylation of phloridzin by acyl donors like methyl *p*-hydroxyphenylacetate and methyl *p*-hydroxyphenylpropionate was also attempted. In that case, only traces of acylation products were observed even after 10 days of reaction. This poor result was probably due to the very low solubility of phloridzin in such media. Furthermore reaction media were observed to turn to brown suggesting oxidation phenomena.

4. Conclusion

The enzymatic acylation of phloridzin by various aromatic substrates like ethyl and methyl cinnamate, methyl benzoate or methyl salicylate, catalyzed by the Novozym 435®, was demonstrated to be very efficient when the acyl donor was used in a large excess and acted as a solvent for the acyl acceptor. The total and rapid conversion of 10 mM phloridzin was obtained at 80 °C, under a pressure of 200 mbar after 4 h. The reaction was perfectly regioselective on the 6″-OH of phloridzin glycoside moiety. With the same concentration of Novozym 435®, about 119 g l^{-1} phloridzin cinnamate (210 mM) were obtained after 80 h. This excellent result represents a significant amelioration comparing with other similar works. In the case of acyl donors like methyl *p*-hydroxyphenylacetate and methyl *p*-hydroxyphenylpropionate only traces of product were obtained due to the poor solubility of phloridzin.

Described process appears to be very efficient to produce original esters of phloridzin. Main drawbacks are the high viscosity of reaction mixtures which is responsible for mass transfer limitations and also the poor solubility of phloridzin in some acyl donors. Nevertheless, phloridzin esters synthesis with such acyl donors is expected to be more effective in media where both substrates are soluble. Reactions in solvent systems are currently under investigation and will be reported in a following paper.

The process is expected to be efficient for the preparation of many other derivatives of flavonoids of interest for cosmetics, pharmaceuticals or food industries.

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