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Synthesis of 3-cyclohexylpropyl caffeate from 5-caffeoylquinic acid with consecutive enzymatic conversions in ionic liquid

Atsushi Kurata[a,*], Shintaro Takemoto[a], Tokio Fujita[a], Kazuya Iwai[b], Mina Furusawa[b], Noriaki Kishimoto[a,**]

[a] Department of Applied Biological Chemistry, Kinki University, 3327-204 Nakamachi, Nara City, Nara 631-8505, Japan
[b] R&D Center, UCC Ueshima Coffee Co. Ltd., 3-1-4 Zushi, Takatsuki-shi, Osaka 569-0036, Japan

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ABSTRACT

We developed a convenient one-pot procedure for conversion of 5-caffeoylquinic acid to 3-cyclohexylpropyl caffeate, which exhibits an antiproliferative effect toward various human tumor cells. The procedure was comprised of two consecutive reactions by chlorogenate hydrolase (EC 3.1.1.42) from *Aspergillus japonicus* and *Candida antarctica* lipase B, and was performed using an ionic liquid, 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide, as the reaction solvent. When various caffeoylquinic acids from coffee beans, namely, 3-caffeoylquinic acid, 4-caffeoylquinic acid, 5-caffeoylquinic acid, 3,5-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid were used, the first alcoholysis reaction with methanol using chlorogenate hydrolase produced methyl caffeate with conversion yields of 60.0%, 61.3%, 86.0%, 92.7%, and 114.0%, respectively, to each individual substrate. Two caffeoyl groups of dicaffeoylquinic acids would be used for the synthesis of methyl caffeate. In the subsequent transesterification reaction by *C. antarctica* lipase B with 3-cyclohexyl-1-propanol, the methyl caffeate produced was converted to 3-cyclohexylpropyl caffeate under reduced pressure to remove the by-product methanol. In the one-pot synthesis, the methyl caffeate was transesterified efficiently to 3-cyclohexylpropyl caffeate by *C. antarctica* lipase B with deactivation of chlorogenate hydrolase by taking advantage of the difference between the optimum temperatures for the two enzymes. This system provided 12.8 mM 3-cyclohexylpropyl caffeate from 15 mM 5-caffeoylquinic acid with conversion yield of 85.3%.

1. Introduction

Caffeic acid esters are widely distributed in plants and propolis [1,2]. Caffeic acid phenethyl ester (CAPE) especially has been found in propolis and has a broad spectrum of biological activities, including antimicrobial, anti-inflammatory, antioxidant, and antitumor activities [3]; it also has an inhibitory effect on HIV-1 integrase, cyclooxygenase, and lipooxygenase [4–7]. It has been reported that the ester part of CAPE is important for the antiproliferative effect on various human tumor cells [8]. Additionally, it was suggested that conversion of the phenyl group to a cyclohexyl group in a CAPE analogue enhanced the antiproliferative effect [9]. Recently, we showed that 3-cyclohexylpropyl caffeate, one of the CAPE analogues, exhibits a strong antiproliferative activity toward various tumor cells that is comparable to that of 5-fluorouracil [10].

Immature green coffee beans are not marketed as coffee because contamination of these beans negatively affects the flavor. However, they contain appreciable amounts of various caffeoylquinic acids, for example, 4.8–5.8 g of 5-caffeoylquinic acid/100 g of immature green coffee beans [11]. These immature beans are notable among unused agricultural resources, and we are therefore currently investigating the enzymatic conversion of their caffeoylquinic acids to valuable products. In recent work, we synthesized CAPE using 5-caffeoylquinic acid and 2-phenylethanol as substrates with chlorogenate hydrolase from *Aspergillus japonicus* by a transesterification reaction in a biphasic aqueous-alcohol state and elucidated the antibacterial, antimutagenic, and anti-influenza virus activities of CAPE [12]. The procedure using chlorogenate hydrolase provided various CAPE analogues, but the maximum conversion yield of CAPE was 50%. The insufficient yield was probably due to the hydrolysis of 5-caffeoylquinic acid by the enzyme to caffeic acid in the aqueous phase. Therefore, a new procedure for the synthesis of CAPE analogues superior to that method in terms of the conversion yield remained to be developed.

Ionic liquids (ILs), which are composed of a bulky asymmetric cation and a small anion, are easily modified with respect to the combination of cation and anion, and therefore, numerous IL

* Corresponding author. Tel.: +81 742 43 7028; fax: +81 742 43 1445.
** Corresponding author. Tel.: +81 742 43 7338; fax: +81 742 43 1445.
E-mail addresses: kurata090401@nara.kindai.ac.jp (A. Kurata), kisimoto@nara.kindai.ac.jp (N. Kishimoto).

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compositions are possible [13]. Unlike conventional organic solvents used for biocatalytic reactions, ILs are able to dissolve many compounds, have a wide temperature range for the liquid phase, and possess no vapor pressures. Thus, ILs have good properties for use as reaction solvents, and extensive studies of enzymatic synthesis using ILs have been carried out [14,15]. We previously developed an efficient procedure for conversion of methyl caffeate to produce various CAPE analogues with *Candida antarctica* lipase B using an ionic liquid, 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([BMIM][NTf_2]), as a solvent [10].

In this study, we found that chlorogenate hydrolase from *A. japonicus* efficiently catalyzed the alcoholysis reaction of caffeoylquinic acids purified from coffee beans, namely, 3-caffeoylquinic acid, 4-caffeoylquinic acid, 5-caffeoylquinic acid, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid, with methanol to produce methyl caffeate in an IL, [BMIM][NTf_2], as the solvent. By using two consecutive reactions by chlorogenate hydrolase and *C. antarctica* lipase B in [BMIM][NTf_2] as the solvent, we developed a convenient one-pot procedure for an enzymatic synthesis of 3-cyclohexylpropyl caffeate from 5-caffeoylquinic acid (Fig. 1). Firstly, methyl caffeate (compound 2) was prepared from 5-caffeoylquinic acid (compound 1) and methanol using chlorogenate hydrolase with the IL. Then, the unreacted methanol was removed *in vacuo* (14 hPa) at 80 °C for 1 h, and 3-cyclohexylpropyl caffeate (compound 4) was obtained using *C. antarctica* lipase B with methyl caffeate and 3-cyclohexyl-1-propanol (compound 3) as the substrates. In order to accelerate the reaction equilibrium to give the desired product, we performed the *C. antarctica* lipase B-catalyzed reaction under reduced pressure (845 hPa) to remove the by-product methanol from the reaction mixture. Additionally, to take advantage of the different optimum temperatures for the two enzymes in the one-pot reaction, the conversion of methyl caffeate to 3-cyclohexylpropyl caffeate was performed by *C. antarctica* lipase B with deactivation of chlorogenate hydrolase. The one-pot two-step method in the IL is a convenient economical preparation of 3-cyclohexylpropyl caffeate with good yield.

2. Experimental

2.1. Enzymes and materials

Chlorogenate hydrolase (without glucose as a stabilizing agent, 0.36 U mg^{-1}) from *A. japonicus* and *C. antarctica* lipase B (Novozyme435, 3530 U mg^{-1}) were kindly donated by Kikkoman (Chiba, Japan) and Novozymes (Bagsvaerd, Denmark), respectively. One unit of chlorogenate hydrolase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of caffeic acid from 5-caffeoylquinic acid per min at 40 °C [11]. One unit of Novozyme435 activity was defined as the amount of enzyme that catalyzes the production of 1 μmol of 2-phenylethyl acetate from vinyl acetate and 2-phenylethanol per min at 25 °C [10]. Caffeoylquinic acids, namely, 3-caffeoylquinic acid, 4-caffeoylquinic acid, 5-caffeoylquinic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, and a mixture of 3,4-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid, were purified from green coffee beans [16]. The purities of the compounds were confirmed with HPLC analysis according to reference [16]. The HPLC analysis of these compounds showed that single peak was occurred at 1.45 min (3-caffeoylquinic acid), 17.7 min (4-caffeoylquinic acid), 14.8 min (5-caffeoylquinic acid), 38.6 min (3,5-dicaffeoylquinic acid), 44.0 min (4,5-dicaffeoylquinic acid), and 35.8 min (3,4-dicaffeoylquinic acid). These compounds were identified with FAB-MS analysis and ^1H NMR analysis according to reference [16]. 3-Cyclohexyl-1-propanol (Tokyo Kasei, Tokyo, Japan) was purchased. 1-Butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([BMIM][NTf_2]), 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF_4]), 1-butyl-3-methylimidazolium trifluoromethanesulfonate ([BMIM][CF_3SO_3]), *N*-methyl-*N*-propylpiperidinium bis(trifluoromethylsulfonyl)imide ([MPPip][NTf_2]), and *N*-methyl-*N*-propylpyrrolidinium bis(trifluoromethylsulfonyl)imide ([MPPro][NTf_2]) were purchased from Kanto Kagaku (Tokyo, Japan). All other reagents were purchased from Merck (Darmstadt, Germany), Sigma–Aldrich Japan (Tokyo, Japan), and Nacalai Tesque (Kyoto, Japan).

2.2. Immobilization of chlorogenate hydrolase

Quaternary ammonium sepabeads (SEPABEADS EC-QA) was donated by Mitsubishi Chemical Co. (Tokyo, Japan). Chlorogenate hydrolase (0.1 g) and quaternary ammonium sepabeads (1.0 g) were dissolved in 4 ml of 20 mM sodium phosphate buffer (pH 6.5), and the mixture were incubated with stirring at 300 rpm using an Invitro shaker Mix-VR (TAITEC, Tokyo, Japan) at 24 °C for 20 h. Then, the supernatant was removed by decanting, and the immobilized enzyme fraction was washed three times with 10 ml of 20 mM sodium phosphate buffer (pH 6.5). The immobilized chlorogenate hydrolase was assayed for protein concentration and enzyme activity. The protein concentration was determined using a Bradford assay kit (Nacalai Tesque) with bovine serum albumin as the standard and by measuring absorbance at 595 nm. The procedure for determining chlorogenate hydrolase activity is described below. The immobilized enzyme (0.02 U mg^{-1}) was prepared with the immobilization yield of 48.8%.

2.3. Enzyme activity

The standard reaction for chlorogenate hydrolase was performed at 40 °C for 4 h with shaking at 200 rpm (Magnetic stirrer SW-RS777D, Nissin, Tokyo, Japan) under ambient pressure in a 1-ml reaction mixture consisting of 15 mM 5-caffeoylquinic acid, 2200 mM methanol, 3.6 U of immobilized chlorogenate hydrolase, 10 μl of 50 mM sodium phosphate buffer (pH 6.5), and [BMIM][NTF_2] as the reaction solvent. Fifty microliters of the reaction mixture was removed, and the reaction with chlorogenate hydrolase was terminated by adding 950 μl of methanol. For quantitative analyses of the substrate and product formed, high-performance liquid chromatography (HPLC) was performed using a Cosmosil 5C18-ARII column (4.6 mm × 250 mm, Nacalai Tesque) on a Hitachi D-2000 Elite HPLC system (Hitachi High-Technologies Corp., Tokyo, Japan) equipped with a UV detector. The substrate and product formed were detected at 330 nm. The column was equilibrated with 0.2% acetate containing 30% methanol at a flow rate of 1.0 ml/min at 40 °C. Elution was performed in a linear gradient of 30–60% methanol for 10 min, followed by an isocratic elution with 100% methanol for 15 min. 5-Caffeoylquinic acid and methyl caffeate were eluted at retention times of 5.5 min and 13.1 min, respectively. The synthesis of 3-cyclohexylpropyl caffeate with methyl caffeate and 3-cyclohexy-1-propanol by Novozyme435 was described previously [10]. The method of quantitative analysis of the 3-cyclohexylpropyl caffeate formed was same as that of methyl caffeate described above. The product 3-cyclohexylpropyl caffeate was eluted at the retention time of 18.5 min.

2.4. Consecutive conversions

For synthesis of 3-cyclohexylpropyl caffeate from 5-caffeoylquinic acid by two consecutive reactions, methyl caffeate was produced from 5-caffeoylquinic acid with shaking at 200 rpm