Enzymology:

Purification and Characterization of Cannabidiolic-acid Synthase from *Cannabis sativa* L.: BIOCHEMICAL ANALYSIS OF A NOVEL ENZYME THAT CATALYZES THE OXIDOCYCLIZATION OF CANNABIGEROLIC ACID TO CANNABIDIOLIC ACID

Futoshi Taura, Satoshi Morimoto and Yukihiro Shoyama

J. Biol. Chem. 1996, 271:17411-17416.
doi: 10.1074/jbc.271.29.17411

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 29, Issue of July 19, pp. 17411–17416, 1996
Printed in U.S.A.

Purification and Characterization of Cannabidiolic-acid Synthase from *Cannabis sativa* L.

BIOCHEMICAL ANALYSIS OF A NOVEL ENZYME THAT CATALYZES THE OXIDOCYCLIZATION OF CANNABIGEROLIC ACID TO CANNABIDIOLIC ACID*

(Received for publication, February 9, 1996, and in revised form, April 26, 1996)

Futoshi Taura, Satoshi Morimoto‡, and Yukihiro Shoyama

From the Faculty of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan

We identified a unique enzyme that catalyzes the oxidocyclization of cannabigerolic acid to cannabidiolic acid (CBDA) in *Cannabis sativa* L. (CBDA strain). The enzyme, named CBDA synthase, was purified to apparent homogeneity by a four-step procedure: ammonium sulfate precipitation followed by chromatography on DEAE-cellulose, phenyl-Sepharose CL-4B, and hydroxylapatite. The active enzyme consists of a single polypeptide with a molecular mass of 74 kDa and a pI of 6.1. The NH_2-terminal amino acid sequence of CBDA synthase is similar to that of Δ^1-tetrahydrocannabinolic-acid synthase. CBDA synthase does not require coenzymes, molecular oxygen, hydrogen peroxide, and metal ion cofactors for the oxidocyclization reaction. These results indicate that CBDA synthase is neither an oxygenase nor a peroxidase and that the enzymatic cyclization does not proceed via oxygenated intermediates. CBDA synthase catalyzes the formation of CBDA from cannabinerolic acid as well as cannabigerolic acid, although the k_{cat} for the former ($0.03\ s^{-1}$) is lower than that for the latter ($0.19\ s^{-1}$). Therefore, we conclude that CBDA is predominantly biosynthesized from cannabigerolic acid rather than cannabinerolic acid.

Cannabinoids are plant secondary metabolites possessing alkylresorcinol (typically olivetol or olivetolic acid) and monoterpene groups in their molecules (Fig. 1). Numerous cannabinoids have been isolated from marijuana or fresh *Cannabis* leaves, and their chemical and pharmacological properties have been extensively investigated (1). Among them, Δ^1-tetrahydrocannabinol is the psychoactive principle of marijuana (2). On the other hand, cannabidiolic acid (CBDA)[1] and cannabidiol do not exert psychotropic effects, but both cannabinoids possess a variety of pharmacological activities. For example, CBDA displays a potent antimicrobial effect (3), while cannabidiol reduces aggressive behavior in the L-pyroglutamate-treated rat, spontaneous dyskinesias in the dystonic rat, and turning behavior in the 6-hydroxyldopamine-treated rat caused by apomorphine (4). Therefore, CBDA and cannabidiol have attracted considerable attention as having therapeutic potential in various disorders (5).

Several pathways have been proposed to explain the biosynthesis of these cannabinoids. Typical biosynthetic schemes have been based on the assumptions that Δ^1-tetrahydrocannabinolic acid (Δ^1-THCA), the precursor of Δ^1-tetrahydrocannabinol, is synthesized by the ring closure of CBDA and that CBDA is formed from cannabigerolic acid (CBGA) via hydroxyl-CBGA (1). To confirm these assumptions, we investigated Δ^1-THCA biosynthesis by enzymological means and established that Δ^1-THCA is actually biosynthesized from CBGA by Δ^1-THCA synthase and not from the presumed precursor, CBDA (6). In contrast, it is still unknown whether the biosynthesis of CBDA proceeds through the above biosynthetic pathway. This lack of a precise understanding of CBDA biosynthesis is mostly due to the fact that the enzymes involved in CBDA formation have not hitherto been studied.

To understand the mechanism of CBDA biosynthesis, we investigated the enzymes involved in the production of CBDA. Consequently, we identified a unique enzyme (named CBDA synthase) that catalyzes the stereoselective oxidocyclization of CBGA to CBDA in the rapidly expanding leaves of the CBDA strain. In this paper, we describe the purification and biochemical properties of CBDA synthase. In addition, we present evidence that CBDA is biosynthesized from CBGA through oxidocyclization without hydroxylation.

EXPERIMENTAL PROCEDURES

Plant Materials—The two *Cannabis* strains (CBDA and Mexican strains) were grown in the herbal garden of the Faculty of Pharmaceutical Sciences, Kyushu University. CBDA synthase was extracted from rapidly expanding leaves of the 15-week-old CBDA strain, while cannabinoids were extracted from mature leaves of both strains (15-week-old plants).

Cannabinoids—CBDA and Δ^1-THCA were purified from mature leaves of the CBDA and Mexican strains, respectively, as described previously (7). Cannabidiol was prepared by heating CBDA at 120 °C for 20 min. CBG was chemically synthesized by a modification of the method of Mechoulam and Yagen (8). Olivetol (2 g; Sigma) and geraniol (3 g; Sigma) were dissolved in 400 ml of chloroform containing *p*-toluenesulfonic acid (80 mg) and stirred at room temperature for 12 h in the dark. The reaction mixture was washed with 400 ml of saturated sodium bicarbonate and then with 400 ml of water. After the chloroform layer was concentrated at 40 °C under reduced pressure, the residue was chromatographed on a 2.0 × 25-cm column of silica gel (Merck). The column was eluted with 1000 ml of benzene to give CBG (1.4 g). CBGA was obtained by carboxylation of CBG with methylmagnesium carbonate as follows (9). Magnesium metal (92 mg) was refluxed in 15 ml of absolute methanol for 2 h, and then the methanol was completely evaporated. The residue (magnesium methylate) was resuspended in dimethylformamide (0.8 ml). Using the described apparatus (10), the magnesium methylate solution was stirred under CO_2 at 4 °C for 2 h, forming a viscous solution containing methylmagnesium carbonate. CBG (120 mg) was added to the methylmagnesium carbonate solution and heated at 120 °C for 1 h. The reaction mixture was dissolved in 100 ml of chloroform/methanol (2:1), adjusted to pH ~2 with diluted HCl, and partitioned with 50 ml of water. After the organic layer was dried

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 81-92-641-1151; Fax: 81-92-641-8154; E-mail: morimoto@shoyaku.phar.kyushu-u.ac.jp.

[1] The abbreviations used are: CBDA, cannabidiolic acid; Δ^1-THCA, Δ^1-tetrahydrocannabinolic acid; CBG, cannabigerol; CBGA, cannabigerolic acid; CBNRA, cannabinerolic acid; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

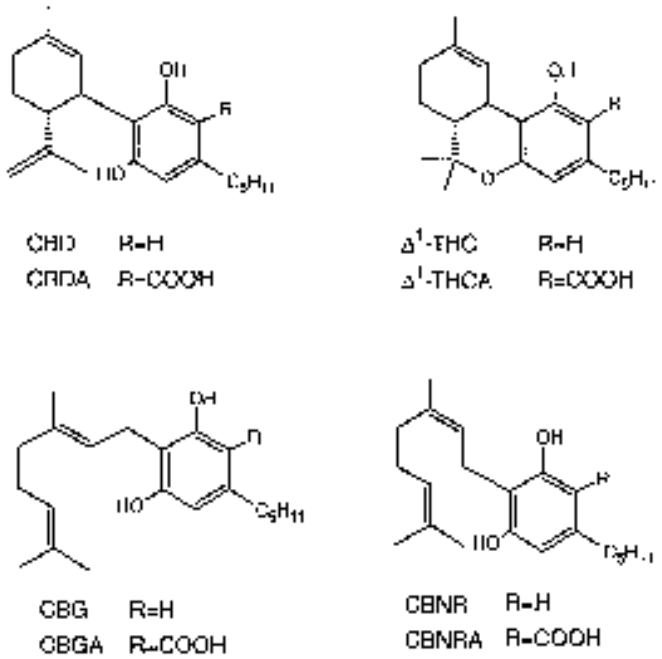

FIG. 1. **Structures of cannabinoids.** *CBD*, cannabidiol; *Δ^1-THC*, Δ^1-tetrahydrocannabinol; *CBNR*, cannabinerol.

with sodium sulfate, the solvent was removed by evaporation, and the residue was chromatographed on a 2.0 × 25-cm silica gel column. Elution with 1000 ml of *n*-hexane/ethyl acetate (2:1) gave CBGA (45 mg). Cannabinerol and cannabinerolic acid (CBNRA) were also synthesized as described above for CBG and CBGA, respectively, except for the use of nerol (Sigma) instead of geraniol (11). The structures of these cannabinoids were confirmed by obtaining their ^{1}H NMR (Varian) spectra.

CBDA Synthase Assay—The standard mixture solution consisted of 200 μM CBGA, 0.1% (w/v) Triton X-100, and 100 mM sodium citrate buffer (pH 5.0) in a total volume of 500 μl. The reaction was started by adding of 100 μl of enzyme, and the mixture was incubated at 30 °C for 2 h. After terminating the reaction with 600 μl of methanol, a 50-μl aliquot was analyzed by analytical HPLC as described below. Although the CBGA concentration was slightly above the K_m (137 μM), CBDA production was linear with respect to time and enzyme concentration. The effects of metal ions and EDTA were determined by dialyzing an enzyme preparation against buffer A (10 mM sodium phosphate buffer (pH 7.0), 3 mM mercaptoethanol) containing 2 mM EDTA (12 h) then against buffer A alone (12 h).

HPLC Conditions—The amount of CBDA in the reaction mixtures was measured using an HPLC system (Tosoh, Tokyo) composed of a CCPM pump and a UV-8000 absorbance detector equipped with a 0.46 × 15-cm column of Cosmosil 5C_{18} AR (Nacalai Tesque, Tokyo). The sample was eluted with 65% (v/v) aqueous acetonitrile containing 50 mM phosphoric acid at a flow rate of 1 ml/min. The eluate was monitored by absorption at 280 nm. The peak intensity was determined with a Chromatocorder 21 (Tosoh). The retention time and concentration of CBDA were verified by comparison with those of authentic CBDA.

Purification of CBDA Synthase—Unless otherwise indicated, all extraction and purification procedures were performed at 4 °C. Rapidly expanding leaves (80 g) of the CBDA strain were homogenized in a Waring blender at high speed together with 800 ml of extraction buffer (100 mM sodium phosphate buffer (pH 7.0), 10 mM mercaptoethanol) containing 8 g of polyvinylpolypyrrolidone (Sigma). After the homogenate was filtered through a nylon screen, the filtrate was centrifuged at 100,000 × *g* for 1 h. The supernatant was then fractionated with ammonium sulfate. Proteins precipitating at 45–75% saturation were collected by centrifugation at 20,000 × *g* for 15 min, resuspended in ~30 ml of buffer A, and dialyzed overnight against three changes of the same buffer. After insoluble materials were removed by centrifugation at 20,000 × *g* for 15 min, the dialyzed sample was applied at a flow rate of 1.7 ml/min to a 2.5 × 30-cm column of DE52 cellulose (Whatman) equilibrated with buffer A. Elution was monitored photometrically at 280 nm, and fractions of 20 ml were collected. CBDA synthase activity was found only in fractions eluted with buffer A, and further elution with 1 M NaCl did not afford any fractions containing CBDA synthase. The most active fractions (fractions 3–5) were pooled and concentrated to ~10 ml by ultrafiltration (Advantec, Tokyo). After ammonium sulfate was added to the concentrated solution to raise the concentration to 0.8 M, the sample was loaded onto a 1.5 × 15-cm column containing phenyl-Sepharose CL-4B (Pharmacia Biotech Inc.) equilibrated with buffer B (buffer A containing 0.8 M ammonium sulfate). The column was rinsed with 3 column volumes of the same buffer, and bound proteins were then eluted with a 500-ml linear gradient of buffer B to buffer A at a flow rate of 1.5 ml/min. The most active fractions (fractions 38–42, each 10 ml) were pooled, concentrated, and dialyzed overnight against three changes of buffer A. The dialysate was applied to a 1 × 10-cm column containing hydroxylapatite (Nacalai Tesque) pre-equilibrated with buffer A. After washing the column with 3 column volumes of the same buffer, bound proteins were eluted with a 200-ml linear gradient of NaCl (0–1.5 M) at a flow rate of 1.0 ml/min. The most active fractions (fractions 10–12, each 10 ml) were pooled, concentrated, and dialyzed overnight against two changes of buffer A. The purity of CBDA synthase was confirmed by SDS-PAGE analysis of the dialysate. The purified enzyme could be stored at 4 °C in buffer A for 2 weeks with a 10–20% loss of activity. Storage of the purified enzyme in 40% (w/v) glycerol at −20 °C was ineffective for its stabilization.

Isolation and Characterization of Enzymatic Product—In the presence of purified CBDA synthase (100 μg), 500 ml of the standard assay solution (see "CBDA Synthase Assay") was incubated at 30 °C for 12 h. The reaction mixture was partitioned with 100 ml of ethyl acetate, and this layer was concentrated under reduced pressure to dryness. The residue was dissolved in 500 μl of 80% (v/v) aqueous acetonitrile, and each of the 25-μl aliquots was applied to preparative HPLC as described under "HPLC Conditions." The column was eluted with 65% (v/v) aqueous acetonitrile to afford an enzymatic product (0.4 mg). Its structure was characterized as (−)-CBDA by comparison of its fast atom bombardment mass (Jeol Ltd., Tokyo) and circular dichroism (Jasco International Co. Ltd., Tokyo) spectra with those of authentic CBDA.

Protein Assay—Protein concentrations were determined according to Bradford (12) using bovine serum albumin as the standard.

Analysis of Proteins by SDS-PAGE and Isoelectric Focusing—The purified enzyme was resolved by SDS-PAGE according to Laemmli (13) using a 10% acrylamide gel (Bio-Rad) of 0.75 mm thickness at 20 mA. Proteins were visualized by Coomassie Brilliant Blue R-250 and silver staining. The subunit molecular mass of the enzyme was determined by comparison with low molecular mass protein standards (Sigma). Isoelectric focusing was conducted according to O'Farrell (14) using 7.5-mm glass tubes (Atto Corp., Tokyo). The ampholyte gradient ranged from pH 4.0 to 7.0. After electrophoresis at 750 V for 2 h, proteins were visualized by Coomassie Brilliant Blue R-250 staining. The pI of the purified enzyme was determined by comparison with marker proteins (Sigma).

Determination of Native Molecular Mass—The native molecular mass was determined by gel filtration on a 2.5 × 75-cm column of Sephacryl S-200 HR equilibrated with buffer A containing 0.1 M NaCl. CBDA synthase was eluted with the same buffer at a flow rate of 0.4 ml/min. The column was calibrated with molecular mass markers from 29 to 700 kDa (Sigma).

NH_2-terminal Sequencing of CBDA Synthase—Purified CBDA synthase (20 μg) was resolved by SDS-PAGE as described above, transferred to a polyvinylidene difluoride membrane, and NH_2-terminally sequenced on an Applied Biosystems 473A protein sequencer by standard Edman degradation. The amino acid sequence of CBDA synthase was compared with those of other known proteins using the SwissProt protein data base.

Substrate Specificity and Kinetic Parameters—CBGA and CBNRA were tested as potential substrates of CBDA synthase using the standard assay, unless otherwise stated. The K_m and V_{max} values for both substrates were determined by Lineweaver-Burk double-reciprocal plots of the velocity curves of the CBDA-producing reaction with an increasing concentration of substrates. The Michaelis-Menten equation was fitted to the data by nonlinear least-squares regression analysis.

RESULTS

Identification of CBDA Synthase in Cannabis sativa—The CBDA strain was used to identify CBDA synthase because this strain produces much more CBDA than other strains (15). Various aerial parts were collected from the 15-week-old CBDA strain and homogenized with 100 mM phosphate buffer (pH 7.0). When CBGA was used as a substrate, the homogenate from the rapidly expanding leaves exhibited potent CBDA syn-

TABLE I
Purification of CBDA synthase

Data are based on the extraction of 80 g of rapidly expanding leaves. Assays were carried out under the standard conditions described under "Experimental Procedures."

Step	Protein	Total activity	Specific activity	Recovery	Purification
	mg	*pkat*[a]	*pkat/mg*	*%*	*-fold*
100,000 × *g* supernatant	672	1957	2.91	100	1
45–70% $(NH_4)_2SO_4$	178	1658	9.31	85	3
DE52	15.8	1211	77	62	26
Phenyl-Sepharose CL-4B	0.9	561	632	29	217
Hydroxylapatite	0.2	299	1510	15	519

[a] pkat, picokatals.

thase activity (29 picokatals/g of fresh leaves). In contrast, there was much less (9 picokatals/g of fresh leaves) and no activity in the mature leaves and stems, respectively. To investigate the subcellular localization of the enzyme, the homogenate from the rapidly expanding leaves was fractionated by differential centrifugation, and the level of enzyme activity was measured in each fraction. CBDA synthase activity was undetectable in the light membrane (100,000 × *g* pellet) and heavy membrane (10,000 × *g* pellet) fractions. However, the cytosolic fraction (100,000 × *g* supernatant) contained >95% of the enzyme activity in the whole homogenate. Therefore, CBDA synthase was considered to be a soluble protein. To extract the enzyme more effectively, we tested several buffers, although extraction with citrate buffer (pH 6.0) or Tris-HCl buffer (pH 7.5) was somewhat less effective (16 or 19 picokatals/g of fresh leaves, respectively) than phosphate buffer (29 picokatals/g of fresh leaves). Based on these results, we extracted CBDA synthase from the rapidly expanding leaves with 100 mM phosphate buffer (pH 7.0).

Purification of CBDA Synthase—A four-step procedure was developed that resulted in the purification of CBDA synthase to apparent homogeneity. Table I summarizes the combination of procedures that yielded a high purification level. Since the enzyme was sensitive to oxidation, mercaptoethanol was required for successful purification of the active enzyme. During the purification of CBDA synthase, the enzyme activity was monitored by quantifying the amount of CBDA produced from the substrate CBGA.

As a first step, the cytosolic fraction from rapidly expanding leaves of the CBDA strain was fractionated by ammonium sulfate saturation. About 85% of the CBDA synthase activity precipitated between 45 and 75% saturation, resulting in a 3-fold purification. The solubilized ammonium sulfate fraction was applied to a DEAE-cellulose (DE52) column, where most of the enzyme activity was not bound to the matrix. However, this procedure substantially increased the specific activity, purifying CBDA synthase ~26-fold. After the DE52 eluate was applied to phenyl-Sepharose CL-4B, the bound enzyme was eluted with a descending gradient of ammonium sulfate from 0.8 to 0 M. This step gave a 217-fold purification, although the most active fractions were still contaminated with small amounts of several proteins. The contaminating proteins were well removed by hydroxylapatite chromatography. Since elution with an increasing gradient of phosphate buffer (0–250 mM) led to low recovery of the enzyme activity, we examined other solvent systems. The enzyme could be successfully eluted with a 0–1.5 M NaCl gradient in phosphate buffer. SDS-PAGE showed that the most active fractions from the hydroxylapatite column were >95% pure (Fig. 2).

Molecular Mass, Isoelectric Point, and NH_2-terminal Sequence of CBDA Synthase—The purified protein displayed a monomeric molecular mass of ~74 kDa by SDS-PAGE (Fig. 2). The native molecular mass was determined by gel filtration on

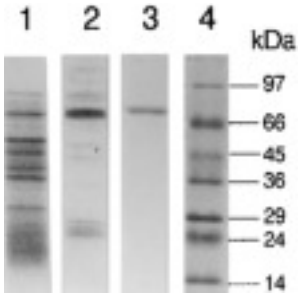

FIG. 2. **SDS-PAGE analysis of CBDA synthase.** Samples were resolved by electrophoresis on a 10% acrylamide gel. Proteins were stained with Coomassie Brilliant Blue. *Lane 1*, DE52 step; *lane 2*, phenyl-Sepharose CL-4B step; *lane 3*, hydroxylapatite step; *lane 4*, molecular standards with the indicated molecular masses.

a Sephacryl S-200 HR column under nondenaturing conditions. CBDA synthase was eluted from the column as a single molecular species with an estimated molecular mass of 75 kDa, indicating that functional CBDA synthase behaves like a monomeric enzyme. The pI for this enzyme was determined to be 6.1 by isoelectric focusing. These properties of CBDA synthase were similar to those of Δ^1-THCA synthase, which catalyzes the conversion of CBGA to Δ^1-THCA (6). In addition, Edman degradation of the purified enzyme revealed that CBDA synthase has an NH_2-terminal sequence similar to that of Δ^1-THCA synthase (Table II). The NH_2-terminal sequence of CBDA synthase shares 87% identity with that of Δ^1-THCA synthase, and the first 11 residues of both enzymes, including an unidentified residue, are identical. We compared the CBDA synthase sequence with those of other proteins in the SwissProt protein data base and found no homologies.

Product Identification—The principal product produced by purified CBDA synthase was identified as CBDA on the basis of its retention time on HPLC. To obtain further structural information, the enzymatic product isolated by preparative HPLC was structurally characterized by analyses of its circular dichroism and fast atom bombardment mass spectra. Consequently, both spectra of the enzymatic product were consistent with those of (−)-CBDA isolated from the CBDA strain. The agreement of the circular dichroism spectra indicates that CBDA synthase catalyzes the stereoselective oxidocyclization of CBGA to CBDA.

Standard Assay Conditions—Owing to the extremely hydrophobic property of cannabinoids, detergent was required to solubilize them in the assay buffer. Therefore, we investigated the effects of various detergents on CBDA synthase activity (Fig. 3). Among three detergents tested, Triton X-100 was the most favorable for the CBDA synthase reaction. The addition of up to 0.1% Triton X-100 increased the CBDA synthase activity, although concentrations over 0.2% inhibited the activity.

We also determined the pH optimum of CBDA synthase. The enzyme activity was maximal at pH 5.0, with half-maximal activities at pH values around 4.0 and 6.0. Thus, the standard

TABLE II
NH_2-terminal amino acid sequences of CBDA synthase and Δ^1-THCA synthase

Protein	Sequence[a]
CBDA synthase	Asn-Pro-Arg-Glu-Asn-Phe-Leu-Lys-*X*-Phe-Ser-Gln-Tyr-Ile-Pro-Asn-
Δ^1-THCA synthase	Asn-Pro-Arg-Glu-Asn-Phe-Leu-Lys-*X*-Phe-Ser-Lys-His-Ile-Pro-Asn-

[a] The letter *X* indicates that the identity of the residue was ambiguous.

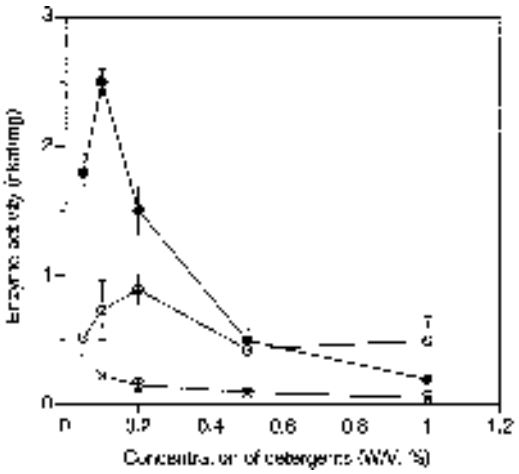

FIG. 3. **Effects of detergents on CBDA synthase activity.** The assay solvents consisted of 100 mM citrate buffer (pH 5.0) containing various concentrations of Triton X-100 (●), Tween 20 (○), and Emulgen 911 (×). The minimal concentrations of detergents required to dissolve CBGA completely in citrate buffer were 0.05% Triton X-100, 0.05% Tween 20, and 0.1% Emulgen 911. In the presence of the purified enzyme (0.66 μg), all assay mixtures were incubated at 30 °C for 2 h. The enzyme activity was detected as described under "Experimental Procedures." Data are means of three replicate assays.

assay conditions included citrate buffer (pH 5.0) containing 0.1% Triton X-100. Under these conditions, the amount of CBDA produced by CBDA synthase was linear with the incubation period for at least 4 h, and the rate of the enzymatic reaction was linear with respect to the amount of enzyme added (data not shown).

Effects of Metal Ions, Coenzymes, and Oxidoreductase Inhibitors on CBDA Synthase Activity—CBDA synthase is an oxidoreductase that catalyzes the cyclization of the monoterpene moiety in CBGA. Since most terpene cyclases require a divalent ion such as Mg^{2+} or Mn^{2+} for the cyclization of substrates (16), we first examined the effects of various metal ions on CBDA synthase activity. However, Mg^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+}, and Cu^{2+} (2 mM each) had no significant effect. In contrast, Hg^{2+} (2 mM) completely inhibited the enzyme activity. This inhibition might be due to the nonenzymatic degradation of the substrate because CBGA is very unstable in assay buffer containing Hg^{2+}.[2] On the other hand, the chelating agent EDTA had little influence on the enzyme activity at concentrations up to 5 mM. Therefore, it seems likely that CBDA synthase does not require metal ions for the oxidocyclization of CBGA.

Furthermore, a variety of cofactors, coenzymes, and inhibitors of oxidoreductases were also examined to understand the mechanism of oxidocyclization catalyzed by CBDA synthase. As shown in Table III, CBDA synthase did not require molecular oxygen for the production of CBDA in the presence or absence of NADPH. The cytochrome P-450 inhibitors CO, triazole, and KSCN (17) also had no effect on CBDA synthase activity. Hydrogen peroxide neither activated nor inhibited the

[2] S. Morimoto and Y. Shoyama, unpublished data.

TABLE III
CBDA synthase activity under various conditions

All assays were conducted using 0.66 μg of purified CBDA synthase and 200 μM CBGA. The relative activity of 100% is 2.0 nanokatals/mg, and the standard deviation was always within 5% of the mean of triplicate determinations.

Conditions	Relative activity	Conditions	Relative activity
	%		%
Standard	100	2 mM 2-methyl-2-nitrosopropane	104
N_2[a]	101	1 mM H_2O_2	100
1 mM NADPH	99	1 mM NAD	94
1 mM NADPH + N_2[a]	102	1 mM NADP	97
1 mM NADPH + CO[b]	99	1 mM FAD	70
2 mM triazole	106	1 mM FMN	24
2 mM KSCN	101		

[a] Molecular oxygen in the assay solution was removed by nitrogen purge for 5 min.
[b] The assay solution was saturated with CO gas (purge for 5 min).

enzyme activity. These experiments show that CBDA synthase is neither an oxygenase nor a peroxidase. Furthermore, it was not inhibited by the spin trap agent 2-methyl-2-nitrosopropane, confirming that radical species do not appear in the enzymatic reaction. Judging from these catalytic properties of CBDA synthase, the enzymatic cyclization of CBGA proceeds through a dehydrogenation process that is not accompanied by oxygenation and radical production. However, this dehydrogenation does not require coenzymes such as NAD, NADP, FAD, and FMN. The more effective inhibition by both flavins was due to nonenzymatic degradation of CBGA (6).

Substrate Specificity—CBDA synthase exhibited Michaelis-Menten kinetics in response to changes in CBGA concentration. The enzyme displayed a maximal specific activity of 2.57 nanokatals/mg for this substrate, corresponding to a k_{cat} of 0.19 s^{-1} (Table IV). This turnover number seemed to be relatively low, but it was almost identical to that for Δ^1-THCA formation by Δ^1-THCA synthase (k_{cat} = 0.20 s^{-1}) (6). CBNRA, the *Z*-isomer of CBGA, was also converted by CBDA synthase to CBDA, although the enzyme showed lower k_{cat} and higher K_m values for CBNRA as compared with CBGA (Table IV). In contrast to the acidic cannabinoids, the neutral cannabinoids, CBG and cannabinerol, did not undergo oxidocyclization by CBDA synthase, indicating that a carboxyl group in the substrate is essential for the enzymatic cyclization of the monoterpene moiety.

DISCUSSION

Despite a lack of experimental evidence, it has been believed that CBDA is biosynthesized from CBGA via hydroxyl-CBGA (1). Several groups have attempted to confirm this hypothesis by feeding experiments, although they could not obtain unequivocal results owing to the low incorporation of labeled precursors into CBDA (15, 18). To definitively establish the biosynthetic mechanism of CBDA, we directly investigated the enzyme (CBDA synthase) that catalyzes the formation of CBDA. Since this enzyme had not been studied, we first attempted to identify CBDA synthase under various conditions. After unsuccessful attempts, CBDA synthase could be ex-

TABLE IV
Kinetic parameters for CBDA synthase activity

Assays were performed using 0.66 μg of purified CBDA synthase. Data are means of three replicate assays.

Cannabinoid	V_{max}	k_{cat}[a]	K_m
	nkat[b]*/mg*	s^{-1}	*mM*
CBGA	2.57	0.19	0.137
CBNRA	0.39	0.03	0.206
CBG	—[c]	—	—
CBNR	—	—	—

[a] k_{cat} was calculated using a subunit molecular mass of 74 kDa and one active site/subunit.
[b] nkat, nanokatals; CBNR, cannabinerol.
[c] —, no activity was detected.

tracted with phosphate buffer from the CBDA strain. Higher enzyme activity was observed in rapidly expanding leaves than in mature leaves. This distribution of CBDA synthase correlates well with the CBDA content; a higher amount of CBDA is found in rapidly expanding leaves (11.9 mg/g of fresh leaves) than in mature leaves (3.3 mg/g of fresh leaves).[2] These findings indicate that CBDA is predominantly biosynthesized by CBDA synthase in rapidly expanding leaves of the CBDA strain. Previously, we demonstrated that biosynthesis of Δ^1-THCA also predominantly occurs in rapidly expanding leaves of the Mexican strain (6). The roles of CBDA and Δ^1-THCA in plants remain largely unclear, but these cannabinoids may play an important role in leaf development.

CBDA synthase was purified 519-fold by a four-step procedure that yielded up to 15% final recovery of the enzyme activity. Purification of this enzyme to homogeneity permitted the characterization of its precise properties, resulting in a variety of new findings. In particular, it is noteworthy that the oxidocyclization of CBGA by CBDA synthase is not accompanied by oxygenation (Fig. 4), contrary to the published hypothesis of CBDA biogenesis. Oxygenase-type enzymes catalyzing the cyclization of terpene groups have been identified in several plants (19), although cyclases that catalyze the direct dehydrogenation of terpene groups have rarely been found in the plant kingdom. Crombie *et al.* (20) have demonstrated that deguelin, the isoflavonoids in *Tephrosia vogelii*, is formed through the prenyl cyclization of rot-2-enoic acid by deguelin cyclase and that, like CBDA formation, this reaction proceeds through direct hydrogenation without a cofactor requirement. However, it is quite difficult to precisely compare the kinetic and physical properties of deguelin cyclase and CBDA synthase since deguelin cyclase was not purified to homogeneity.

Concerning the substrate specificity, CBDA synthase catalyzes the formation of CBDA from CBNRA as well as CBGA (Fig. 4). Since the C-1–C-2 double bond of CBDA has the same configuration as that of CBNRA, CBDA formation could proceed from CBGA through CBNRA to CBDA. However, CBDA synthase displayed much higher activity for CBGA than CBNRA, indicating that CBNRA is not an intermediate in the oxidocyclization of CBGA into CBDA. In addition, the lower substrate specificity for CBNRA suggests that CBDA is biosynthesized predominantly from CBGA rather than CBNRA. This is supported by the fact that the content of CBNRA in the CBDA strain is much lower than that of CBGA (0.08 *versus* 2.8 mg/g of rapidly expanding leaves).[2]

Kinetic properties similar to those of CBDA synthase have been described for limonene synthase, which mediates the formation of limonene with higher V_{max} and lower K_m values for geranyl pyrophosphate as compared with neryl pyrophosphate (18). However, the cyclization catalyzed by limonene synthase is not accompanied by oxidation (16). Moreover, all terpene cyclases, including limonene synthase, require either Mg^{2+} or

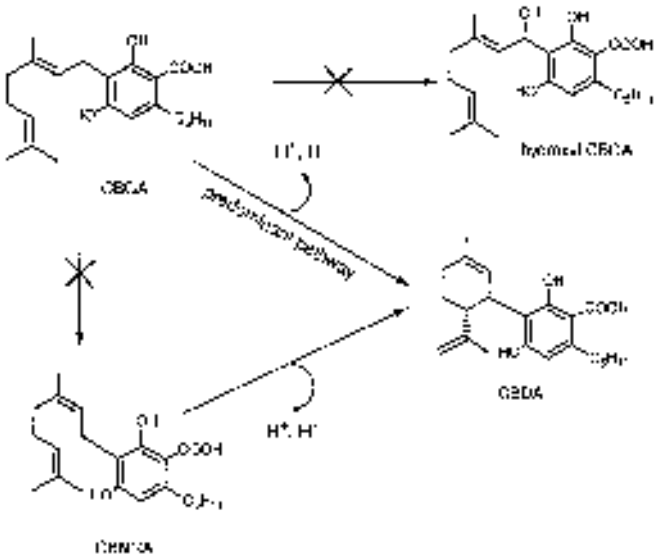

FIG. 4. **CBDA biosynthesis catalyzed by CBDA synthase.**

Mn^{2+}, contrary to CBDA synthase. To explain the roles of Mg^{2+} and Mn^{2+} in terpene cyclization, Croteau (16) has proposed that these metal ions might neutralize the negative charge of the diphosphate moiety and assist in ionization of the allylic diphosphate substrate. Since CBGA has no allylic diphosphate moiety, it is reasonable that CBDA synthase has no requirement for Mg^{2+} and Mn^{2+}. Although the low turnover number for CBGA (0.19 s^{-1}) suggests that CBDA synthase also may require some cofactors, we could not demonstrate either cofactors or coenzymes that activate the enzyme activity. However, since a much lower or a similar turnover number (k_{cat} = 0.01–0.3 s^{-1}) has been reported for some terpene cyclases (16, 21–24), it is understandable that cofactors and coenzymes are not essential for the CBDA synthase reaction.

Many biochemical properties of CBDA synthase are closely related to those of Δ^1-THCA synthase. As reported (6), Δ^1-THCA synthase catalyzes the oxidocyclization of CBGA with a higher turnover number (0.20 s^{-1}) for CBGA than for CBNRA, and this reaction has no requirement for cofactors, coenzymes, and molecular oxygen. In addition, the molecular mass, pI, and NH_2-terminal sequence of both enzymes are quite similar. Although CBDA has a different ring system from Δ^1-THCA, these similarities suggest that both cannabinoids are formed by a similar reaction mechanism.

Acknowledgments—We thank Yoshitsugu Tanaka, Kyoko Soeda, and Dr. Ryuichi Isobe for NMR and mass measurements of cannabinoids. We acknowledge Dr. Yuji Ito for helpful advice and discussions.

REFERENCES

1. Mechoulam, R. (1970) *Science* **168,** 1159–1166
2. Gaoni, R., and Mechoulam, R. (1964) *J. Am. Chem. Soc.* **86,** 1946–1947
3. Petri, G. (1988) in *Biotechnology in Agriculture and Forestry: Medicinal and Aromatic Plants I* (Bajaj, Y. P. S., ed) Vol. 4, pp. 333–349, Springer-Verlag, Heidelberg, Germany
4. Consroe, P., Sandyk, R., and Snider, S. R. (1986) *Int. J. Neurosci.* **30,** 277–282
5. Consroe, P., Laguna, J., Allender, J., Snider, S. R., Stern, L., Sandyk, R., Kennedy, K., and Schram, K. (1991) *Pharmacol. Biochem. Behav.* **40,** 701–708
6. Taura, F., Morimoto, S., Shoyama, Y., and Mechoulam, R. (1995) *J. Am. Chem. Soc.* **117,** 9766–9767
7. Shoyama, Y., Yamauchi, T., and Nishioka, I. (1970) *Chem. & Pharm. Bull. (Tokyo)* **18,** 1327–1332
8. Mechoulam, R., and Yagen, Y. (1969) *Tetrahedron Lett.* **1969,** 5349–5352
9. Mechoulam, R., and Ben-Zvi, Z. (1969) *J. Chem. Soc. D* **1969,** 343–344
10. Shoyama, Y., Hirano, H., and Nishioka, I. (1978) *J. Labelled Compd. Radiopharm.* **14,** 835–842
11. Taura, F., Morimoto, S., and Shoyama, Y. (1995) *Phytochemistry (Oxf.)* **39,** 457–458

12. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
13. Laemmli, U. K. (1970) *Nature* **227,** 680–685
14. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250,** 4007–4021
15. Shoyama, Y., Yagi, M., Nishioka, I., and Yamauchi, T. (1975) *Phytochemistry* (*Oxf.*) **14,** 2189–2192
16. Croteau, R. (1987) *Chem. Rev.* **87,** 929–954
17. Imai, Y., and Sato, R. (1967) *Eur. J. Biochem.* **1** 419–426
18. Kajima, M., and Piraux, M. (1982) *Phytochemistry* (*Oxf.*) **21,** 67–69
19. Tahara, S., and Ibrahim, R. K. (1995) *Phytochemistry* (*Oxf.*) **38,** 1073–1094
20. Crombie, L., Rossiter, J. T., Van Bruggen, N., and Whiting, D. A. (1992) *Phytochemistry* (*Oxf.*) **31,** 451–461
21. Rajaonarivony, J. I. M., Gershenzon, J., and Croteau, R. (1992) *Arch. Biochem. Biophys.* **296,** 49–57
22. Alonso, W. R., and Croteau, R. (1991) *Arch. Biochem. Biophys.* **286,** 511–517
23. Hohn, T. M., and Plattner, R. D. (1989) *Arch. Biochem. Biophys.* **272,** 137–143
24. Cane, D. E., and Pargellis, C. (1987) *Arch. Biochem. Biophys.* **254,** 421–429