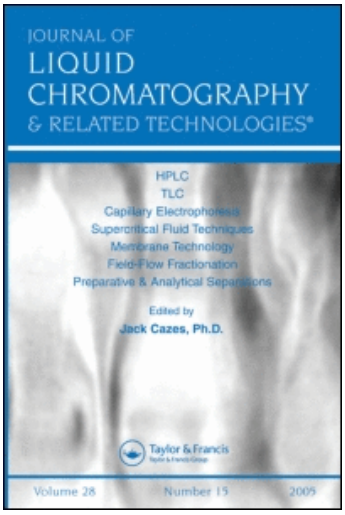

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Preparation and Purification of Epigallocatechin by High-Speed Countercurrent Chromatography (HSCCC)

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ABSTRACT

Epigallocatechin (EGC) was prepared by the degallation of Epigallocatechin-*O*-3-gallate (EGCG). EGCG was completely converted to EGC and gallic acid by adding 4 mg tannase/100 mg EGCG at the concentration of 2 mg/mL under pH 6.0, at 35°C for 30 min. EGC was then separated from the reaction mixture by HSCCC, using a two-phase solvent system composed of hexane–ethyl acetate–water (1/9/10, v/v/v). Finally, 1.3 g of EGC at 97% purity was prepared from about

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2.3 g of EGCG at 85% purity, indicating that EGC was almost completely recovered by HSCCC.

Key Words: Preparation; Purification; Epigallocatechin; HSCCC.

INTRODUCTION

Catechins (flavan-3-ols and their gallates) are the predominant polyphenols in green tea, constituting 15–35% of dry weight.[1] Catechins have been proven to have antioxidant, antimutagenic, and anticarcinogenic properties and can also prevent cardiovascular diseases.[2,3] The positive health effects of catechins have attracted much more attention of chemists and biochemist during recent years. However, because of the shortage of pure catechin compounds, it is important to develop highly efficient purification methods for individual catechins for a number of purposes, including their quality control, model fermentation studies aiming to achieve understanding of the formation of thearubigins from various precursors, and further biological activity studies, such as adsorption, distribution, metabolism, and elimination, etc. Several papers have reported the application of HSCCC on the separation of polyphenols, including catechins from various natural products.[4–6] Although epigallocatechin (EGC) is one of the important catechins, it is contained in natural green tea preparations in a relatively low amount compared with its gallate, epigallocatechin-*O*-3-gallate (EGCG), which is the most abundant catechin in green tea.

This paper describes the preparation of EGC from the degallation of EGCG by tannase and following purification of EGC by HSCCC. A preparative separation method of gallic acid was also achieved.

EXPERIMENTAL

Reagents

All solvents used in this study were of analytical grade and purchased from Sigma Chemicals. Epigallocatechin gallate (EGCG, 95%) and epigallocatechin (EGC, 98%) were purchased from Sigma Company, St. Louis, MO, USA, while the crude EGCG sample was prepared in our laboratory.

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Apparatus

A Quattro CPC manufactured by Brunel Institute for Bioengineering (Uxbridge, UK) was used for the HSCCC separation of catechins. It is equipped with two opposite bobbins containing two coils made of l.6 mm I.D. Teflon tubing in each side. Several different coil volumes can be made by joining the coils in series through the flying leads. The total coil volume is 585.5 mL and the minimum coil volume is 105.1 mL. The system is equipped with a Perkin–Elmer Series 200 pump. The eluents were monitored by a Waters 486 tunable absorbance detector and collected by a Waters fraction collector. The data was collected by a chromatographic system.

HPLC analysis was performed using a Dionex summit system equipped with a model P580 pump, a GINA 50 autosampler, an UVD340S diode array detector and a Chromeleon data system was used.

HSCCC Separation

The separations were performed at a revolution speed of 800 rpm and at 30°C. Both elution modes were used, i.e., lower phase eluted in the head-to-tail mode and the upper phase in the tail-to-head mode. Usually, in each separation, the coiled column was first filled with the stationary phase, then the CPC was rotated at 800 rpm, while the mobile phase was pumped into the column at a flow-rate of 3.0 mL. After the mobile phase front emerged and the system established hydrodynamic equilibrium, the sample solution was loaded through an injection valve (with injection volume 2–6 mL). The sample solutions were made by dissolving samples in the mobile phase, and in some cases, a little amount of stationary phase was added to increase the solubility.

In the preliminary HSCCC separation for EGCG, after the target peak is eluted, the mobile phase was switched to the upper organic phase by eluting it in the opposite direction (by switching the inlet and outlet of the column). The run was continued until other major components were eluted from the column.

HPLC Analysis

The analysis of catechins was performed on Luna Phenyl-Hexyl column (5 μm, 250 × 4.60 mm, Phenomenex, UK) by the following elution programme. The mobile phases are composed of solvent A, 2% acetic acid in acetonitrile, and solvent B, 2% acetic acid in high pure water. Elution programme: initial, 95% B; gradient to 69% B in 50 min; isocratic at 69% B

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for 5 min; at 55.01 min, back to initial condition 95% B and isocratic for 10 min; Flow rate, 1.0 mL/min. Peak detection was carried out at 274 nm.

Preparation of EGC

The starting material is a brown solid material rich in EGCG. At first, this material was subjected to preliminary purification by HSCCC for enrichment of EGCG. Then, 500 mg of this purified EGCG was dissolved in 100 mL water and placed in a water bath at 35°C for 5 min., and then 10 mL of tannase in buffer (pH = 6, 2 mg/mL) was added to the solution. After 30 min of stirring, the reaction mixture was taken out from the water bath and cooled down at room temperature, followed by extraction with 100 mL of ethyl acetate 6–8 times. The ethyl acetate fractions were combined, concentrated, and freeze-dried. Finally, this sample was subjected to HSCCC for separation of EGC.

RESULTS AND DISCUSSION

Purification of EGCG

Based on our previous study,[6] two solvent systems composed of hexane–ethyl acetate–water and ethyl acetate–ethanol–water can be used flexibly with a wide range of volume ratios to obtain different patterns of separations. Here, the two-phase solvent system composed of hexane–ethyl acetate–water (1/9/10, v/v/v) was employed for quick preparative separation of EGCG from the above mentioned crude material. By using a two-mode elution described earlier, almost all residuals retained in the column were eluted out by the reversed second mobile phase, leaving a clean solvent in the column. Consequently, the run can be repeated for several times without renewing the column contents. This can greatly increase the productivity of EGCG with low solvent cost. Figure 1 gives a profile of this two-mode HSCCC separation of EGCG. Starting with about 1 g of raw material, about 730 mg of EGCG can be obtained each day. HPLC analysis shows the purity of EGCG is about 85% by an external standard method, while no other peak is observed in the chromatogram.

Preparation of EGC from EGCG

EGC can be prepared by the degallation of its gallate, EGCG, using tannase as described earlier (Fig. 2, upper diagram). HPLC analysis of the

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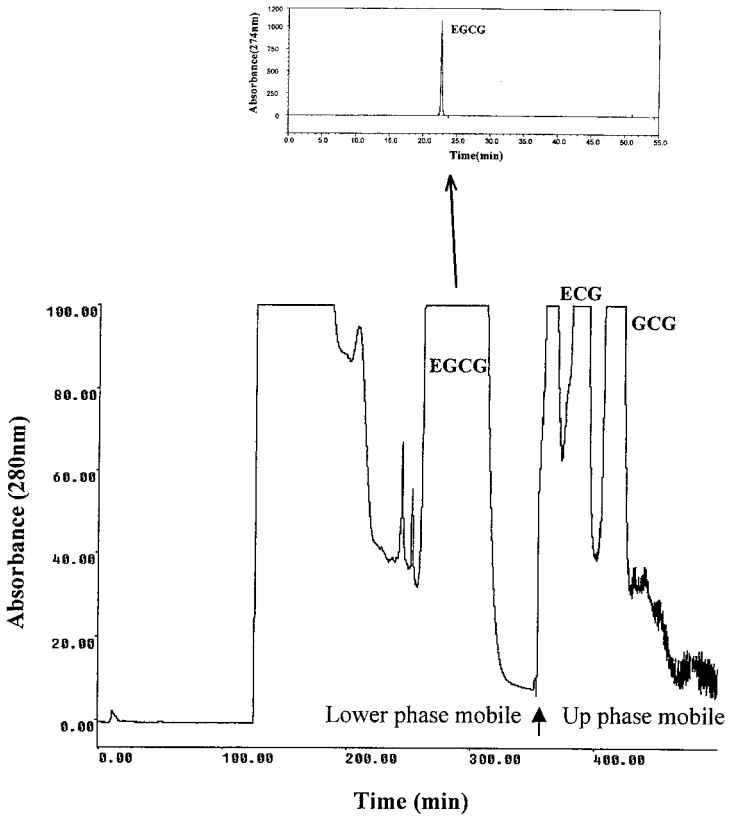

Figure 1. Profile of two-mode HSCCC separations of EGCG and HPLC analysis of the target peak fraction. Solvent system: hexane/ethyl acetate/water (1 : 9 : 10, v/v/v); sample: 1.0 g; mobile phase: lower phase until 340 min when switched to the upper phase; flow rate: 3 mL/min; revolution: 800 rpm. HPLC conditions: Luna phenyl-hexyl column (5 μm, 250 × 4.60 mm, Phenomenex, UK). The mobile phase: solvent A (2% acetic acid in acetonitrile) and solvent B (2% acetic acid in pure water). Elution programme: initial 95% B; gradient to 69% B in 50 min; isocratic at 69% B for 5 min; at 55.01 min back to initial condition of 95% B and isocratic for 10 min; flow rate: 1.0 mL/min; detection: 274 nm.

reaction products (Fig. 2, lower diagram) indicated that EGCG could be completely converted to EGC and gallic acid by adding 4 mg of tannase/100 mg EGCG at the concentration of 2 mg/mL under the conditions mentioned above.

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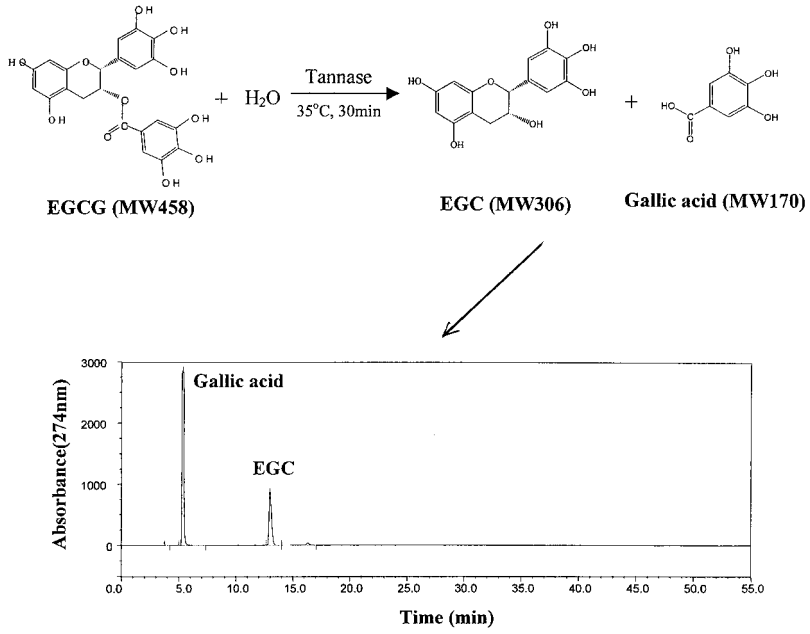

Figure 2. Degallation reaction of EGCG by tannase (top) and HPLC analysis of the reaction mixture (bottom). HPLC conditions: see the Fig. 1 caption.

Separation of EGC by HSCCC

The separation of EGC from gallic acid in the product mixture was also fulfilled by the same solvent system of hexane-ethyl acetate-water (1/9/10, v/v/v). Figure 3 (bottom) shows a chromatogram of EGC using HSCCC. Starting with about 500 mg EGCG, after degallation and HSCCC separation, about 290 mg of EGC was obtained. HPLC analysis shows the average purity of EGC is about 97% by the external standard quantification (Fig. 3, top). In addition, about 150 mg of gallic acid was obtained as a by-product.

Productivity of EGC

Totally, 1.3 g of EGC were prepared from about 2.3 g of EGCG. This result is reasonable compared to the result computed according to the following equation:

$$\text{EGC Amount} = \text{EGCG Amount} \times \% \text{ purity} \times \frac{\text{MW}_{\text{EGC}}}{\text{MW}_{\text{EGCG}}}.$$

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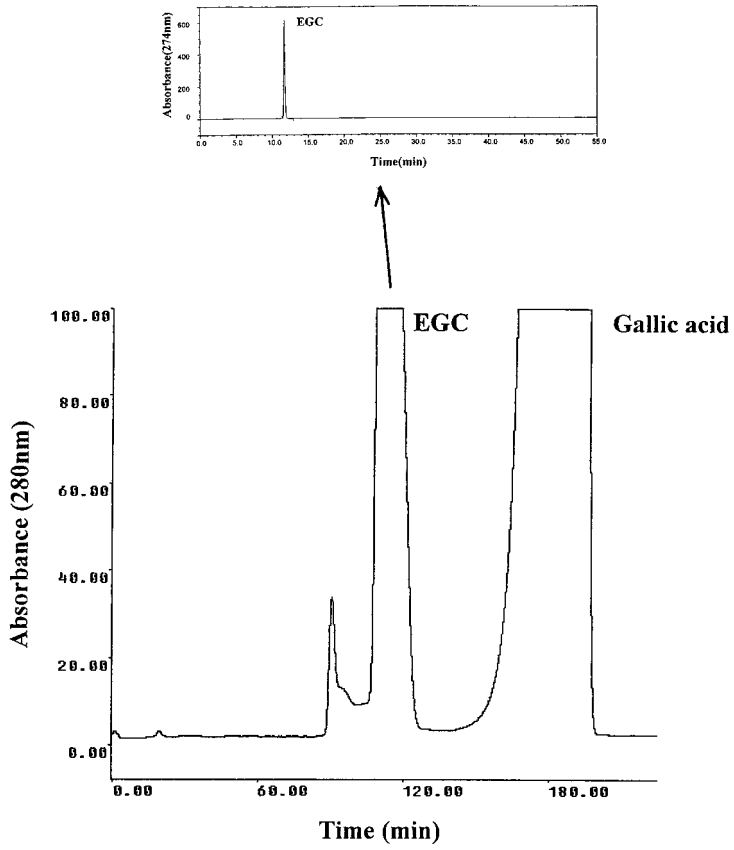

Figure 3. HSCCC chromatogram of EGC. Solvent system: hexane/ethyl acetate/water (1 : 9 : 10, v/v/v); sample: reaction mixture derived from 500 mg of EGCG. Other conditions are same as those described in the Fig. 1 caption. HPLC conditions: see the Fig. 1 caption.

EGCG was completely transferred to EGC by the treatment with tannase, and EGC was completely recovered by HSCCC.

CONCLUSIONS

The results demonstrate that EGC can be successfully prepared from the degallation of EGCG by tannase, and completely recovered by HSCCC separation with high purity. The separation and purification of EGCG and

EGC in this paper has proven again that HSCCC is a powerful preparative technique. The solvent systems used in this and previous papers will be useful for the separation of other catechins such as catechin (C), epicatechin (EC), etc.

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