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Short communication

Purification of (−)-epigallocatechin from enzymatic hydrolysate of its gallate using high-speed counter-current chromatography

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Abstract

Epigallocatechin gallate (EGCG) was hydrolyzed at various concentrations of tannase under pH 6.0 at 35°C, and the reaction mixtures were separated by high-speed counter-current chromatography with a two-phase solvent system composed of hexane–ethyl acetate–water (1:13:20). The best results were obtained when 2 mg/ml of the enzyme buffer solution was added to 0.3 *M* EGCG buffers at a rate of 0.1 ml/min. Using 10 mg of the enzyme, 342 mg of epigallocatechin were obtained at a purity of 99.1%.

1. Introduction

(−)-Epigallocatechin (EGC) is an important natural product from tea leaves, since it possesses various medicinal properties including antioxidant effect, inhibition of tumor growth and prevention of cardiovascular sclerosis [1–3]. However, purification of EGC from tea extract requires two steps, i.e. Sephadex LH-20 column chromatography followed by HPLC [4]. We are currently preparing (−)-epigallocatechin gallate (EGCG) which can be hydrolyzed by tannase to produce EGC. For this purpose we conducted a series of experiments to maximize a yield of EGC from the hydrolysis of EGCG by tannase. In order to purify EGC from the hydrolysate, we selected high-speed counter-current chromatography (HSCCC), because of its inherent advantage over conventional liquid chromatography by eliminating loss of samples by irreversible adsorption onto the solid support [5–7].

2. Experimental

2.1. Apparatus

HSCCC experiments were performed using a coil planet centrifuge equipped with a multilayer coil column that was designed and fabricated at the Beijing Institute of New Technology Application, China. The multilayer coil was prepared by winding a 1.6 mm I.D. polytetrafluoroethylene (PTFE) tube coaxially onto the column

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holder hub. The total column capacity measured 230 ml. The HSCCC centrifuge was rotated at 800 rpm with an 8 cm revolution radius. The system was equipped with an FMI pump (Zhejiang Instrument Factory, Hangzhou, China), a variable-wavelength UV detector (UV-752 made in Shanghai, China), a recorder and an injection valve.

2.2. Reagents

All of the organic solvents were of an analytical grade and purchased from Shanghai Chemical Factory, Shanghai, China. Tannase (tannin acylhydrolase, EC.3.1.1.20), 5000 U/g, was purchased from Kikkoman, Japan. The enzyme was stored at 4°C. EGCG was prepared using a new chromatographic method (Chinese patent pending) in our laboratory and stored at −20°C. Its purity was determined to be greater than 99% by HPLC analysis.

2.3. Tannase hydrolysis reaction

According to Thomas and Murtagh [8], tannase has an optimum pH at 6.0 and optimum temperature at 35°C. The tannase hydrolysis reaction reached the equilibrium in about 2 minutes though the enzyme concentration were different. We have selected five enzyme concentrations of 0.4, 0.8, 1.2, 1.6 and 2.0 mg/ml to initiate the reaction under pH 6.0 at 35°C (Fig. 1).

Five tubes, each containing 5 ml of 0.1 *M* NaH_2PO_4 buffers (pH 6.0) —in which the enzyme concentrations are 0.4, 0.8, 1.2, 1.6 and 2.0 mg/ml —were incubated in a shaking water bath at 35°C. Then, 0.2 ml of 0.3 *M* EGCG in a buffer was added to each of the tubes at 2-min intervals until the hydrolysis process was halted. TLC was used to monitor the degree of hydrolysis at each step.

2.4. TLC analysis

Polyacrylamide plates used in thin-layer chromatography (TLC) were purchased from Huangyie Chemical Factory, China. Acetone–water (2:1) was used for development, and three analytes —EGCG, EGC and gallic acid— were detected by purple spots on the TLC plate after the color reaction with 0.1% $FeCl_3$. Each analyte in CCC fractions was identified using the respective standard samples.

2.5. HSCCC procedure

HSCCC experiments were performed with a two-phase solvent system composed of hexane–ethyl acetate–water (1:13:20, v/v/v). The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated. Both the upper and the lower phases were degassed separately by ultrasonication.

In each separation, the multilayer coil column was first entirely filled with the upper stationary phase. Then the lower mobile phase was pumped into the inlet of the column at a flow rate of 1.6 ml/min, while the apparatus was rotated at 800 rpm. After the mobile phase front emerged and the two phases had established the hydrodynamic equilibrium throughout the column, the sample solution was injected through the in-

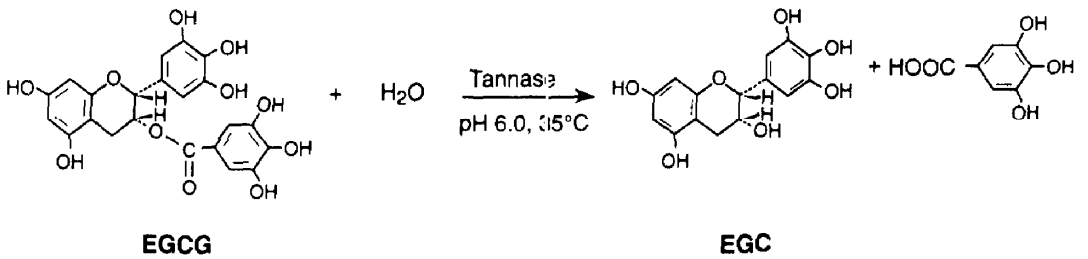

Fig. 1. Hydrolysis reaction of EGCG by tannase.

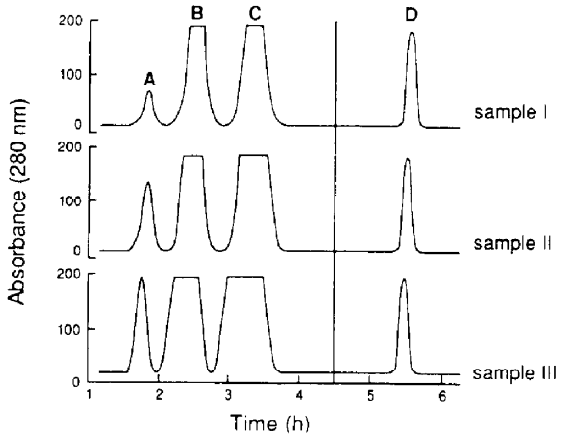

Fig. 2. Chromatograms of three reaction mixtures. Samples I, II and III were obtained from 2, 6 and 10 mg of tannase, respectively (see Table 1). Sample sizes: I = 165 mg/6.2 ml; II = 360 mg/7.6 ml; III = 550 mg/9.0 ml.

jection valve. The effluent from the outlet of the column was continuously monitored with a UV detector at 280 nm and the peak fractions were collected (Fig. 2). After three peaks were eluted, the centrifuge was stopped and the column contents were fractionated by continuously eluting the column with the mobile phase.

3. Results and discussion

Table 1 shows the results of hydrolysis studies of the reaction mixture for five different enzyme concentrations. These data indicate that 2, 4, 6, 8 and 10 mg tannase can be used to hydrolyze 0.30 mmol EGCG in 10 min, 0.48 mmol EGCG in 16 min, 0.72 mmol EGCG in 24 min, 0.96 mmol EGCG in 32 min, and 1.14 mmol EGCG

Table 1
Analyses of reaction mixtures by five different tannase concentrations

Reaction time (min)	EGCG added (mmol)	Initial tannase concentrations in the reaction mixture (mg/5 ml)				
		2	4	6	8	10
2	0.60	+	+	+	+	+
4	0.12	+	+	+	+	+
6	0.18	+	+	+	+	+
8	0.24	+	+	+	+	+
10	0.30	+	+	+	+	+
12	0.36	+/−	+	+	+	+
14	0.42	+/−	+	+	+	+
16	0.48	+/−	+	+	+	+
18	0.54	+/−	+/−	+	+	+
20	0.60	+/−	+/−	+	+	+
22	0.66	+/−	+/−	+	+	+
24	0.72	+/−	+/−	+	+	+
26	0.78	+/−	+/−	+/−	+	+
28	0.84	+/−	+/−	+/−	+	+
30	0.90	+/−	+/−	+/−	+	+
32	0.96	+/−	+/−	+/−	+	+
34	1.02	+/−	+/−	+/−	+/−	+
36	1.08	+/−	+/−	+/−	+/−	+
38	1.14	+/−	+/−	+/−	+/−	+
40	1.20	+/−	+/−	+/−	+/−	+/−

+ = Hydrolyzed completely, two spots appearing on TLC plate; +/− = hydrolyzed incompletely, the third spot (EGCG) detected on TLC plate.

in 38 min, respectively. All five reaction mixtures were subjected to HSCCC separation after cooling down to room temperature. Fig. 2 shows chromatograms of three reaction mixtures obtained by HSCCC. We found that peak resolution between three eluted analytes (A, B and C) was reduced as the sample volume was increased, reaching almost its limit for sample III (Fig. 2).

As indicated earlier, peaks A, B and C were eluted with the mobile phase while peak D was collected from the column after stopping the centrifugation. In TLC analysis, fractions corresponding to peaks B, C and D each produced a single spot and were identified as gallic acid, EGC, and EGCG, respectively. Each analyte produced a monochromatic purple color reaction with 0.1% $FeCl_3$ on the TLC plate. Fraction corresponding to peak A showed a negative color reaction to 0.1% $FeCl_3$ suggesting that it is probably the enzyme protein.

HSCCC of sample III produced 342 mg of EGC with a high purity of 99.1% as determined by HPLC. The overall results of the present studies demonstrated that HSCCC can be effectively used for the preparation of EGC from the EGCG hydrolysate by tannase.

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