Isolation of (−)-Epigallocatechin from Green Tea Extract by Means of Immobilized Tannase

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Abstract
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(−)-Epigallocatechin is a chemoprotective polyphenolic compound found in green tea. Its low abundance in tea increases the cost and difficulty of obtaining sufficient (−)-epigallocatechin to conduct research on (−)-epigallocatechin-mediated health benefits. We developed a convenient and economical method for preparing (−)-epigallocatechin from a commercial green tea extract in a sufficient quantity to carry out mechanistic studies of the pure compound. Crude epigallocatechin gallate was isolated from the tea extract by silica gel column chromatography, and was then hydrolyzed with immobilized tannase. The (−)-epigallocatechin found in the hydrolyzed products was extracted into ethyl acetate and purified by Sephadex LH-20 to produce (−)-epigallocatechin. Very pure (−)-epigallocatechin (93.7%) was obtained and its identity was confirmed by ESI-MS and HPLC. The overall yield was 10% by mass of the initial green tea extract obtained as pure (−)-epigallocatechin. No specialized equipment was required, and the solvents and chromatographic supports used were inexpensive and readily available.

Key words

Camellia sinensis · Theaceae · catechin · flavonoid · polyphenol · antioxidant · tea

Abbreviations
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EGC: (−)-epigallocatechin
EGCg: (−)-epigallocatechin gallate

EGC is a polyphenolic compound commonly found in tea, *Camellia sinensis* L. (Theaceae). Polyphenols including flavonoids may contribute to the health-promoting benefits of plant-based foods and beverages such as tea via bioactivities such as antioxidant activity, protein precipitation, and metal chelation [1–3]. However, identifying the compounds responsible for specific bioactivities has been difficult, in part due to the expense of some purified flavonoids, particularly those like EGC that are minor constituents of complex mixtures in natural sources [4].

Although methods have been developed for isolating EGC directly from tea, two factors limit their application [5]. One is the very low content of EGC in green tea. The other limitation is an instrumental requirement. Although fractions enriched in specific catechins can be prepared by solvent extraction [6], most methods for the purification of EGC use countercurrent chromatography [4,7–9]. This instrumentation is relatively uncommon, and thus many laboratories cannot obtain pure EGC using existing methods. We developed a method for the preparation of EGC that is convenient and economical, and relies only on conventional extractions and chromatography.

We chose to use a commercial green tea extract as a convenient source of EGCg, which can be hydrolyzed to yield the desired product, EGC (○ **Fig. 1**). The green tea extract is rich in EGCg (○ **Fig. 2 a**), and 85% of the original preparation is soluble in ethanol. We used silica gel chromatography to fractionate the ethanol solution, recovering about two-thirds of the initial green tea extract as EGCg (○ **Table 1** and **Fig. 2 b**).

EGCg was hydrolyzed into EGC and gallic acid by immobilized tannase. The reaction was allowed to proceed for 30 min to obtain the maximal amount of EGC. Although some unhydrolyzed EGCg remained in the product, longer hydrolysis resulted in the loss of EGC to undefined side products. By using the enzyme incorporated into alginate beads, it was easy to recover the polyphenols from the enzyme by ethyl acetate extraction (○ **Fig. 2 c**). The ethyl acetate extraction was important for EGC enrichment, enhancing the purity 4.5-fold compared to samples that were not extracted. During the hydrolysis, some tannase beads appeared to dissolve in the reaction buffer, diminishing the purity of the product. Ethyl acetate extraction helps separate the desired product from the very water-soluble alginates that comprise the tannase beads [10]. In addition, the solubility of gallic acid is much higher in water than in ethyl acetate [11], so extraction in ethyl acetate helped remove the undesirable product gallic acid from the EGC. About half of the EGCg was recovered as EGC after the hydrolysis and ethyl acetate fractionation (○ **Table 1**).

EGC was further purified by Sephadex LH-20 chromatography, using slightly acidified aqueous ethanol as the mobile phase to stabilize EGC [12]. Three peaks eluted from the column, and were identified by their retention times on HPLC and their characteristic UV-visible features (○ **Fig. 3**). About 10% of the starting crude green tea extract was recovered as EGC (○ **Table 1**).

The purity of the isolated EGC was 93.7% based on HPLC analysis (○ **Fig. 2 d**). The HPLC retention time (t_R 19.6 min) for our purified compound was identical to authentic EGC (Sigma-Aldrich). The UV spectrum of the EGC peak has a high A_{220} to A_{280} ratio that is quite distinct from the UV spectrum of GA or EGCg (○ **Fig. 2**). The characteristic molecular ion peak (m/z 305) is the dominant peak in the mass spectrum (○ **Fig. 4**). A peak at m/z 611 was an EGC dimer based on the clear anion signal for EGC (m/z 305) in the MS/MS spectrum. Both the MS spectrum (○ **Fig. 4**) and the MS/MS spectrum of the parent ion matched that of an authentic standard [13]. No signal was detected for EGCg (m/z 458) or gallic acid
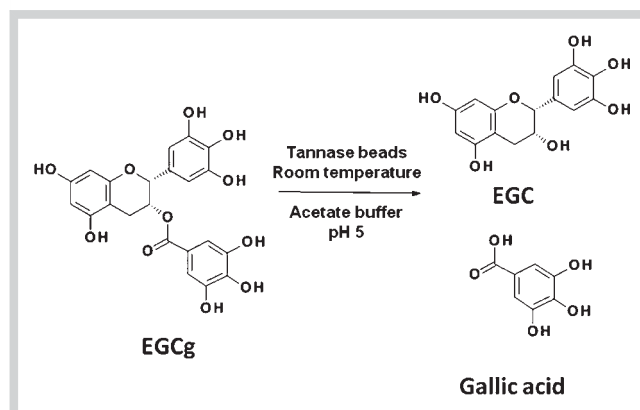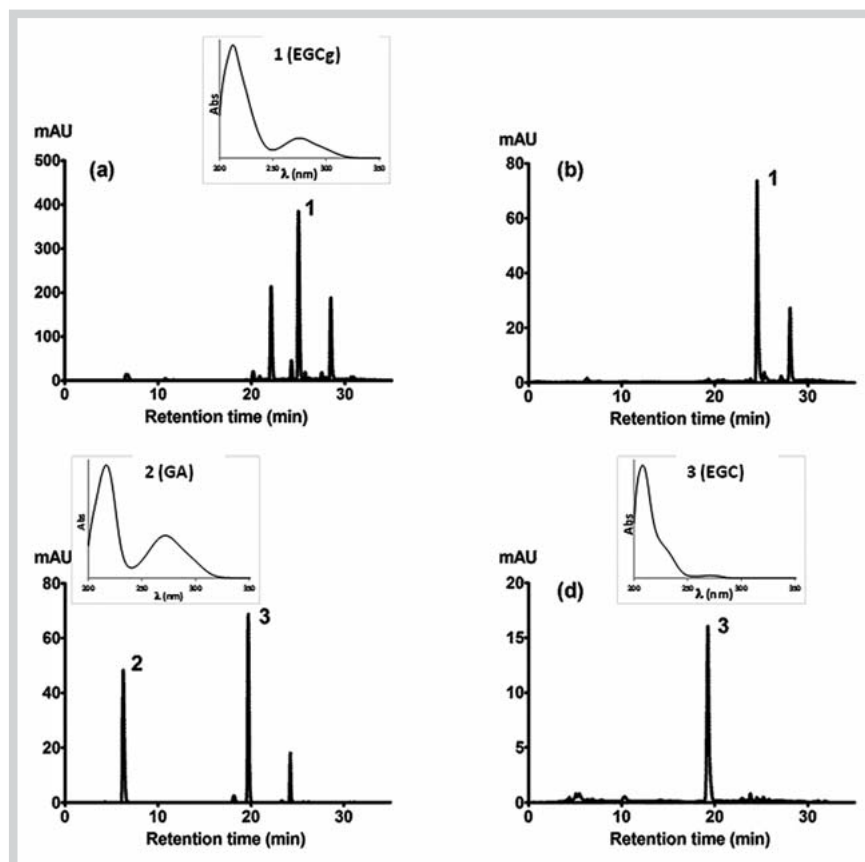

Fig. 1 Structural formulas of EGCg and its hydrolysis products EGC and gallic acid.

Fig. 2 HPLC chromatogram (280 nm) of products at each step of the purification. Commercial green tea extract (**a**), products from the silica gel column (**b**), hydrolysis products (**c**), and purified EGC from the Sephadex LH-20 column (**d**). The spectrum of each peak collected with the diode array detector on the HPLC is shown as an inset to the appropriate chromatogram. Peak 1 is EGCg, peak 2 is gallic acid, and peak 3 is EGC.

**Table 1** Yield and purity of samples from each preparation step.

	EGCg from silica gel	EGC in hydrolysis products	EGC from Sephadex LH-20
Yield[a] (%)	70 ± 12	32.9 ± 2.7	10.4 ± 1.0
Purity (%)	29.3 ± 1.3	42.4 ± 7.9	93.7 ± 1.7

[a] Yield is calculated by mass compared to the starting mass of the crude green tea extract

(m/z 169), which is in agreement with the HPLC data (○ **Fig. 2 d**). In conclusion, we have developed a convenient method for purifying the bioactive flavan-3-ol EGC from a commercial green tea extract. We have used the method to provide sufficient material for bioactivity and mechanistic testing [14]. The method could be scaled up if EGC is found to have bioactivities or properties that warrant production on a larger scale.

Materials and Methods
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Green tea (*C. sinensis*) extract was kindly donated by Silvateam. The EGCg standard and tannase powder were provided by Lipton. The EGC standard and Sephadex LH20 were purchased from Sigma-Aldrich. Whatman filter paper (#40), silica gel (0.060–0.200 mm, 60 Å), and all other chemicals (analytical grade) were purchased from Fisher Scientific. If not otherwise specified, all experiments were performed at room temperature.

Immobilized tannase [15] was prepared by mixing tannase (25 mL, 0.1 mg protein/mL) with 67 mL of 3 % aqueous sodium alginate by magnetic stirring for 30 min and dripping the solution into 1 L of 2 % CaCl₂ with a syringe. Once formed, tannase-con-
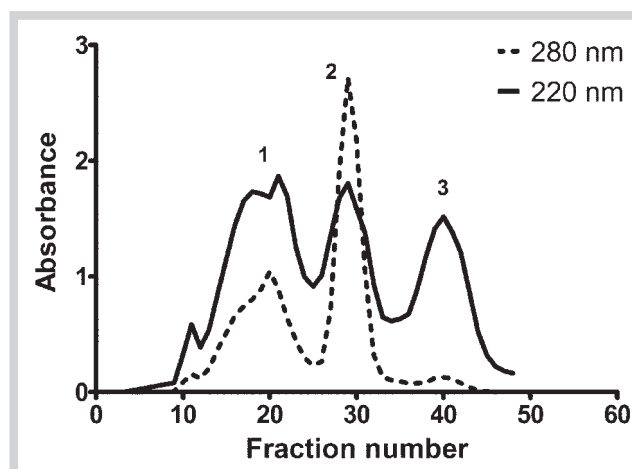
Fig. 3 Isolation and purification of EGC with Sephadex LH-20 chromatography. The absorbance of each sample was measured at both 220 nm and 280 nm, and the data were plotted against the fraction number. Peak 1 is gallic acid, peak 2 is EGCg, and peak 3 is EGC.

taining beads were transferred to 1 L of fresh 2 % CaCl₂ solution for 1 h of hardening and were then cross-linked in 1 L of 0.025 % glutaraldehyde for 2.5 h. After incubation, unreacted glutaraldehyde was removed by rinsing the tannase beads twice with 500 mL of 50 mM sodium acetate buffer, pH 5.0, before storing the beads in that buffer at 4 °C.

The powdered, dark green crude tea extract (100 mg) was mixed with 5 mL of ethanol with rotation for 10 min before centrifuging
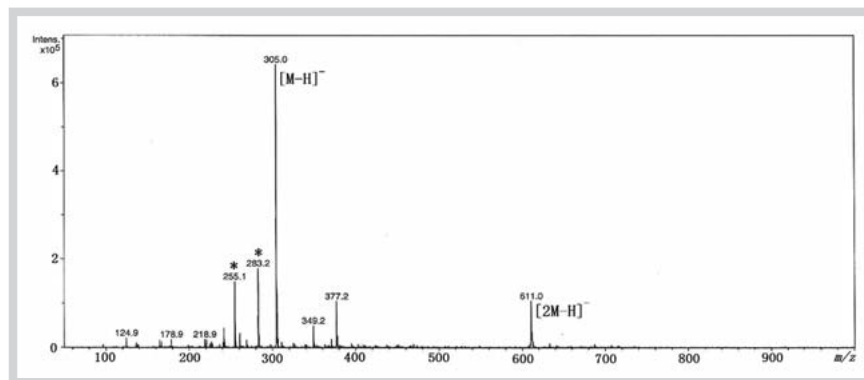
Fig. 4 ESI-MS of purified EGC. *Indicates background signals. MS-MS of the parent ion, 304.9, yielded the following ions (spectrum not shown): 124.9, 136.8, 138.9, 164.8, 166.8, 178.9, 218.9, 220.9, 261.0. This peak list concurs with that of authentic EGC [13].

for 5 min at 800 × g. The supernatant was collected and the pellet was resuspended in 5 mL of ethanol. The extraction was repeated three times in total, with substantial amounts of the original powder dissolving during the extractions. The combined supernatants were rotary evaporated, redissolved in 10 mL of dichloromethane : ethanol (50 : 50, v/v), and kept at − 20 °C for purification by silica gel chromatography.

The extract obtained from 100 mg of crude tea was loaded onto a silica gel column (2 cm i.d. × 25 cm) that was equilibrated with 300 mL of 85 : 15 dichloromethane : ethanol (v/v). The column was first eluted with 200 mL of that solvent mixture, and then with 200 mL of pure ethanol. The last 100 mL of the ethanol eluate was EGCg-rich and was rotary evaporated to dryness. The residue was redissolved in water, filtered through a double layer of filter paper, lyophilized, and kept at − 20 °C for tannase-catalyzed hydrolysis.

The lyophilized solid (100 mg) was dissolved in 20 mL of 50 mM sodium acetate buffer (pH 5.0) and mixed with ~ 10 g tannase beads (~ 5 mL) plus buffer to achieve a 50-mL reaction volume. After 30 min at room temperature, the tannase beads were removed from the solution by filtering the suspension. EGC was enriched by extracting the aqueous filtrate with 100 mL of ethyl acetate. The ethyl acetate layer was collected, rotary evaporated, reconstituted in water, filtered, lyophilized, and quantified. HPLC was employed to characterize the samples before and after hydrolysis. The lyophilized hydrolysis products were kept at − 20 °C for isolation and purification with Sephadex LH-20 chromatography.

The lyophilized hydrolysis product (100 mg) was dissolved in 7 mL of 80% ethanol containing 0.2% acetic acid, and was filtered with a 0.22-μm nitrocellulose filter. A Sephadex LH-20 column (i.d. 2 cm × 27 cm) equilibrated in 80% ethanol containing 0.2% acetic acid was used for EGC isolation and purification. A Spectra/Chrom® CF-2 fraction collector was used to collect 50 fractions of eluate (8.5 min/fraction). The absorbance of each fraction was determined at 220 nm and at 280 nm with an Agilent 8453 spectrophotometer. Fractions rich in EGC were combined, rotary evaporated, reconstituted in water, lyophilized, and kept at − 20 °C for HPLC and ESI-MS characterization. The Sephadex LH-20 column was cleaned and regenerated with 150 mL of 80% ethanol containing 0.2% acetic acid.

Sample characterization was performed with an Agilent 1100 series HPLC system equipped with a diode array detector (Agilent Technologies) and Chemstation A.09.03 software. Separation was performed on a Zorbax Eclipse XD8 C8 column (5 μm, 4.6 × 150 mm i.d.) at a flow rate of 0.5 mL/min. Samples were eluted with a gradient of 0.1% trifluoroacetic acid in water (solvent A) and 0.13% trifluoroacetic acid in acetonitrile (solvent B) as follows: 0–10 min, 5% B; 10–20 min, 20% B; 20–30 min, 35% B; 30–33 min, 95% B; 33–35 min, 5% B. Samples were filtered through 0.22 μm cellulose acetate spin filters before injecting 10 μL on the HPLC. The product EGC (t_R 19.6 min) and starting material EGCg (t_R 24.7 min) were detected using the diode array detector to collect data from 200 nm to 400 nm. The spectrum of each peak was generated with the Chemstation software.

Mass spectra were obtained with an Esquire LC ion trap instrument (Bruker Daltonics) with Data Analysis 3.0 software. The ESI source was operated in the negative mode. A nominal target mass was set to 306 before fine-tuning. The capillary, skimmer 1, and trap drive voltages were 100.2, -29.2, and 46.1 V, respectively. The ion charge control was on with a target of 20 000. The 300 °C nitrogen dry gas flow rate was 4 L/min and the nebulization gas pressure was 11 psi. A syringe pump was used to infuse the sample at 10 μL/min. Each data point in the spectrum consisted of an average of 20 scans over a mass range of 50 to 1000 *m/z*.

The yield (mass) and purity (HPLC) of the product were calculated from four independent replications of the purification, and are reported as the average ± SD. The purity was determined by comparing the integrated peak areas of EGC or EGCg to calibration curves prepared with the respective authentic standard.

Conflict of Interest
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The authors declare no conflict of interest.

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