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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

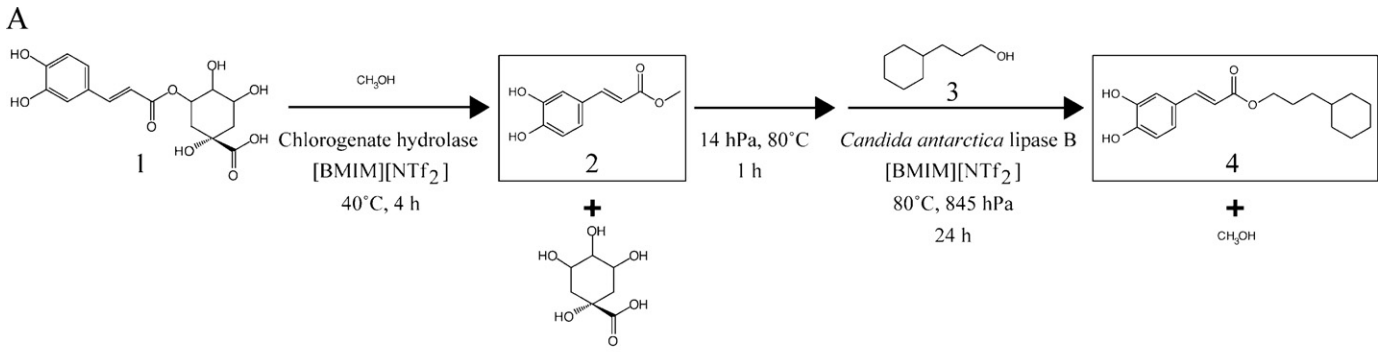
Quaternary ammonium sepabeads (SEPABEADS EC-QA) was donated by Mitsubishi Chemical Co. (Tokyo, Japan). Chlorogenate hydrolase (0.1 g) and quaternary ammonium sepabeads (1.0 g) were dissolved in 4 ml of 20 mM sodium phosphate buffer (pH 6.5), and the mixture were incubated with stirring at 300 rpm using 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.

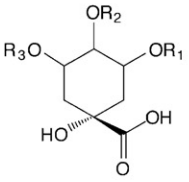
2.4. Consecutive conversions

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



B

	Quinic acid		
	R_1	R_2	R_3
3-Caffeoylquinic acid	Caffeoyl group	H	H
4-Caffeoylquinic acid	H	Caffeoyl group	H
5-Caffeoylquinic acid	H	H	Caffeoyl group
3,4-Dicaffeoylquinic acid	Caffeoyl group	Caffeoyl group	H
3,5-Dicaffeoylquinic acid	Caffeoyl group	H	Caffeoyl group
4,5-Dicaffeoylquinic acid	H	Caffeoyl group	Caffeoyl group


Quinic acid

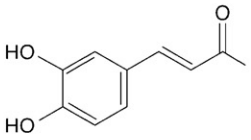

Caffeoyl group

Fig. 1. Consecutive enzymatic reactions for synthesis of 3-cyclohexylpropyl caffeate from 5-caffeoylquinic acid, and chemical structures of caffeoylquinic acids from coffee beans. (A) In all reaction steps, [BMIM][NTf_2] was used as the solvent. Compound 1: 5-caffeoylquinic acid, 2: methyl caffeate, 3: 3-cyclohexyl-1-propanol, and 4: 3-cyclohexylpropyl caffeate. (B) Structures of substrates for synthesis of methyl caffeate with chlorogenate hydrolase.

(Magnetic stirrer SW-RS777D, Nissin) 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 solvent. After the reaction at 40 °C for 4 h, the reaction mixture was gently stirred by a rotary evaporator (RE600, Yamato Science Co., Ltd., Tokyo, Japan) under reduced pressure (14 hPa) at 80 °C for 1 h to remove unreacted methanol. Next, 400 mM 3-cyclohexane-1-propanol and 120,000 U of Novozyme435 were added to the reaction mixture, and 3-cyclohhexylpropyl caffeate was synthesized at 80 °C under reduced pressure (845 hPa) to remove methanol, which was produced as a byproduct. Chlorogenate hydrolase is deactivated at 80 °C. Then, fifty microliters of the reaction mixture was removed, and the reaction was terminated by adding 950 μl of methanol. The conditions for quantitative analysis of 5-caffeoylquinic acid, methyl caffeate, and 3-cyclohexylpropyl caffeate were described above.

2.5. Purification of reaction products

The purification procedures for methyl caffeate and 3-cyclohexylpropyl caffeate were almost identical. As a representative example, the purification procedure for methyl caffeate is described. The reaction mixture was extracted with diethyl ether, and the extract containing the substrates and methyl caffeate was concentrated *in vacuo*. The concentrate was applied to a silica gel column (26 mm × 400 mm, Wako gel C-200, Wako Pure Chemical) equilibrated with chloroform. Then, the product was eluted with chloroform:methanol (9:1). The product formed was detected by HPLC as described above and thin-layer chromatography (TLC) with a silica gel 60F254 plate (Merck no. 5715, Darmstadt, Germany) using chloroform:methanol (9:1) and detected by UV-radiation at 254 nm.

2.6. Structure determination

The structure of 3-cyclohexylpropyl caffeate was previously determined by ^{1}H and ^{13}C NMR analysis [10]. For determination of the structure of the methyl caffeate produced, the ^{1}H and ^{13}C NMR spectra were recorded on a JEOL FT-NMR JNM-270EX (270 MHz) spectrometer with tetramethylsilane as the internal standard.

2.6.1. Methyl caffeate (compound 2)

^{1}H NMR (CD_3OD): δ 3.72 (d, 3H, J = 1.00 Hz), 6.25 (d, 1H, J = 1.31 Hz), 6.90 (d, 1H, J = 1.86 Hz), 6.92 (d, 1H, J = 1.86 Hz), 6.93 (d, 1H, J = 1.86 Hz), 7.54 (d, 1H, J = 2.03 Hz); ^{13}C NMR (CD_3OD): δ 51.99 (C_1), 114.83 (C_3), 115.10 (C_7), 116.48 (C_6, C_{10}), 127.68 (C_5), 146.82 (C_4), 146.95 (C_9), 149.59 (C_8).

3. Results and discussion

3.1. Effect of aqueous solution on chlorogenate hydrolase in IL

We found that immobilized chlorogenate hydrolase catalyzes the conversion of 5-caffeoylquinic acid to methyl caffeate with methanol using [BMIM][NTf_2] as a solvent under a non-aqueous condition. Accordingly, we investigated an enzymatic synthesis of 3-cyclohexylpropyl caffeate from 5-caffeoylquinic acid *via* methyl caffeate. In various enzymatic syntheses, water can shift the equilibrium towards the direction of hydrolysis; therefore, methods with lipase for removal of the water produced as a by-product were applied [17,18]. However, the catalytic activity in a non-conventional solvent depends on the amount of water associated with the enzyme forming its native conformation [19]. First, the effect of the addition of an aqueous solution on the production of methyl caffeate was examined in [BMIM][NTf_2] by varying the additional volume of 50 mM sodium phosphate buffer (pH 6.5) from 0 to 5% (v/v) (Fig. 2). When the concentration of the aqueous solution was 1% or lower, the production of methyl caffeate increased. How-

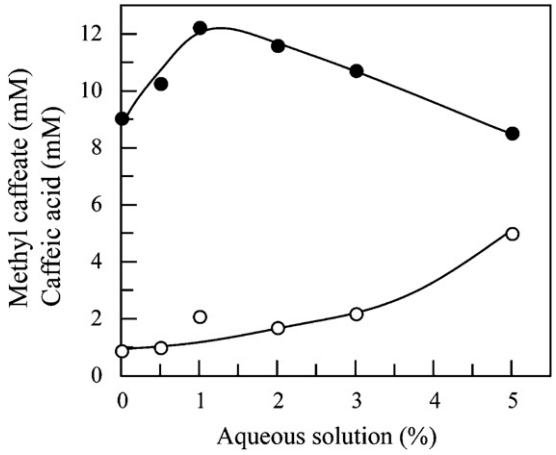


Fig. 2. Effects of water concentration on conversion of 5-caffeoylquinic acid to methyl caffeate by chlorogenate hydrolase. The reaction was performed according to the standard method described in Section 2, except that the reaction was carried out with a 0–5% (v/v) aqueous solution of 50 mM sodium phosphate (pH 6.5). Each symbol indicates methyl caffeate (closed circle) and caffeic acid (open circle).

ever, the production of methyl caffeate was decreased in greater than 2% aqueous solutions. The production of caffeic acid was increased with addition of the buffer, indicating that the enzyme probably catalyzed hydrolysis of 5-caffeoylquinic acid to produce caffeic acid rather than the alcoholysis to produce methyl caffeate. Thus, our results indicated that addition of 1% aqueous solution was suitable for the production of methyl caffeate. Using lipase from *C. antarctica* for synthesis of isoamylacetate in a biphasic 1-butyl-3-methylimidazolium hexafluorophosphate [BMIM][PF_6]-alcohol system produced a similar optimum curve, and thus the 1% (w/w) initial water concentration is suitable for the reaction [20]. It was suggested that ILs are able to maintain the active structures of enzymes with the monomolecular layer of water [21]. Thus, chlorogenate hydrolase maintains the active structure with the layer of the buffer in [BMIM][NTf_2].

3.2. Conversion of 5-caffeoylquinic acid with chlorogenate hydrolase

We examined the production of methyl caffeate or 3-cyclohexylpropyl caffeate using immobilized chlorogenate hydrolase in [BMIM][NTf_2] (Fig. 3A, B). After a 10-h reaction using 15 mM 5-caffeoylquinic acid and 2200 mM methanol, 11.3 mM methyl caffeate and 3.2 mM caffeic acid were produced with conversion yields of 75.4% and 21.5%, respectively (Fig. 3A), whereas 13.1 mM caffeic acid and 1.21 mM 3-cyclohexylpropyl caffeate were produced with conversion yields of 87.6% and 8.1%, respectively, using 15 mM 5-caffeoylquinic acid and 2200 mM 3-cyclohexyl-1-propanol (Fig. 3B). The result showed that methanol is a better substrate for chlorogenate hydrolase than 3-cyclohexylpropyl caffeate to produce methyl caffeate with a high conversion yield. Additionally, Novozyme435 did not catalyze the conversion of 5-caffeoylquinic acid to 3-cyclohexylpropyl caffeate or methyl caffeate (data not shown), but efficiently catalyzed the conversion of methyl caffeate to 3-cyclohexylpropyl caffeate with a conversion yield of 93.8% [10]. Thus, consecutive conversions of 5-caffeoylquinic acid to 3-cyclohexylpropyl caffeate *via* methyl caffeate using chlorogenate hydrolase and Novozyme435 would be a more effective procedure than a single conversion of 5-caffeoylquinic acid to 3-cyclohexylpropyl caffeate.

3.3. Selection of IL for chlorogenate hydrolase

In order to investigate the activities of chlorogenate hydrolase in various ILs, the alcoholysis of 5-caffeoylquinic acid with methanol was examined (Fig. 4). The reactions were performed in five ILs, namely, [BMIM][NTf_2], two [BMIM] cation-containing ILs, 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF_4]) and 1-butyl-3-methylimidazolium trifluoromethanesulfonate ([BMIM][CF_3SO_3]), and two [NTf_2] anion-containing ILs, *N*-methyl-*N*-propylpyrrolidinium bis(trifluoromethylsulfonyl)imide ([MPPro][NTf_2]) and *N*-methyl-*N*-propylpiperidinium bis(trifluoromethylsulfonyl)imide ([MPPip][NTf_2]). Although chlorogenate hydrolase catalyzed the alcoholysis reaction in [BMIM][NTf_2] to produce 13.7 mM methyl caffeate, the reaction scarcely proceeded in [BMIM][BF_4] and [BMIM][CF_3SO_3]. Although Novozyme435 catalyzed transesterification of methyl caffeate to 3-cyclohexylpropyl caffeate in [BMIM][NTf_2], the transesterification scarcely proceeded in [BMIM][BF_4] and [BMIM][CF_3SO_3] [10]. Lipase from *Candida rugosa* was found to be active in [BMIM][PF_6] for the transesterification, but inactive in ILs including [BMIM][acetate] and [BMIM][nitrate] [22]. Lipase from *Pseudomonas aeruginosa* was more stable in [BMIM][PF_6] than in [BMIM][BF_4] [23]. Thus, the nature of the anion in IL plays a critical factor in determining the enzyme activity and stability. It was reported that the hydrogen-bond basicities of [BMIM][BF_4] and [BMIM][CF_3SO_3] are larger than that of [BMIM][NTf_2] [22,24]. Additionally, it was suggested that the

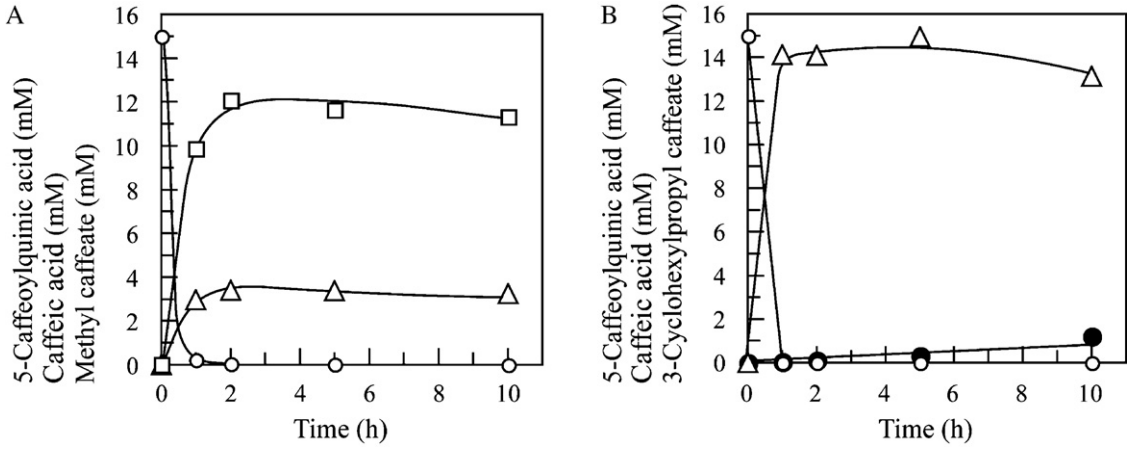


Fig. 3. Conversion of 5-caffeoylquinic acid to methyl caffeate and caffeic acid (A) and. 3-cyclohexylpropyl caffeate and caffeic acid (B) by chlorogenate hydrolase. The reaction was performed according to the standard method described in Section 2. The reaction mixture consisted of methanol (A) or 3-cyclohexyl-1-propanol (B) as the substrate. Symbols indicate 5-caffeoylquinic acid (open circle), caffeic acid (open triangle), methyl caffeate (open square), and 3-cyclohexylpropyl caffeate (closed circle).

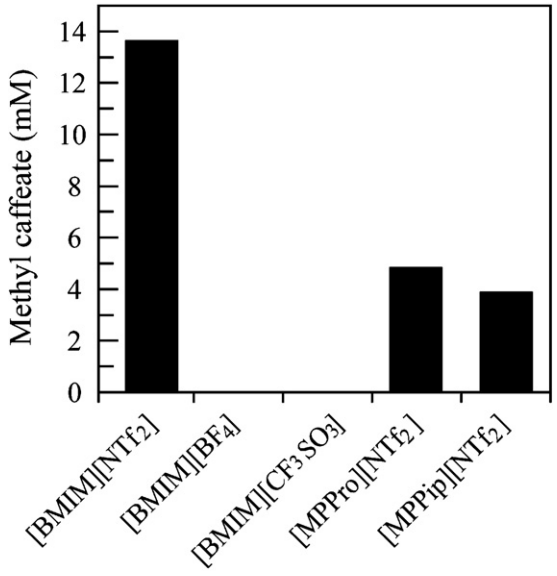


Fig. 4. Selection of IL for chlorogenate hydrolase. The reaction was performed according for the standard method described in Section 2.

$[BF_4]$ and $[CF_3SO_3]$ anions are more nucleophilic than the $[NTf_2]$ anion, and that the $[BF_4]$ and $[CF_3SO_3]$ anions coordinate more strongly to positively charged sites in the structure of an enzyme. In consequence, the enzyme is deactivated by a conformation change in the enzyme structure due to these anions. As shown Fig. 4, chlorogenate hydrolase was deactivated in $[BMIM][BF_4]$ and $[BMIM][CF_3SO_3]$. Additionally, the ability to decrease the enzyme activity was in the order of cations: $[MPPip]^+ > [MPPro]^+ > [BMIM]^+$. In the case of chlorogenate hydrolase, the enzyme activity was affected by the anions and the cations.

Because both chlorogenate hydrolase and Novozyme435 were active in $[BMIM][NTf_2]$, it is suitable for the consecutive conversion of 5-caffeoylquinic acid to 3-cyclohexylpropyl caffeate *via* methyl caffeate.

3.4. Effect of temperature

The effect of temperature on the activity of chlorogenate hydrolase for alcoholysis reaction with 5-caffeoylquinic acid and methanol was examined in $[BMIM][NTf_2]$ at temperatures from 20 to 100 °C (Fig. 5). With increased temperature, the amounts of the product initially increased. However, at higher than 40 °C, the amounts of the product decreased, indicating that 40 °C is the optimum temperature for chlorogenate hydrolase. Additionally, the enzyme is deactivated at temperatures higher than 80 °C. In contrast, we previously showed that the optimum temperature of Novozyme435 is 80 °C [10].

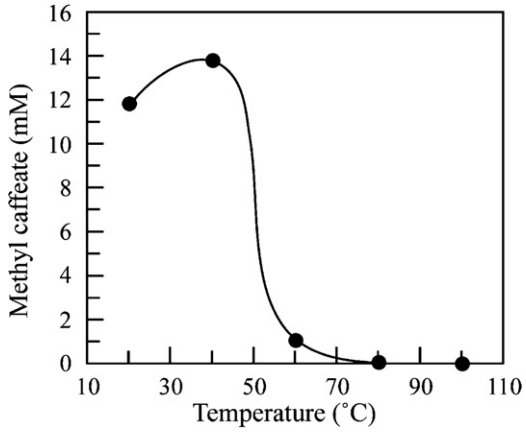


Fig. 5. Effect of temperature on conversion of 5-caffeoylquinic acid to methyl caffeate by chlorogenate hydrolase. The reaction was performed according to the standard method described in Section 2.

3.5. Effect of substrate concentration

For determination of the optimum concentrations of 5-caffeoylquinic acid and methanol, the conversion yields of methyl caffeate toward 5-caffeoylquinic acid were measured (Fig. 6). Fig. 6A shows the conversion yields of 5–30 mM 5-caffeoylquinic acid and 2200 mM methanol in $[BMIM][NTf_2]$. When the 5-caffeoylquinic acid concentration was 15 mM or lower, about 85% conversion yield was observed. However, the conversion yield decreased at concentrations of 5-caffeoylquinic acid greater than 15 mM. When the methanol concentration was 2200 mM or less, the conversion yield was markedly increased. However, the conversion yield was decreased at concentrations more than 2200 mM. High concentrations of 5-caffeoylquinic acid and methanol possibly have an inhibitory effect on chlorogenate hydrolase. Our results indicated that 15 mM 5-caffeoylquinic acid and 2200 mM methanol were suitable for the enzyme reaction.

3.6. Substrate specificity

We examined the production of methyl caffeate with alcoholysis reaction by chlorogenate hydrolase in $[BMIM][NTf_2]$. Methyl caffeate were produced using various 15 mM caffeoylquinic acids (15 μmol) and 2200 mM methanol (2200 μmol). Using 3-caffeoylquinic acid, 4-caffeoylquinic acid, 5-caffeoylquinic acid, 3,5-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid, methyl caffeate was synthesized at concentrations of 9.0 mM (9.0 μmol), 9.2 mM (9.2 μmol), 12.9 mM (12.9 μmol), 13.9 mM (13.9 μmol), and 17.1 mM (17.1 μmol), respectively. Because dicaffeoylquinic acid and caffeoylquinic acid have two and one caffeoyl groups, respectively, the volume of methyl caffeate prepared from 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid were greater than that of methyl caffeate prepared from 3-caffeoylquinic acid, 4-caffeoylquinic acid, and 5-caffeoylquinic acid. In the cases of 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid, both caffeoyl groups would be used for synthesis of methyl caffeate. When 3-caffeoylquinic acid, 4-caffeoylquinic acid, 5-caffeoylquinic acid, 3,5-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid were used as the substrate, the conversion yields of methyl caffeate to each substrate were 60%, 61.3%, 86.0%, 92.7%, and 114%, respectively. Additionally, using a mixture of 3,4-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid, which is a crude fraction prepared from coffee beans, HPLC analysis showed that all peaks of caffeoylquinic acids disappeared and that the peak of methyl caffeate occurred after a 4-h reaction with chlorogenate hydrolase in $[BMIM][NTf_2]$. Chlorogenate hydrolase acted on caffeoylquinic acids and dicaffeoylquinic acids. Thus, using this procedure, methyl caffeate was produced from various caffeoylquinic acids prepared from coffee beans.

3.7. Effect of methanol on transesterification by Novozyme435

During conversion of 5-caffeoylquinic acid with methanol, the reaction mixture probably contained unreacted methanol. Additionally, when methyl caffeate prepared from caffeoylquinic acids was used as the acyl donor to produce 3-cyclohexylpropyl caffeate, methanol was produced as a byproduct. In the conversion of methyl caffeate to 3-cyclohexylpropyl caffeate in the consecutive synthesis, therefore, the reverse reaction with methanol would occur easily to decrease the amount of the desired product. We tested the

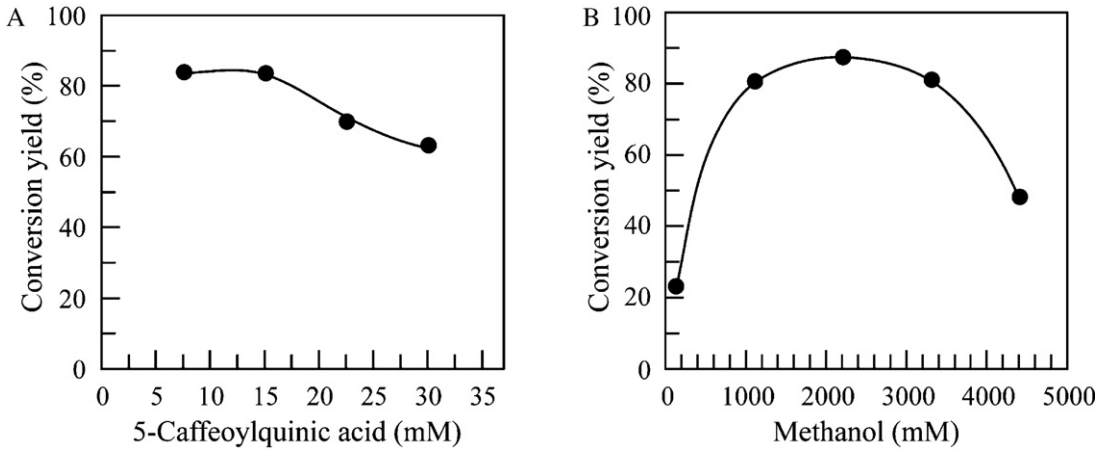


Fig. 6. Effects of 5-caffeoylquinic acid (A) and methanol (B) concentrations on conversion yields of methyl caffeate. The reaction with chlorogenate hydrolase was performed according to the standard method described in Section 2, except that the reaction was carried out using 7.5–30 mM 5-caffeoylquinic acid (A) and 120–4400 mM methanol (B).

effect of methanol on Novozyme435, and observed that methanol had an inhibitory effect on the production of 3-cyclohexylpropyl caffeate (Fig. 7). For development of the two-step procedure, we attempted to avoid this difficulty by removing the unreacted methanol *in vacuo* (14 hPa) and the transesterification of methyl caffeate by Novozyme435 under reduced pressure (845 hPa), so that methanol could be removed immediately from the reaction mixture [25].

3.8. Consecutive conversion of 5-caffeoylquinic acid with chlorogenate hydrolase and Novozyme435

For the development of a convenient procedure for synthesis of 3-cyclohexylpropyl caffeate from caffeoylquinic acids *via* methyl caffeate, we tested the consecutive enzymatic reactions by chlorogenate hydrolase and Novozyme435 using [BMIM][NTf_2] as the solvent (Fig. 8). In the one-pot synthesis, methyl caffeate was first prepared from 5-caffeoylquinic acid by a 4-h reaction at 40 °C. Next, the unreacted methanol was removed under reduced pressure (14 hPa) for 1 h at 80 °C, and chlorogenate hydrolase was deactivated at 80 °C. Then, 3-cyclohexyl-1-propanol and Novozyme435 were added to the reaction mixture. The transesterification of methyl caffeate to 3-cyclohexylpropyl caffeate was performed under reduced pressure (845 hPa) for removal of methanol as the by-product. In the one-pot two-step synthesis, 15 mM 5-caffeoylquinic acid (15 μmol) was converted, *via* 13.1 mM methyl caffeate (13.1 μmol, 87.3%), to 12.8 mM 3-cyclohexylpropyl caffeate (12.8 μmol, 97.7%). The conversion yield of 3-cyclohexylpropyl caffeate toward 5-caffeoylquinic acid was

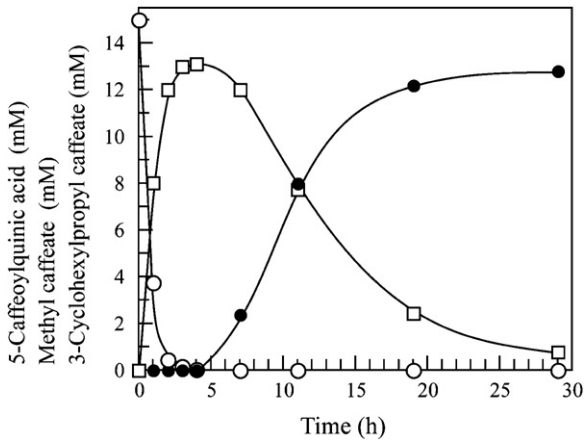


Fig. 8. Consecutive reactions for conversion of 5-caffeoylquinic acid to 3-cyclohexylpropyl caffeate *via* methyl caffeate. The reaction conditions are described in Section 2. The symbols indicate 5-caffeoylquinic acid (open circle), methyl caffeate (open square), and 3-cyclohexylproyl caffeate (closed circle).

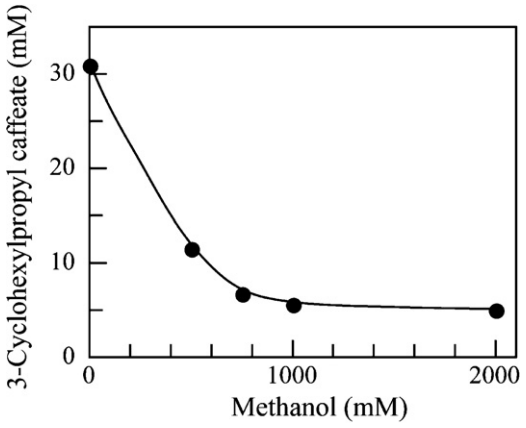


Fig. 7. Effect of methanol on conversion of methyl caffeate to 3-cyclohexylpropyl caffeate by Novozyme435. Transesterification of methyl caffeate was performed at 40 °C for 24 h with shaking in a 1-ml reaction mixture consisting of 50 mM methyl caffeate, 400 mM 3-cyclohexyl-1-propanol, 120,000 U of Novozyme435, 0–2200 mM methanol, and [BMIM][NTf_2] as the reaction solvent. After the reaction, aliquots of the reaction mixture were removed, and the 3-cyclohexylpropyl caffeate produced was quantified by HPLC analysis as described in Section 2.

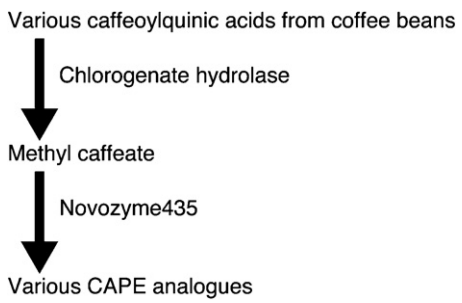


Fig. 9. One-pot two-step synthesis of various CAPE analogues from various caffeoylquinic acids prepared from coffee beans *via* methyl caffeate. Using chlorogenate hydrolase, Novozyme435, and [BMIM][NTf_2] as the reaction solvent, caffeoylquinic acids from coffee beans were converted to various CAPE analogues, which have antimicrobial, anti-inflammatory, antioxidant, and antitumor activities.

85.3%. In the synthesis of CAPE analogues, the conversion yield of 3-cyclohexylpropyl caffeate toward 5-caffeoylquinic acid (85.3%) was superior to that of CAPE toward 5-caffeoylquinic acid (50%) [11]. CAPE analogues were obtained by single transesterification systems catalyzed by Novozyme435 using [BMIM][NTf_2] or isooctane as the solvent with conversion yields of 93.8% and 91.65%, respectively [10,26]. The conversion yield of 3-cyclohexylpropyl caffeate produced by the consecutive conversion system was comparable to those of CAPE produced by single transesterification systems.

4. Conclusion

We developed a convenient procedure for conversion of 5-caffeoylquinic acid to 3-cyclohexylpropyl caffeate, which exhibits an antiproliferative activity toward various human tumor cells [10]. The procedure uses [BMIM][NTf_2] as the reaction solvent, and is composed of consecutive reactions catalyzed by chlorogenate hydrolase and Novozyme435 (Fig. 1). In this study, we showed that chlorogenate hydrolase acts on the caffeoylquinic acids in coffee beans, namely, 5-caffeoylquinic acid, 4-caffeoylquinic acid, 3-caffeoylquinic acid, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid, to produce methyl caffeate in [BMIM][NTf_2]. Additionally, Novozyme435 catalyzed the conversion of methyl caffeate to various CAPE analogues [10]. The procedure developed in this study may be useful for the exploitation of immature coffee beans that contain various caffeoylquinic acids (Fig. 9).

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