Efficient enzymatic modification of epigallocatechin gallate in ionic liquids

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ABSTRACT

Epigallocatechin gallate (EGCG), the main polyphenolic substance in tea, exhibits well-known biological benefits. In order to improve fat solubility and bioavailability, a novel path for the lipase enzymatic transesterification synthesis of acylated EGCG derivatives in an ionic liquid solvent was established. The optimal reaction parameters were determined and a maximum conversion of the transesterification reaction was achieved at 98.65%. [Bmim][BF\textsubscript{4}] was the best reaction medium and the immobilized lipase Novozym 435 was the best catalyst. The enzyme was added to a final concentration of 2% (w/w, EGCG), and the reaction was performed at an optimum temperature of 70°C stirring for 10 h at 250 rpm. The most suitable acyl donor, vinyl acetate, and EGCG were mixed at a molar ratio of 90:1 for the reaction. The structure of the purified acetylated EGCG was determined to be 5\textsuperscript{′′}-O-acetyl-EGCG and 3\textsuperscript{′},5\textsuperscript{′′}-2-O-acetyl-EGCG by mass spectrometry, NMR, and infrared analyses.

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1. Introduction

Epigallocatechin gallate (EGCG), which is mainly derived from green tea, has exhibited excellent antioxidant, anti-diabetic by activated protein kinase (1, 2), anti-cancer (3), anti-virus (4), and anti-angiogenesis (5) activities, which has been widely confirmed. However, its low fat solubility and instability under neutral or alkaline conditions have limited its use in lipid foods and efficient use in vivo. To overcome this limitation, various types of fat-soluble molecular modifications have been made to EGCG, including methylation, esterification, and acylation of phenolic hydroxyl groups. The bioavailabilities of these EGCG derivatives were significantly improved. In some cases, the derivatives showed stronger biological activity than that of natural EGCG. Kazuaki et al (6) showed that palmitoylated EGCG effectively decreased cancer cell growth and inhibited tumor growth in vivo by inhibiting epidermal growth factor receptor (EGFR) activation. In addition, Lam et al (7) showed that the stability of peracetylated EGCG was better than that of unmodified EGCG, and its inhibition activity of the proteasome and apoptosis of MCF7 breast cancer cells in leukemia cell lines was also stronger. Introduction of an alkyl group at the 4\textsuperscript{′′}-O position of AcEGCG significantly increased the biological activity (8).

For the synthesis of acylated EGCG, early methods were based on long-chain fatty acids for EGCG phenolic hydroxyl modification (9, 10), improving the fat solubility to some extent, but the modified products easily aggregated and precipitated with poor stability. The use of short-chain alkanes as acyl donors allows for the synthesis of more stable and higher-yield acylated EGCGs.
in non-aqueous systems. Lam et al. treated EGCG with acetic anhydride catalyzed by pyridine to synthesize acetylated EGCG obtaining derivatives with a conversion of 82%, and this method has been widely used (8, 11). Mori (12) and Zhu (13) obtained acylated derivatives by lipase catalyzed reactions in dimethylformamide (DMF) or acetonitrile, with conversions of 39% and 87.37%, respectively. This generally lower conversion should attract attention for improvement efforts. In addition, the toxic and volatile organic reagents used during the synthesis and separation process are extremely harmful to the environment, and certain restrictions have been placed on their application in the food industry.

Therefore, ionic liquids (ILs) were applied for the synthesis of EGCG derivatives, which has rarely been reported. Higher conversion and more stable product could be obtained under the catalysis of lipase. Applying ILs to the synthesis of some phenolic derivatives has also been reported. For instance, Pang et al. (14) used the immobilized lipase Novozym 435 to catalyze the transesterification of methyl caffeate and propanol, achieving a high conversion of propyl caffeate (98.5 ± 3.24%) in [Bmim][CF3SO3].

As a novel green solvent, ILs exhibit many excellent properties including thermal stability, low vapor pressure, low melting point, non-volatility, and reusability (15). However, the efficiency of reaction could still be improved and reaction period shortened. In addition, enzymatic synthesis is often more effective in ILs. Studies have shown that lipases are not easily deactivated in ILs, even though they are similar in polarity to certain lower alcohols, resulting in higher viability, stability, and selectivity (16).

This paper focuses on the enzymatic acylation using lipase of EGCG in an IL system, providing a feasible method for optimizing these homologous reactions. Different environmentally friendly ILs and immobilized lipases were selected. The optimum conditions for the synthesis of acylated EGCG were determined by investigating the effects of different transesterification conditions, including reaction time, temperature, substrate molar ratio, enzyme loading, acyl donor, and agitation rate. Furthermore, structural analysis of the purified acetylated EGCG was performed to determine the specific acylation site.

2. Materials and methods

2.1. Materials

EGCG (purity >99%) was obtained from Hangzhou Hetian Biotechnology Co., Ltd (Hangzhou, China). The ILs used herein were purchased from the Lanzhou Institute of Material Chemistry, including 1-ethyl-3-methylimidazolium tetrafluoroborate ([Emim][BF4], >99%), 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim][BF4], >99%), 1-octyl-3-methylimidazolium tetrafluoroborate ([Omim][BF4], >99%), 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim][PF6], >99%), 1-butyl-3-methylimidazolium hydrogen sulfate ([Bmim] [HSO4], >99%). Immobilized Candida Antarctica lipase (Novozym 435), Rhizomucor miehei lipase (Lipzyme RM), and Thermomyces lanuginosus lipase (Lipzyme TLIM) were obtained as gifts from Novozymes (Bagsvaed, Denmark). Vinyl acetate (AR, >99%) was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Vinyl butyrate (purity >99%), vinyl octylate (purity >99%), vinyl laurate (purity >99%), and vinyl stearate (purity >99%) were purchased from Tokyo Chemical Industry Ltd. (Tokyo, Japan).

2.2. Lipase-catalyzed synthesis of EGCG derivatives in ILs

The lipase-catalyzed synthesis reaction of EGCG with vinyl acetate in ILs was performed in 25 mL screw caps at 50°C. The reaction substrates consisted of 0.218 mM EGCG and 10.9 mM vinyl acetate sequentially dissolved in 5 mL of the IL, which were continuously magnetically stirred at 300 rpm with 5% (w/w, based on EGCG) Novozym 435 for 12 h. After incubation, the lipase was removed via filtration through a 0.45 μm nylon membrane filter (Millipore, Bedford, MA, USA), and 20 μL of the reaction solution was added to an EP tube and diluted with 980 μL of methanol.

The conversion of EGCG was calculated using the following equation

\[
\text{Conversion (\%)} = \frac{A_0 - A_1}{A_0} \times 100
\]

where \(A_0\) is the content of EGCG before the reaction in μg/mL and \(A_1\) is the content of EGCG at the time of sampling in μg/mL.

2.3. HPLC analysis

The formed product was quantitatively analyzed via high performance liquid chromatography (HPLC) and the sample after the pre-reaction analysis was filtered using a 0.45 μm filter. The sample was removed for HPLC analysis with 0.5 mg/mL EGCG (dissolved in 50% methanol solution) as a standard.

HPLC was performed using an Agilent 1260 system (Santa Clara, CA, USA) with a constant pump and UV
detector. The reaction mixture was separated using a C18 column (4.6 mm × 250 mm, i.d.; 5 μm, Waters, USA) with automatic injection of 5 μL and was continuously analyzed for 35 min. The elution was performed using mobile phase A (methanol/water/phosphate, 5:95:0.05, v/v/v) and B (methanol/water/phosphate, 65:35:0.05, v/v/v) at a flow rate of 0.8 mL/min at 30°C at a detection wavelength of 280 nm. The experiments were performed in triplicate in parallel.

2.4. Structural determination

To obtain the substituted product of an individual acetylated EGCG, the reaction mixture was separated by Waters 2545 series preparatory HPLC (Milford, MA, USA) with a UV detector at 280 nm. The operating conditions were as follows: separation column, Waters XBridge C18 column (19 mm × 250 mm, i.d.; 10 μm); mobile phase A, methanol/water/formic acid (5:95:0.1, v/v/v); mobile phase B, methanol/water/formic acid (80:20:0.1, v/v/v); gradient elution procedure, 0–20 min, 40–50% B, 20–30 min, 50–80% B, 30–35 min, 80–40% B; flow rate, 10 mL/min. The organic solvent was removed by rotary evaporation and lyophilizing yielded powdery acetylated EGCG, which was then subjected to structural identification.

2.4.1. Infrared spectroscopy

Using a Fourier transform infrared (FTIR) spectrometer (Nicolet Nexus 470, Thermo, USA), the FTIR spectra of EGCG derivatives were obtained at a resolution of 2 cm⁻¹, scanning from 400 to 4000 cm⁻¹ 32 times. The sample was finely ground with pure KBr in an agate mortar, then pressed into a transparent sheet using a hydraulic press with a pressure of 5-10 MPa.

2.4.2. Analysis by LC-MS

The composition of the mixed EGCG derivatives was characterized by Waters Synapt HDMS system (Milford, MA, USA). Analytical separation was performed using a BEH C18 column (2.1 mm × 100 mm, i.d.; 1.7 μm, Waters) at 30°C using mobile phase A (water/formic acid, 99.9:0.1, v/v) and mobile phase B (acetoni trilate) at a flow rate of 0.3 mL/min. The gradient elution procedure was conducted with 5–40% (v/v) B from 0 to 10 min, and then from 40% to 100% (v/v) B at 10–15 min, until ending with 5% (v/v) B at 20 min. The UV detection wavelength was 280 nm and the MS conditions were as follows: ion mode, ESI⁻; capillary voltage, 3.5 kV; cone voltage, 45 V; ion source temperature, 100°C; solvent degassing temperature, 300°C; collision energy, 6 eV; mass range, 50–2000m/z.

2.4.3. NMR

Further structural identification of specific acylation sites of EGCG derivatives were analyzed in CD₃OD, as a vintage solvent for EGCG derivatives, and were confirmed by ¹H-NMR and ¹³C-NMR spectroscopy.

2.5. Statistical analysis

Statistical analysis of the enzymatic transesterification optimization was performed using a one-way analysis of variance (ANOVA) with the Microsoft Excel program. All experimental results were obtained in triplicate and the data are represented as mean ± SD. P values less than 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Selection of optimal ILs for transesterification

ILs are formed by the integration of various inorganic anions and organic cations. The combination of different ions seriously affects the resulting physical and chemical properties, including the solubility of ILs in the matrix, anion hydrogen bond basicity, hydrophobicity, and viscosity (17). Therefore, a combination of a typical mononuclear anion and an imidazolium cation was used as the reaction solvent. From Figure 1, Novozym 435 exhibited the highest conversion of EGCG (42.10 ± 1.45%) in the IL containing [BF₄]⁻ compared to that in the ILs containing [PF₆]⁻ and [HSO₄]⁻. In general, Novozym 435 generally achieved higher conversion in [Bmim][PF₆] than that in [Bmim][BF₄], similar to the enzymatic synthesis of phenethyl catechol analogs (18) and pyridine nucleosides via transesterification of glycosides (19). However, EGCG was almost
insoluble in [Bmim][PF₆] and the reaction system formed a milky white suspension, which was attributed to strong hydrophobicity of [Bmim][PF₆] where the reaction failed. Additionally, [Bmim][BF₄] and [Bmim][PF₆] with the same alkyl carbon chain cations exhibited similar E₄ values (0.670 and 0.669, respectively), but the anion polarity cannot explain the poorly soluble of EGCG in the ILs (20, 21). With its strong acidity, [HSO₄]⁻ was irreversibly destructive to the structure of lipase and EGCG, resulting in a black viscous reaction system.

Hydrogen bond basicity can affect enzyme activities (16, 22). Stronger hydrogen bond basicity of the anion results in easier formation of hydrogen bonds with the enzyme, resulting in destroying the structure of the enzyme. Studies have shown that enzymes exhibit better stability in anions containing low hydrogen bond basicities (23). Compared to other types of anions in ILs, [Bmim][BF₄] showed low hydrogen bond basicity without strong interaction with water, ensuring optimal hydration of the lipase. Therefore, for the enzymatic transesterification of EGCG, it was suitable to select an IL containing [BF₄]⁻ as a reaction medium.

In addition, higher viscosity can retard the conformational transition of protein, maintaining the enzyme structure and preserving its activity (16). In contrast, the viscosity highly affects the rate of mass transfer by increasing mass transfer resistance. From Figure 2, it is clear that as the alkyl carbon chain of the cation increased, the conversion of EGCG increased initially and subsequently decreased. Despite its short chain length and viscosity, the conversion of EGCG in [Emim][BF₄] was still lower than that achieved in [Bmim][BF₄], likely because [Emim]⁺ has a higher coordination number than that of [Bmim]⁺ (24). The transmission characteristics in ILs are related to the ion coordination number (25). Larger coordination number often leads to more anions coordinating with Lys-290 of the lipase structure, resulting in partial deactivation of the enzyme. In [Omim][BF₄], the high hydrophobicity could lead to a significant loss of secondary structure of the α-10 helix and exposure of the catalytic triad to the ILs (24). Thus, high hydrophobicity and high viscosity caused by the long alkyl chain length synergistically inhibit the enzymatic activity and increased the mass transfer resistance, resulting in a much lower conversion of EGCG in [Omim][BF₄] compared to that in [Emim][BF₄] or [Bmim][BF₄]. Therefore, [Bmim][BF₄] was selected as the reaction solvent for subsequent reaction optimization.

### 3.2. Selection of optimal lipase

Lipase exhibits good stability in ILs and shows higher activity and stereoselectivity after immobilization (26). Therefore, Novozym 435, Lipozyme RM, and Lipozyme TL IM were studied for their influence on enzymatic synthesis of acetylated EGCG. Comparison of catalytic performance of the three lipases with the same transesterase activity was shown in Figure 3. The conversion of EGCG catalyzed by Lipozyme RM and Novozym 435 after 10 h were 53.95% and 54.88%, respectively, compared to 46.19% for Lipozyme TL IM. The catalytic performance of Novozym 435 was higher than the other lipases evaluated. Pan et al. also reported that Novozym 435 had better catalytic properties in ILs compared with lipase RM (27). In addition, we found that the

![Figure 2](image2.png) **Figure 2.** Effects of ILs with different alkyl chain lengths on the conversion of EGCG with vinyl acetate catalyzed by Novozym 435. Experimental parameters: EGCG to vinyl acetate ratio of 1:50 (mol/mol); lipase load of 5% based on EGCG (mg); esterification performed in 25 mL screw caps under magnetic stirring at 300 rpm at 50°C.

![Figure 3](image3.png) **Figure 3.** Effects of the immobilized lipase screening on the conversion of EGCG with vinyl acetate catalyzed by Novozym 435, Lipozyme RM, and Lipozyme TL IM. Experimental parameters: EGCG to vinyl acetate ratio of 1:50 (mol/mol); lipase load of 5% based on EGCG (mg); esterification performed in 25 mL screw caps under magnetic stirring at 300 rpm at 50°C.
EGCG conversion gradually increased with the extension of reaction time, and reached the maximum value at 10 h (Figure 3). After 10 h, the conversion exhibited a downward trend. This may be due to product accumulation that balanced the acylation reaction in the opposite direction of EGCG acylation product formation, therefore decreasing the conversion with an extended reaction time. Considering the conversion of EGCG and catalytic effects, the lipase Novozym 435 was selected as the optimal enzyme.

### 3.3. Selection of the optimal acyl donor

The optimal reaction substrate was determined using vinyl esters of different chain lengths as acyl donors. Vinyl acetate, vinyl butyrate, vinyl octanoate, and vinyl laurate were used for the enzymatic acylation of EGCG in [Bmim][BF₄]. Figure 4 shows that short chain substituted derivatives can achieve higher conversion compared to medium and long chain substitutions. Salem et al. (28) showed that the conversion of isoquercitrin-ethylated derivatives gradually decreased with extension of the substituent carbon chain. Katsoura et al. (29) reported similar results for free fatty acids and vinyl esters in ILs. Studies of acylation of naringin and rutin with CALB lipase showed that higher conversion (approximately 65%) was observed for short chain acyl donors. Long chain acyl donors exhibited low substitution capacity, likely due to the large nonlinear steric hindrance caused by the nonlinear molecular bending. The spatial collision of the long chain acyl groups is detrimental to the formation of the transition state product during the enzymatic reaction (10, 30). From Figure 4, we also found that for the influence of medium or long chain acyl donors on acylation reaction, with that increase of carbon chain, the conversion first increased and then decreased, which may be due to the low solubility of medium or long chain acyl donors in ILs, forming two phase system, with more products gathered in the upper layer, and the possibility of EGCG contacting with acyl donors increased, thus promoting the reaction. Similar to our experimental results, in the acylation reaction with naringin as substrate, the conversion of naringin first increased and then decreased when the carbon chain was increased from C8 to C12 (31). Therefore, short chain vinyl esters are more favorable for the enzymatic acylation substitution reaction of EGCG.

### 3.4. Parameter analysis of the transesterification synthesis procedure

Temperature is important for enzymatic reactions in terms of enzyme stability and reaction kinetics. The reaction was performed at 30, 40, 50, 60, 70, and 80°C and the results are shown in Figure 5(a), demonstrating a dependence of the conversion on temperature. The conversion of EGCG reached a maximum (85.49 ± 0.66%) at 70°C because increasing temperature causes the IL viscosity to decrease, which is beneficial for mass transfer. On the other hand, temperature is directly related to enzyme activity, which affects the enzymatic reaction. Meanwhile, immobilization of the lipase Novozym 435 improved thermal stability, as was observed previously for many other reactions (32, 33). Figure 5(a) also shows that almost no conversion occurred at 80°C, but Novozym 435 was not deactivated even at 80°C (27, 34). The declining conversion can be ascribed to the volatile absence of vinyl acetate. Therefore, 70°C was determined to be the optimum reaction temperature.

Figure 5(b) shows the dependence between the amount of enzyme loading and EGCG conversion at 70°C in [Bmim][BF₄]. As the catalyst load was changed between 1% and 2%, the conversion of EGCG slightly improved. However, when the enzyme loading exceeded 2%, the conversion decreased gradually. These results indicated that within a certain range of lipase concentration, higher enzyme loading can cause faster reaction rates. Previous studies have reported that the aggregation of immobilized enzyme in a solvent-free system increased the viscosity in the system and reduced the efficiency of substrate transfer to the enzyme active site (35, 36). This may explain the sharp decline after exceeding 2% enzyme loading. Therefore, the optimal enzyme loading was selected as 2%.
Figure 5(c) shows the effect of the varied molar ratio of substrates on enzymatic synthesis of acetylated EGCG at 70°C. This indicated that increased amounts of vinyl acetate resulted in higher conversion of EGCG. With the limited setting interval, a trend toward a lower conversion was not observed, but a reduced rate of growth was clearly found. Excess vinyl acetate addition could increase the overall viscosity of the system and reduce mass transfer, making it more feasible to enter the lipase active site as an acyl donor (37).

Because of the relatively high viscosity of ILs, the effect of stirring speed on the reaction is non-negligible. Keeping the other factors constant, the effect of rotating speed from 100 to 350 rpm on the conversion of EGCG was investigated. Figure 5(d) shows that with increasing rotational speed, the conversion of EGCG initially increased and subsequently decreased. At low rotation speeds, the reaction proceeded slowly in terms of mass transfer with a small stirring force. The contact between the substrate and enzyme was insufficient, resulting in a reduced conversion of EGCG. As the rotation speed was increased, the conversion of EGCG gradually increased to a maximum (45.59 ± 3.74%) at 250 rpm. However, excessive agitation failed to result in higher conversion. Some of the enzyme adhered to the bottle wall due to the fast stirring. Thus, the decreased reaction rate was likely due to the removal of enzymes and was independent of mass transfer (38).

3.5. Structural identification of the EGCG derivatives

Herein, we attempted to enhance the fat solubility of EGCG by introducing an acetyl group into a water-soluble EGCG molecule in an environmentally-friendly IL system. The crude reaction product was separated...
into different components by preparative HPLC, identified, and the main components collected were analyzed (named compounds 1, 2, and 3).

The mass spectra of the three compounds obtained by LC-MS are shown in Figure 6. Quasi-molecular ion peaks were obtained via soft ionization in ESI negative ion mode. For compound 1, the quasi-molecular ion peak [M-H]⁻ with m/z 457.0 appeared in mass spectrum (a), corresponding to the EGCG with a relative molecular weight of 458 Da. Therefore, Figure 6(a) was determined to be the mass spectrum of EGCG. The quasi-molecular ion peak at m/z = 169.0 was [M-289]⁻, possibly due to additional cleavage of the epigallocatechin (mass 289) portion.

For compound 2, mass spectrum (b) shows that the highest intensity signal peak of the quasi-molecular ion peak [M-H]⁻ with m/z = 499.0. This was assigned to one hydroxyl group in the EGCG molecule being substituted with an acetyl group, and the compound was identified as a monoacylated EGCG derivative molecule (C_{24}H_{20}O_{12}). The m/z = 457.0 and 211.0 peaks observed in mass spectrum (b) correspond respectively to the loss of one acetyl group in the monosubstituted EGCG molecule and the quasi-molecular ion peak formed after additional cleavage inside the molecule ([M-42]⁻ and [M-289]⁻). The peak at m/z = 441 indicates that the monosubstituted EGCG molecule lost the acetyl group and oxygen atom due to ionization. Similarly, for compound 3, mass spectrum (c) shows a [M-H]⁻ quasi-molecular ion peak with the strongest signal peak at m/z = 541.0 and was attributed to the substitution of two OH groups in EGCG by acetyl groups. Therefore, the

**Figure 6.** Mass spectra corresponding to the three separate products, (a) compound 1, (b) compound 2, and (c) compound 3.
observed at 1810 cm\(^{-1}\) of compounds 2 and 3, two absorption peaks were observed at 2250 cm\(^{-1}\). Absorption vibrations of aromatic rings were also present in the infrared spectrum, compounds 2 and 3 showed a distinct absorption peak at 1700 cm\(^{-1}\), corresponding to the carbonyl group. In addition, compared to the EGCG infrared spectrum, the hydroxyl group of EGCG was substituted by an acetyl group. In addition, compared to the EGCG infrared spectrum, the hydroxyl group of EGCG was substituted by an acetyl group.

In the infrared spectrum shown in Figure 7, it is clear that the three compound spectra exhibited absorption vibrations of unsaturated C–H at 3300–3000 cm\(^{-1}\). Absorption vibrations of aromatic rings were also observed at 2250–1450 cm\(^{-1}\). In addition, it should be noted that in the vibrational range of carbonyl C = O at 1800–1600 cm\(^{-1}\), EGCG exhibited a single peak with strong absorption at 1691 cm\(^{-1}\), corresponding to the carbonyl group in the EGCG structure. In the IR spectrum of compounds 2 and 3, two absorption peaks were observed at 1810–1700 cm\(^{-1}\), indicating that both compounds contained two or more C = O groups. That is, the hydroxyl group of EGCG was substituted by an acetyl group. In addition, compared to the EGCG infrared spectrum, compounds 2 and 3 showed a distinct absorption peak at 2970–2955 cm\(^{-1}\), which is characteristic of methyl (-CH\(_3\)) stretching vibrations in alkanes. No such absorption peak was observed in the molecular structure of EGCG, indicating that no methyl group was present in EGCG. However, the acetyl group contains a methyl group, indicating the successful modification and production of compounds 2 and 3.

In addition to the LC-MS analysis, due to the presence of eight phenolic hydroxyl groups in the EGCG molecule, the specific acylation position must be determined. The substitution sites of the two substitution products were determined by \(^1\)H and \(^{13}\)C NMR comparing the chemical shifts to that of the bulk EGCG molecule. In Table 1, it is clear that most protons were shifted to the low field and EGCG shows a low-field shift of all proton signals upon acylation substitution (10). Compared to the EGCG bulk, the single-substituted EGCG exhibited large low-field chemical shifts (\(\Delta\delta \approx 0.03\)–0.07) of the H-2” and H-6” signals, and minor displacement at other proton sites (\(\Delta\delta \leq 0.03\)). Thus, it can be inferred that an acylation reaction occurred on the B-ring. In addition, the chemical shift difference at the H-6” position was significantly larger than that at H-2”. It was preliminarily determined that the acylation substitution occurred on C-5” of the B-ring. Similarly, for the disubstituted product, the acylation site was assigned to the A-ring because H-2” and H-6” showed a low field shift of \(\Delta\delta \approx 0.09\). Specific substitution sites may also occur at C-3” and C-5” of the A-ring (position H-6, H-8) and D-ring (positions H-2’, H-6’) with small shifts of \(\Delta\delta \approx 0.03\) and \(\Delta\delta \approx 0.01\), respectively, indicating that the A- and D-rings were not acylation sites.

The specific site of acetylation of EGCG was further confirmed by \(^{13}\)C NMR. Hydroxyacylation can displace the carbon signal to a low field and the adjacent carbon to a high field (10). In Table 1, it is clear that for single-substituted derivatives, the chemical shifts of C-3” and C-5” were reduced (\(\Delta\delta \approx 0.43\)) compared to those of the other carbon-substituted sites (\(\Delta\delta \leq 0.07\)), indicating the possibility of substitution at these sites. The absence of the high field shift of the C-4” signal may be due to the influence of the introduced acetyl space structure on the chemical shift. In addition, the chemical shift of C-6” showed a slight trend toward high field (\(\Delta\delta \approx 0.02\)), indicating that substitution likely occurred at C-5”.

Thus, the structure of compound 2 (monosubstituted

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**Figure 7.** FT-IR spectra of (a) compound 1, (b) compound 2, and (c) compound 3.

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**Table 1.** \(^1\)H and \(^{13}\)C chemical shifts (\(\delta\)) of EGCG and its derivatives.

| C/H position | Compound 1\(^a\) | Compound 2\(^b\) | Compound 3\(^c\) |
|-------------|-----------------|-----------------|-----------------|
| \(^1\)H      | \(^{13}\)C      | \(^1\)H         | \(^{13}\)C      | \(^1\)H         | \(^{13}\)C      |
| 2           | 77.15           | 4.97            | 77.07           | 4.97            | 76.82           | 4.95            |
| 3           | 68.56           | 5.53            | 68.57           | 5.54            | 68.55           | 5.55            |
| 4           | 25.41           | 2.86            | 25.43           | 2.84            | 25.43           | 2.84            |
|             |                 | 2.96            |                 | 2.91            |                 | 2.91            |
| 5           | 155.77          | 5.96            | 155.49          | 5.97            | 155.49          | 5.99            |
| 6           | 144.88          | 5.96            | 145.31          | 5.97            | 145.31          | 5.99            |
| 7           | 156.39          | 5.96            | 155.83          | 5.97            | 155.83          | 5.99            |
| 8           | 95.22           | 5.96            | 95.15           | 5.97            | 95.15           | 5.99            |
| 9           | 156.39          | 5.96            | 155.91          | 5.97            | 155.91          | 5.99            |
| 10          | 95.06           | 5.96            | 95.04           | 5.97            | 95.04           | 5.99            |
| 1’          | 129.30          | 2.91            | 129.43          | 2.94            | 129.44          | 2.94            |
| 2’          | 105.50          | 6.5             | 105.50          | 6.5             | 105.67          | 6.49            |
| 3’          | 145.24          | 6.5             | 144.90          | 6.5             | 144.89          | 6.49            |
| 4’          | 132.4           | 6.5             | 132.41          | 6.5             | 132.39          | 6.5             |
| 5’          | 145.24          | 6.5             | 144.90          | 6.5             | 144.89          | 6.5             |
| 6’          | 108.90          | 6.92            | 108.88          | 6.95            | 108.89          | 7.01            |
| 1”          | 120.17          | 6.5             | 120.15          | 6.5             | 120.15          | 6.5             |
| 2”          | 144.88          | 6.5             | 145.31          | 6.5             | 145.30          | 6.5             |
| 3”          | 138.39          | 6.5             | 138.39          | 6.5             | 138.39          | 6.5             |
| 4”          | 144.88          | 6.5             | 145.31          | 6.5             | 145.30          | 6.5             |
| 5”          | 108.90          | 6.92            | 108.88          | 6.99            | 108.89          | 7.01            |
| COO         | 166.26          | 6.92            | 166.28          | 6.99            | 166.26          | 6.99            |

Notes: \(^{1}\)H and \(^{13}\)C chemical shifts (\(\delta\)) of EGCG.  
\(^{1}\)H and \(^{13}\)C chemical shifts (\(\delta\)) of the monosubstituted product.  
\(^{1}\)H and \(^{13}\)C chemical shifts (\(\delta\)) of the disubstituted product.
acetylated EGCG) was identified as 5′-O-acetyl-EGCG. For the substituted derivatives, the chemical shifts of the C-3′ and C-5′ signals were also reduced (Δδ 0.43), and the chemical shifts of the C-2′ and C-6′ signals were showed a slight trend toward high field shift (Δδ 0.01). No significant change in the C-spectrum chemical shifts at different positions of the A and B rings (Δδ ≤ 0.06) were observed, indicating that no hydroxyl groups were acylated. This result agrees well with the 1H NMR results. Compound 3 (disubstituted EGCG) was identified as 3′, 5′-O-2-acetyl-EGCG.

4. Conclusions

Herein, we modified the EGCG monomer molecule to determine the optimal conditions for the synthesis of acetylated EGCG in an IL system by analyzing various process parameters, including IL species, immobilized lipase, temperature, molar ratio, lipase loading, and stirring rate. In [Bmim][BF$_4$], EGCG and vinyl acetate were catalyzed by 2% immobilized lipase Novozym 435 at 70°C for 10 h at 250 rpm, achieving a maximum reaction conversion of 98.65%. The presence of mono- and di-acetylated derivatives in acetylated EGCG were identified as 5′-O-acetyl-EGCG and 3′, 5′-O-2-o-acetyl-EGCG, among which the proportion of the monosubstituted product and the disubstituted product was 43.56% and 54.79% respectively.

Disclosure statement

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