Production of Hydroxycitric Acid by Microorganisms

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Received March 7, 2005; Accepted May 18, 2005

Hydroxycitric acid (HCA) is a major acid component of the tropical plants Garcinia cambogia and Hibiscus subdariffa. (2S,3S)-HCA from G. cambogia was shown to be a potent inhibitor of ATP citrate lyase (EC4.1.3.8), which catalyzes the extramitochondrial cleavage of citrate to oxaloacetate and acetyl-CoA. (2S,3R)-HCA from H. subdariffa inhibits α-amylase and α-glucosidase, leading to reduction of carbohydrate metabolism. The availability of HCA is limited by the restricted habitat of the plants as well as the difficulty of stereoselective organic synthesis. Hence, we screened microorganisms producing HCA to find an alternative source of optically pure bulk HCA. Two strains, Streptomyces sp. U121 and Bacillus megaterium G45C, were screened by HPLC analysis. Particular metabolites were purified from their culture broths and compared with authentic HCA from plants. NMR studies indicated that the products are identical to Hibiscus-type HCA. This is the first report showing isolation of microorganisms producing HCA.

Key words: hydroxycitric acid; Garcinia cambogia; Hibiscus subdariffa; Streptomyces sp.; Bacillus megaterium

Tropical plants are a rich source of valuable secondary metabolites, and some of them are useful as medicines and food additives. Hydroxycitric acid (HCA) is a major acid component in the rinds of Garcinia cambogia, which thrives prolifically on the Indian subcontinent and in western Sri Lanka. The rinds are used in combination with salt for curing fish in Southeast Asian countries.1,2 HCA is also enriched in the calyces of Hibiscus subdariffa, which is cultivated in several tropical and semitropical countries. The dried flowers are used as roselle herb tea. Roselle juice is a popular beverage and herbal medicine.3,4 Since HCA was identified, its effects have been studied extensively because of its curious bioactivities.5,6

HCA contains two diastereomers forming two chiral centers in their molecules. Each of these diastereomers forms a lactone ring. Therefore there are eight stereoisomers including lactone forms and their pairs of enantiomers (Fig. 1). G. cambogia and H. subdariffa produce (2S,3S) and (2S,3R)-HCA respectively. The former is a competitive inhibitor of ATP citrate lyase (EC4.1.3.8), which catalyzes the extramitochondrial fission of citrate to oxaloacetate and acetyl-CoA.5,7 The subsequent decrease in acetyl-CoA represses fatty acid synthesis and lipogenesis.8,9 Animal studies support these effects, and have shown that (2S,3S)-HCA suppresses food intake,10 thereby inducing weight loss.11 (2S,3R)-HCA has been shown to inhibit pancreatic α-amylase and small intestine α-glucosidase.6,12 In a human cell model system, it reduced carbohydrate digestion.13 Hence natural HCA is considered a safe diet supplement or a possible food additive for diabetes treatment. Only certain species of plants are known to produce HCA, and their growth is limited to tropical and semitropical areas. These facts suggest that production of natural HCA is restricted by plant distribution area and climate. Furthermore, stereoselective organic synthesis is difficult due to the presence of two asymmetric carbons in the molecule. Hence an alternative methodology is required to supply large amounts of an optically pure natural-type HCA economically.

In the present study, we attempted to isolate microorganisms that produce HCA. Screening was performed by HPLC analysis using authentic HCA prepared from G. cambogia and H. subdariffa as standard samples to confirm their retention times. HCA purified from the culture broth of isolated strains was identified by NMR analysis. These results clearly indicated the production of Hibiscus-type HCA by microorganisms.

Materials and Methods

Chemicals. Mannitol and citric acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). trans-Aconitic acid was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). 2-Oxoglutaric acid and glucose were purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals and solvents were of analytical grade.

Synthesis of trans-epoxy aconitic acid. Trans-epoxy-aconitic acid was prepared from trans-aconitic acid by
oxidation with H$_2$O$_2$ in the presence of a catalytic amount of sodium tungstate in H$_2$O at pH 5.0 at 70°C. The yield was about 85%.

**Purification of HCA from plant tissues.** Authentic (2S,3S)-HCA was purified as its methyl ester from dry rinds of *G. cambogia*, which were a gift from Iwata Chemical Co., Ltd. (Iwata, Japan). Dry rinds (10 g) were stirred in 100 ml of methanol-sulfuric acid (10% v/v) for 2 h at 60°C. After it was cooled to room temperature, the reaction mixture was diluted with saturated NaCl solution (100 ml) and extracted three times with dichloromethane (100 ml). The dichloromethane layers were combined and dried with anhydrous sodium sulfate. Dichloromethane was evaporated after filtration. The residue was purified by open column chromatography using silica gel (10 g). Crude HCA methyl ester dissolved in dichloromethane was adsorbed on silica gel (1 g) and dried. The silica gel was suspended in a small volume of n-hexane and placed in the top of a column equilibrated with n-hexane. After it was washed with n-hexane (100 ml), HCA methyl ester was eluted with 300 ml of n-hexane/ethyl acetate (1/1), and the elute was fractionated into 5 ml fractions. All the fractions were analyzed by thin-layer chromatography (TLC) on a silica gel sheet (Silica gel 60 F$_{254}$, Merck, Darmstadt, Germany) with a solvent system of n-hexane/ethyl acetate (1/1). Fractions were combined and then concentrated. The purified product was analyzed and identified by NMR. Authentic (2S,3S)-HCA was purified as a methyl ester from dried calyxes (5 g) of *H. subdariffa* (Fukurodo Pharmacy, Tokyo). The purification procedure was the same as that described for (2S,3S)-HCA from dry rinds of *G. cambogia*.

**Isolation of microorganisms from nature.** The main sources of microorganisms were plants in botanical gardens and soils in western Japan. Pieces of plant leaf, fruit rind, and stem were placed directly on the surface of agar plates containing a possible HCA precursor such as citric acid (50 g), trans-aconitic acid (20 g), trans-epoxyaconitic acid (20 g), mannitol (25 g) or *Garcinia* extract (a gift from Iwata Chemical Co., Ltd.) (5 g) and inorganic components (NaNO$_3$, 2 g; K$_2$HPO$_4$, 1 g; MgSO$_4$, 1 g; KCl, 0.5 g; FeSO$_4$, 0.01 g per liter). Alternatively, parts of plants and soil samples were suspended in sterilized 0.9% NaCl. The supernatant was diluted and spread on agar plates. Microorganisms from several culture collections were also tested for HCA production.

**Screening of microorganisms producing HCA.** An isolated single colony was inoculated into 5 ml of screening media in a 30-ml test tube containing (per liter) 1 g of KH$_2$PO$_4$, 0.25 g of MgSO$_4$, 3 g of corn steep liquor (Nihon Shokuhin Kako Tokyo) and a carbon source such as 100 mM glucose, 10 mM citric acid, 10 mM 2-oxoglutaric acid, 10 mM trans-aconitic acid or 10 mM trans-epoxyaconitic acid. Test tubes were incubated on a reciprocal shaker at 30°C for 48 h. The culture broth was boiled for 10 min and centrifuged. The supernatant was filtrated with a syringe filter unit (Millex-LG 0.20μm, Millipore, Bedford, MA) and analyzed by HPLC (Model L-7100; Hitachi High Technologies, Tokyo) at a flow rate of 1.0 ml/min on a 10.7 x 300 mm GLC610 column (Hitachi) with 0.1% H$_3$PO$_4$ and a column temperature of 60°C. HCA and other organic acids were detected by absorbance at 210 nm.

**Purification of HCA from culture broth.** Microorganisms were cultured in 400 ml medium containing (per liter) 1 g of KH$_2$PO$_4$, 0.25 g of MgSO$_4$, 3 g of corn steep liquor and 100 mM glucose, using 2 liter-buffed conical
Results

flasks on a rotary shaker at 30°C for 72 h. The culture broth (2-liter) was centrifuged (5000 x g, JLA-10,500, Beckman Coulter, Palo Alto, CA) to remove microbial cells. Calcium carbonate (5 g) was added to the supernatant to remove citrate by precipitation as a calcium salt. The filtrate was treated with 10 ml of cation exchanger (Amberlite IR120; Organo, Tokyo) to remove calcium ions. Then, the solution was loaded onto a column of Diaion HP-20 (100 ml, Mitsubishi Chemical Co., Tokyo). The flow-through fraction was applied to column of anion exchanger resin (100 ml, Amberlite IRA410JCL; Organo) and washed with 1 liter of H2O. The adsorbed acidic portion was eluted with 100 ml of 25% formic acid solution and concentrated. The residue was dissolved in 100 ml of methanol-sulfuric acid (10% v/v) and stirred at 60°C for 30 min for esterification. Purification of HCA methyl ester was performed as described in authentic plant HCA. At the final purification step of HCA methyl ester from Streptomyces sp. U121, a preparative HPLC system was utilized (Model LC-908, Recycling Preparative HPLC, Japan Analytical Industry, Osaka; flow rate, 5.0 ml/min; Column, 20 x 250 mm Inertsil ODS, GL Science, Tokyo; Solvent system, CH3CN/H2O (65/35)).

Spectrometric analysis. Structures of purified HCA methyl esters were determined by 1H-NMR. NMR signals were recorded on a JNM-LA series AL300 for 300 MHz (Japan Electron Optics Laboratories, Tokyo). The molecular masses of HCA and its methyl ester were estimated in negative ion mode by ESI-MS (Mariner™, Applied Biosystems, Foster City, CA).

Measurement of HCA concentration. The concentration of HCA was determined by HPLC analysis. Calibration was linear at concentrations between 2 and 2,000 mg/liter with an r² value of 0.999.

Results

Preparation of authentic HCA from G. cambogia and H. subdariffa

Prior to isolation of microorganisms producing HCA, we purified HCA from G. cambogia and H. subdariffa. These HCA were used as authentic samples for screening by HPLC analysis and for identifying the microbial products by NMR analysis. About 200 mg of (2S,3S) and (2S,3R)-HCA methyl ester were obtained from 10 g of dried rinds of Garcinia and 5 g of dried calyxes of Hibiscus respectively.

The results of 1H-NMR (in CDCl₃, 300 MHz) of (2S,3S) and (2S,3R)-HCA methyl ester are shown in Tables 1 and 2, and the structures are illustrated in Fig. 2. The coupling constants of Ha and Hb were nearly identical to those in the previous reports. Moreover, methanolation of HCA lactone methyl ester in the presence of a small amount of sodium methoxide led to increased signals of Hd and He and decreased signals of Ha and Hb (data not shown). These results suggest that Hd and He belong to a nonlactone form, and that both lactone and nonlactone forms exist in purified samples. HCA methyl esters were subjected to hydrolysis with NaOH. The 1H-NMR spectrum (in D₂O, 300 MHz) of (2S,3S)-HCA from G. cambogia was lactone form: 2.79 ppm (Ha, d, J = 18 Hz), 3.14 ppm (Hb, d, J = 18 Hz), 5.30 ppm (Hc, s); nonlactone form: 2.91 ppm (He, d, J = 16.5 Hz) and 3.01 ppm (Hd, d, J = 16.5 Hz), 4.90 ppm (Hf, s). That of (2S,3R)-HCA from H. subdariffa was lactone form: 2.70 ppm (Hb, d, J = 16.5 Hz) and 3.14 ppm (Hc, s), and nonlactone form: 2.91 ppm (He, d, J = 16.5 Hz) and 3.01 ppm (Hd, d, J = 16.5 Hz), 4.90 ppm (Hf, s).

Table 1. 1H-NMR Data for HCA Methyl Ester from G. cambogia

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<tr>
<th>H</th>
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<th>J in Hz</th>
<th>Chemical shift (ppm)</th>
<th>J in Hz</th>
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Three CH₃O-protons belonging to the lactone and nonlactone forms appeared at 3.70–3.90 ppm.

Table 2. 1H-NMR Data for HCA Methyl Ester from H. subdariffa

<table>
<thead>
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<th>J in Hz</th>
<th>Chemical shift (ppm)</th>
<th>J in Hz</th>
</tr>
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<td>He</td>
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<td>s</td>
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Three CH₃O-protons belonging to the lactone and nonlactone forms appeared at 3.70–3.90 ppm.
17.7 Hz), 3.24 ppm (Ha, d, J = 17.7 Hz), 5.11 ppm (Hc, s); nonlactone form: 2.84 and 2.97 ppm (He, Hd, d, J = 16.2 Hz), 4.37 ppm (Hf, s). The coupling constants of Ha and Hb were consistent with those described previously. On ESI-MS, both HCA methyl esters gave [M – H]− ions at m/z 203 and 235, indicating that they included the lactone of monomethyl ester and the nonlactone of dimethyl ester. HCA obtained by alkaline hydrolysis showed [M – H]− ions at m/z 189 and 207, which correspond to those of HCA lactone and nonlactone respectively.

(2S,3S) and (2S,3R)-HCA showed almost identical retention times at 9.30 (peak 1) and 10.20 min (peak 2) on an HPLC chromatogram (Fig. 3B). The HCA sample present in the alkaline solution produced peak 2, suggesting that peaks 1 and 2 correspond to lactone and nonlactone forms respectively. Additionally, they were detected in crude extracts of dried rinds of G. cambogia (data not shown) and dried flowers of H. subdariffa (Fig. 3A) as major acid components.

Screening microorganisms producing HCA

Isolation of microorganisms from various ecological niches was carried out using agar plates containing possible HCA precursors as a carbon source. About 2,000 strains were screened by HPLC analysis. There were 30 strains generating two metabolites with retention times similar to those of HCA lactone and nonlactone. Of these, two strains, U121 and G45C, which were selected on agar plates with Garcinia extract, showed apparently higher production of the metabolites (Fig. 4). The products were purified, and finally 11 and 13 mg of the methyl esters were obtained respectively. The 1H-NMR spectrum (in CDCl3, 300 MHz) of the methylester of U121 exhibited signals at 2.98 and 3.16 ppm (J = 16.4 Hz) and 4.36 ppm, identical to those of the authentic nonlactone form of Hibiscus-type HCA methyl ester diastereoisomer (Tables 2 and 3). That of the methyl ester of G45C showed signals at 2.87 and 3.07 ppm (J = 17.5 Hz) and 5.11 ppm, identical to those of the lactone form of Hibiscus-type HCA methyl ester (Tables 2 and 3). Furthermore, chemical shifts in the nonlactone form were found as well, suggesting that the purified product includes both lactone and nonlactone respectively. In the 1H-NMR spectra of the U121 and G45C products, there was no signal corresponding to the methyl ester of Garcinia-type diastereoisomer HCA (Table 3). These results indicate that strains U121 and G45C produced pure HCA of Hibiscus-type diastereomers.

Identification of isolated strains

The isolated strain U121 had substrate mycelium, aerial mycelium, arthrospores in verticals, and chains of spores. The other isolated strain, G45C, was gram-positive, motile, and rod-shaped, and showed spore formation, and catalase-positive and oxidase-negative reactions. Phenotypic analysis and morphological char-
characteristics strongly suggested that the strains were *Streptomyces* and *Bacillus megaterium* respectively. With these results, the isolated strains were tentatively designated *Streptomyces* sp. U121 and *Bacillus megaterium* G45C respectively.

**Growth curve and production of HCA**

The growth of *Bacillus megaterium* G45C and its HCA production were investigated. Production of HCA paralleled cell growth. The strain yielded HCA after 12 h and reached a maximum level of 22 mg/l at about 44 h (Fig. 5). *Streptomyces* sp. U121 showed slower growth and HCA production (data not shown). The maximum production was 30 mg/l at about 48 h.

**Discussion**

In this report, we describe the isolation of microorganisms producing HCA. Authentic HCA was prepared from *G. cambogia* and *H. subdariffa*, and microorganisms were screened from nature by HPLC analysis. The product was purified from culture broth and identified as the methylester of HCA by NMR analysis. To our knowledge, this is the first report of production of HCA by microorganisms.

NMR and ESI-MS data for purified authentic samples indicate that they included a nonlactone form of dimethyl ester and a lactone form of monomethyl ester. The lactone form data were consistent with those of previous studies. HCA prepared by hydrolysis of the purified esters showed retention times similar to major acid components of the extracts of Garcinia rinds and Hibiscus calyxes (Fig. 3). These results strongly indicate that authentic HCA samples were successfully prepared.

Among more than 2,000 microbial strains, *Streptomyces* sp. U121 and *Bacillus megaterium* G45C generated metabolites showing retention times similar to authentic HCA on HPLC (Fig. 4). We supposed that they were possible HCA producers. The products were purified from their culture broths, and NMR analysis verified them as HCA (Table 3). From the culture broth of *Streptomyces* sp. U121, only nonlactone form HCA methyl ester was obtained. In this case, we used a preparative HPLC system for the final purification process of HCA. Lactone-form HCA was probably eliminated by this step. We isolated three strains that

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Table 3. $^1$H-NMR Data for Products 1 and 2

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<th>Product 1</th>
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<td>$^1$H</td>
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<tr>
<td>Ha</td>
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<td>s</td>
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</table>

*Products 1 and 2 were obtained from *Streptomyces* sp. U121 and *Bacillus megaterium* G45C respectively.*
showed metabolite with a retention time of 10.20 min on the HPLC chromatogram, but NMR data indicated that the products were not HCA (data not shown). Therefore, selection of strains that produced both HCA lactone and nonlactone forms of metabolites appeared to be critical for efficient screening in this HPLC system. Our finding of two HCA producers is not surprising, because the HPLC system has higher sensitivity and resolution than paper chromatography or TLC, which were used to screen producers of particular organic acids. Effective HPLC analysis combined with NMR data allowed the identification of HCA produced by microorganisms.

The two microbial strains isolated in this study proved to yield Hibiscus-type HCA (diastereoisomers). It has been reported that Hibiscus HCA inhibited pancreatic α-amylase and small intestine α-glucosidase.6,12) Administration of these enzyme inhibitors was shown to repress increases in blood glucose and insulin levels.17) Acarbose, an α-glucosidase inhibitor, is widely used as a therapeutic agent for diabetes treatment.17–19) However, it must be used carefully due to side effects. Hibiscus HCA is expected to be a safe food additive for prevention of diabetes and obesity.21) Microbial production is an alternative source of optically pure Hibiscus-type HCA. Isolation of microorganisms producing Garcinia-type HCA is a future project. In our HPLC screening, we obtained 28 additional strains which were found to be possible HCA producers besides Streptomyces sp. U121 and Bacillus megaterium G45C. Their production levels were estimated to be 2 to 10 mg/l for HCA, and structural identification is ongoing. Thus we cannot exclude the possibility that some of them may produce Garcinia-type HCA. At present, the maximum HCA production is 30 mg/l by Streptomyces sp. U121 and 22 mg/l by Bacillus megaterium G45C, as estimated by HPLC. The yields were lower than those of other organic acid fermentations, such as citric acid by Aspergillus niger (30–74 g/l),20) tartaric acid by Gluconobacter suboxydans (5–12 g/l)21) and abscisic acid by Cercospora rosicola (120 mg/l).22) Medium optimization and selective breeding using mutagen and genome shuffling are promising methods to improve HCA production. Construction of HCA hyperproducers is under way in our laboratory.

The biosynthetic pathway of HCA in plants and microorganisms remains unclear. Enhancement of HCA production due to the addition of possible HCA precursors such as TCA cycle intermediates and other analogs to their culture broth was not observed. Resting cell reaction with candidate HCA precursors did not elicit HCA production either (data not shown). There are several examples indicating that plants and microorganisms generate common secondary metabolites. Higher plants and fungi have different routes for the biosynthesis of abscisic acid.23,24) Gibberellin biosynthesis in Gibberella fujikuroi is different from that in higher plants.25) On the other hand, Phaeosphaeria sp. L487 produces GA1 through a pathway similar to that in higher plants.26) Based on observation of the chemical structure of HCA, the biosynthetic pathway is probably not complicated, but it must be carefully explored. Construction of HCA hyperproducers might help to establish its biosynthetic principles.

In conclusion, we discovered microorganisms that produce HCA, providing a potential alternative source to Hibiscus HCA.

**Acknowledgments**

We are grateful to Dr. H. Funahashi and M. Nakao in Iwata Chemical Co., Ltd., for the gift of Garcinia extract and their invaluable advice. We thank S. Goto and T. Sambe for screening of microorganisms. We would like to acknowledge the ESI-MS analysis of Dr. K. Tsurusaki
in the Department of Environment and Information Sciences of Fukuyama University. This work was in part supported by Iwata Chemical Co., Ltd., and “High-Tech Research Center” Project for Private Universities on Evolution of Green Science for Quality Improvement of Environmental and Health: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology), 2004–2008.

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