Enzymological Evidence for Cannabichromenic Acid Biosynthesis

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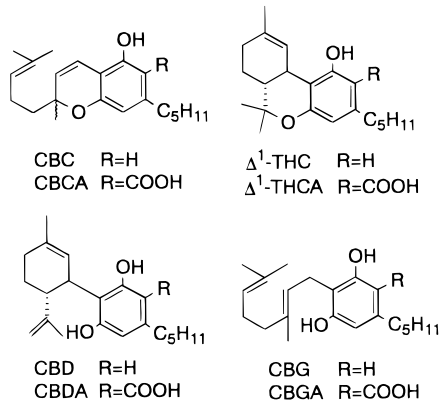
An enzyme involved in the biosynthesis of cannabichromenic acid was identified in young leaves of *Cannabis sativa*. The enzyme, named cannabichromenic acid synthase, catalyzed the oxidocyclization of cannabigerolic acid to cannabichromenic acid. The biosynthetic mechanism for the formation of cannabichromenic acid was similar to those of Δ^1-tetrahydrocannabinolic acid and cannabidiolic acid.

Cannabichromene (CBC) is a cannabinoid commonly found in various strains of marijuana (*Cannabis sativa*).[1] This cannabinoid has no psychotropic effect, but interesting pharmacological activities such as anti-inflammatory, antifungal and antimicrobial effects have been reported.[2] However, the biosynthetic mechanism of CBC remained largely unclear. Mechoulam proposed that cannabichromenic acid (CBCA), the precursor of CBC, is derived biosynthetically from cannabigerolic acid (CBGA) and that this biosynthetic reaction proceeds nonstereospecifically, contrary to the biosynthesis of the optically active cannabinoids Δ^1-tetrahydrocannabinolic acid (Δ^1-THCA) and cannabidiolic acid (CBDA).[3] Moreover, it has long been believed that a



nonenzymatic reaction may be involved in CBCA biosynthesis.[3] However, these hypotheses lack experimental support. In order to reveal the mechanism of CBCA biosynthesis, we attempted to investigate an enzyme (CBCA synthase) catalyzing the formation of CBCA, and we detected CBCA synthase activity in crude enzyme extracts from young leaves of *C. sativa* (CBDA strain). This report deals with the identification and partial characterization of CBCA synthase. In addition, the stereoselectivity of the enzymatic reaction is also described.

CBCA occurs as a minor cannabinoid in mature leaves of various *Cannabis* strains. Our previous study demonstrated that young leaves of the 1- or 2-week-old CBDA strain produce far more CBCA (>2 mg/g dried leaves) than CBDA (<0.1 mg/g dried leaves) and that the CBCA content decreases with plant growth.[4] Therefore, young leaves of the 2-week-old plants were used to identify CBCA synthase. The CBCA synthase activity was assayed with CBGA as a substrate. After young leaves of the 2-week-old CBDA strain were homogenized and fractionated by differential centrifugation, the CBCA synthase activity in each fraction was measured. The soluble fraction (100000*g* supernatant) exhibited potent CBCA-producing activity, confirming that CBCA is enzymatically biosynthesized from CBGA. In contrast, CBCA synthase activity from the light (10000*g* pellet) and heavy membrane (100000*g* pellet) fractions was not detected using various detergents. These results suggested that CBCA synthase is a soluble enzyme. In order to extract the enzyme more effectively, various solvents were tested. Among them, NaCl solution, which is often used to extract proteins ionically bound to cell walls,[5] afforded a soluble fraction containing higher enzyme activity (16.8 pkat/g fresh leaves) than Tris−HCl (pH 8.0), phosphate (pH 7.0) or citrate (pH 6.0) buffers (6.9, 10.5, and 8.6 pkat/g fresh leaves, respectively). On the basis of these results, we extracted CBCA synthase using 1 M NaCl.

In order to obtain precise information on the properties of CBCA synthase, the purification of the enzyme was carried out. As a first step, the soluble fraction from young leaves of the CBDA strain was fractionated with ammonium sulfate. More than 80% of the enzyme activity precipitated on 30−75% saturation of ammonium sulfate. The CBCA-synthase-active fraction was applied to column chromatography on DEAE−cellulose and then CM−cellulose. Sodium dodecyl sulfate (SDS) gel electrophoresis demonstrated that the CM−cellulose eluate was still contaminated with several proteins, although the specific activity was markedly increased by these three steps, resulting in about 60-fold purification.

Using this partially purified enzyme, the optimal conditions for the CBCA synthase assay were investigated. Our previous study reported that the activity of CBDA synthase catalyzing the formation of CBDA depends on the properties and concentrations of the detergents (Triton X-100, Tween 80, and Emulgen 911) required for solbilization of the substrate CBGA and that 0.1% Triton X-100 was the most effective detergent for the CBDA synthase reaction.[6] Therefore, the effects of these three detergents and of SDS on the CBCA synthase activity were examined. As shown in Figure 1, SDS was an effective detergent as compared to the

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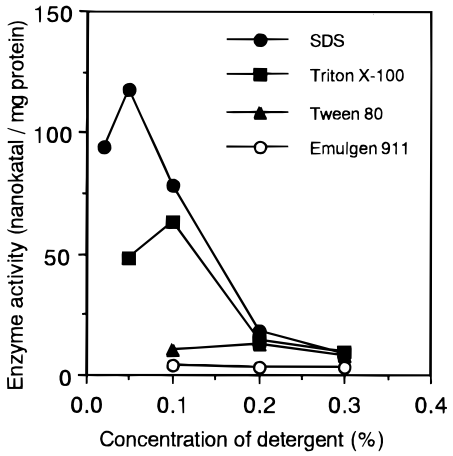

Figure 1. Effects of detergents on CBCA synthase activity. The enzyme activity was detected as described in the Experimental Section. The minimal concentrations of detergents required to completely dissolve CBGA in phosphate buffer were 0.02% (SDS), 0.05% (Triton X-100), 0.05% (Tween 20), and 0.1% (Emulgen 911). Data are means of three replicate assays.

other detergents. The addition of 0.05% SDS resulted in the highest CBCA synthase activity, which was about two times higher than that with 0.1% Triton X-100, whereas concentrations of SDS over 0.2% inhibited the enzyme activity. Concerning the optimal pH of CBCA synthase, the enzyme activity was measured using a series of citrate and phosphate buffers of overlapping pH. The enzyme activity was maximal at pH 6.5 with half-maximal velocities at pH 5.6 and 7.3. On the basis of these results, a standard assay was performed with phosphate buffer (pH 6.5) containing 0.05% SDS.

Under the standard assay conditions, CBCA was enzymatically derived from CBGA, and its optical activity was carefully measured to determine whether the enzymatic reaction proceeded stereoselectively. Although the optical rotation of CBCA formed in the enzymatic reaction was almost 0°, an apparent Cotton effect was found in its circular dichroism (CD) spectrum. Since this evidence suggested that CBCA synthase catalyzes the stereoselective formation of CBCA, the optical purity of CBCA was analyzed by chiral HPLC. Preliminarily, various HPLC conditions were tested with chemically synthesized CBCA and CBC,[3,7] which were confirmed by the analyses of their CD spectra to be racemic. However, the resolution of CBCA was not successful under numerous HPLC conditions. In contrast, the enantiomers of CBC were well resolved by HPLC equipped with a Chiralcel OD-R column, and two peaks with equal intensity were observed in the chromatogram (Figure 2A). Therefore, the stereoselectivity of the CBCA synthase reaction was evaluated by HPLC analysis of CBC prepared by decarboxylation of enzymatically synthesized CBCA. As shown in Figure 2B, two peaks eluting at 16 and 17 min were observed, and the peak intensity of the latter was about 5-fold higher than that of the former. In order to reveal whether or not this partial racemization is due to the decarboxylation reaction, we synthesized optically pure CBCA by carboxylation of the latter enantiomer with methylmagnesium carbonate[8] and analyzed the optical purity of CBC derived from it under the same decarboxylation condition. HPLC showed only one peak eluting at 17 min, indicating that racemization does not take place during the decarboxylation of CBCA. From these

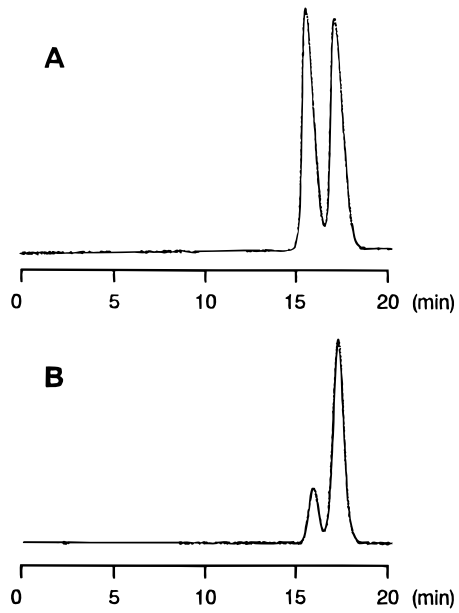

Figure 2. Optical resolution of CBC by chiral HPLC. Chromatogram A: CBC chemically synthesized from CBG. Chromatogram B: CBC prepared by decarboxylation of the enzymatically synthesized CBCA.

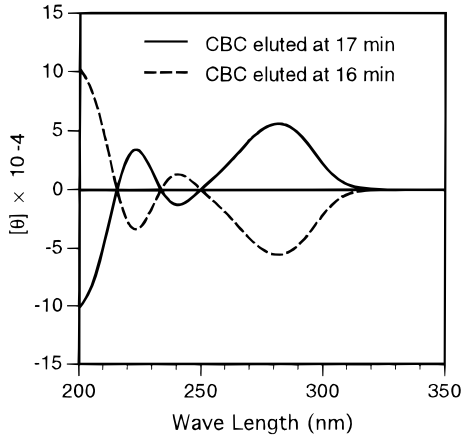

Figure 3. CD spectra of CBC purified by chiral HPLC.

results, it was concluded that the enantiomers of CBCA are also formed in a ratio of 5:1 by the CBCA synthase reaction. The CD spectra of both enantiomers isolated by preparative HPLC displayed opposite curves to each other (Figure 3), but these CD data provided little evidence for the absolute configuration of CBC.

CBCA synthase was identified in young leaves of CBDA strains, but enzymes catalyzing conversion of the neutral cannabinoid cannabigerol (CBG) to CBC could not be detected using various conditions. This finding provides important evidence for the mechanism of CBC formation in *C. sativa*. It has previously been postulated that CBC is derived either by cyclization of CBG or by decarboxylation of CBCA.[3] Previously, we suggested the latter hypothesis for the mechanism of CBC formation based on the fact that fresh leaves of *C. sativa* contain only acidic cannabinoids.[9] The absence of enzymes catalyzing the formation of CBC also supported the latter hypothesis. Since THC and CBD are also derived by the decarboxylation of Δ^1-THCA and CBDA,[6,10] most neutral cannabinoids may be artificially formed by the decarboxylation of the corresponding acidic cannabinoids.

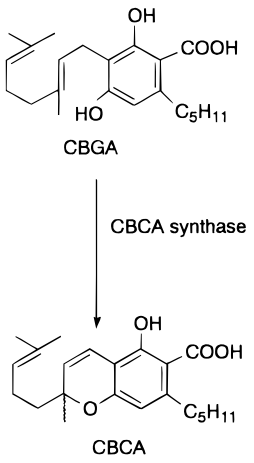

Figure 4. Biosynthetic pathway of CBCA.

In conclusion, our study demonstrates that CBCA is enzymatically formed by the cyclization of CBGA (Figure 4), contrary to the generally presumed biogenesis of CBCA. In a previous paper, we reported that Δ^1-THCA and CBDA are also biosynthesized by oxidocyclization of CBGA.[6,10] It is noteworthy that cannabinoids having different ring systems are biosynthesized from the common substrate CBGA by similar mechanisms.

Experimental Section

General Experimental Procedures. CD spectra were recorded on a JASCO J720W spectropolarimeter. ^{1}H-NMR spectra were obtained on a Varian Unity 500P spectrometer with standard pulse sequences operating at 500 MHz. All NMR spectra were measured in $CDCl_3$. HPLC was conducted using a CCPM pump and a UV-8000 absorbance detector (Tosoh). The peak intensity was calculated using a Chromatocorder 21 (Tosoh). Si gel 60 (70−230 mesh, Merck), DEAE−cellulose (DE-52, Whatman), and CM−cellulose (CM-52, Whatman) were used for column chromatography.

Plant Materials. *C. sativa* (CBDA strain) was grown in the herbal garden and greenhouse of the Faculty of Pharmaceutical Sciences, Kyushu University. CBCA synthase was extracted from young leaves of the 2-week-old CBDA strain.

Preparation of Cannabinoids. CBCA and CBC were isolated from the dried leaves of the 15-week-old CBDA strain as previously reported.[9] Chemically synthesized CBCA and CBC were derived from CBGA and CBG, respectively, as follows. CBGA (10 mg) was dissolved in dioxane (3 mL) containing DDQ (90 mg) and then stirred at room temperature for 1 h in the dark.[7] The solvent was evaporated under nitrogen flow, and the residue was redissolved in $CHCl_3$−MeOH (2:1) (10 mL). After the solution was washed with water three times, the organic layer was evaporated under reduced pressure. The residue was separated by preparative HPLC on Cosmosi $5C_{18}$ AR (Nacalai Tesque) using 90% CH_3CN to afford CBCA (2 mg). CBC was also synthesized as described for CBCA, except for the use of CBG instead of CBGA. CBG was chemically synthesized from olivetol (Sigma) and geraniol by a modification of the method of Mechoulam and Yagen.[11] CBGA and optically pure CBCA were obtained by carboxylation of CBG and chiral-HPLC-purified CBC with methylmagnesium carbonate, respectively.[6,8] These cannabinoids were identified by comparison of their ^{1}H-NMR spectra with those of authentic cannabinoids.[12]

Assays of CBCA Synthase. CBCA synthase activity was assessed by incubating enzyme (50 μL) in substrate buffer (400 μL) containing 200 μM CBGA, 0.05% (w/v) SDS, and 100 mM sodium phosphate (pH 6.5) at 30 °C for 2 h. After the reaction was terminated with 450 μL of methanol, a 50-μL aliquot was applied to analytical HPLC equipped with Cosmosi $5C_{18}$ AR (Nacalai Tesque). CBCA was eluted with 95% aqueous CH_3CN containing 50 mM phosphoric acid at a flow rate of 1 mL/min. The eluate was monitored by absorption at 254 nm, and the peak intensity was calculated using a Chromatocorder 21 (Tosoh). The retention time and concentration of CBCA were verified by comparison with those of authentic CBCA. The enzyme activity (katal) was defined as the amount (mol) of CBCA formed per second.

Extraction and Partial Purification of CBCA Synthase. Unless otherwise indicated, all extraction and purification procedures were performed at 4 °C. Young leaves (30 g) of the CBDA strain were homogenized in a Waring blender at high speed together with 300 mL of 1 M NaCl containing 10 mM mercaptoethanol. After the homogenate was filtered through a Nylon screen, the filtrate was centrifuged at 100000*g* for 1 h. The supernatant was then fractionated with ammonium sulfate. Proteins precipitating at 30−75% saturation were collected by centrifugation at 20000*g* for 15 min, resuspended in about 30 mL of buffer A (10 mM sodium phosphate buffer (pH 7.0), 3 mM mercaptoethanol), and dialyzed overnight against three changes of the same buffer. Insoluble materials were removed by centrifugation at 20000*g* for 15 min. The supernatant was applied to a DE-52 cellulose column (Whatman) equilibrated with buffer A. CBCA synthase was eluted with buffer A. The CBCA-synthase-active fraction was concentrated by ultrafiltration and applied to a CM-52 cellulose column (Whatman) equilibrated with buffer A. The most active fraction was used to characterize the properties of CBGA synthase reaction.

Preparation of CBCA and CBC by CBCA Synthase Reaction. The substrate buffer (see "Assays of CBCA Synthase) (50 mL) was incubated at 30 °C for 12 h in the presence of partially purified enzyme (102 μg). The reaction mixture was partitioned with 50 mL of AcOEt. The AcOEt layer was dried with sodium sulfate (5 g), and then the solvent was removed under reduced pressure. The residue was dissolved in 500 μL of aqueous CH_3CN, and each of the 100-μL aliquots was applied to preparative HPLC on Cosmosil $5C_{18}$ AR. CBCA was eluted with CH_3CN, and the CD spectrum was measured in the eluate. CBC was derived by heating the reaction mixture at 120 °C for 5 min, followed by preparative HPLC as described above.

Optical Resolution of CBC. Chiral HPLC was conducted with Chiralcel OD-R (4.6 × 250 mm, Daicel). CBC was eluted at flow rate of a 1 mL/min with 70% aqueous CH_3CN.

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