PURIFICATION AND CHARACTERIZATION OF CANNABICHROMENIC ACID SYNTHASE FROM *CANNABIS SATIVA*

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Abstract—Cannabichromenic acid synthase was purified to apparent homogeneity by sequential column chromatography including DEAE-cellulose, phenyl-Sepharose CL-4B, and hydroxylapatite. The enzyme catalysed the oxidocyclization of cannabigerolic acid and cannabinerolic acid to cannabichromenic acid. The K_m values for both substrates were in the same order of magnitude although the V_{max} value for the former was higher than that for the latter. These results suggested that cannabichromenic acid is predominantly formed from cannabigerolic acid rather than cannabinerolic acid. The enzyme required neither molecular oxygen nor hydrogen peroxide, indicating that the cannabichromenic acid synthase reaction proceeds through direct dehydrogenation without hydroxylation. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Cannabinoids are novel secondary metabolites found only in marijuana (*Cannabis sativa* L.), and more than sixty cannabinoids have been isolated and structurally elucidated (Fig. 1). Among them, the biological activities of the psychoactive cannabinoid, Δ^1-tetrahydrocannabinol have been thoroughly investigated [1]. Other cannabinoids also show a variety of interesting pharmacological activities. For example, cannabichromene (CBC) and its related cannabinoids possess anti-inflammatory, anti-fungal and anti-microbial effects [2], while the analgetic effect induced by Δ^1-tetrahydrocannabinol is potentiated by CBC [3]. Thus, CBC has attracted considerable attention, whereas little was known about the biosynthesis of cannabichromenic acid (CBCA), the precursor of CBC.

Recently, we have identified an enzyme (CBCA synthase) catalysing the highly stereoselective conversion of cannabigerolic acid (CBGA) to CBCA in young leaves of *C. sativa* [4]. However, since a crude enzyme preparation was used to characterize the properties of CBCA synthase, the mechanism of CBCA biosynthesis has remained ambiguous. In order to evaluate the precise properties of CBCA synthase, we attempted to purify the enzyme from young leaves of *C. sativa* and characterize its biochemical properties. In this report, we describe the purification and characterization of CBCA synthase.

RESULTS AND DISCUSSION

Purification of CBCA synthase

The purification of CBCA synthase from the cannabidiolic acid (CBDA) strain was carried out using the extraction and assay methods as previously established [4]. Consequently, we developed a four-step procedure that resulted in the purification of CBCA synthase to homogeneity. The typical data from the purification of CBCA synthase are shown in Table 1. Since the presence of CBDA synthase, which catalyses the formation of CBDA from CBGA, was confirmed in the CBDA strain [5], its activity as well as CBCA synthase activity was assayed to avoid the contamination of CBDA synthase.

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

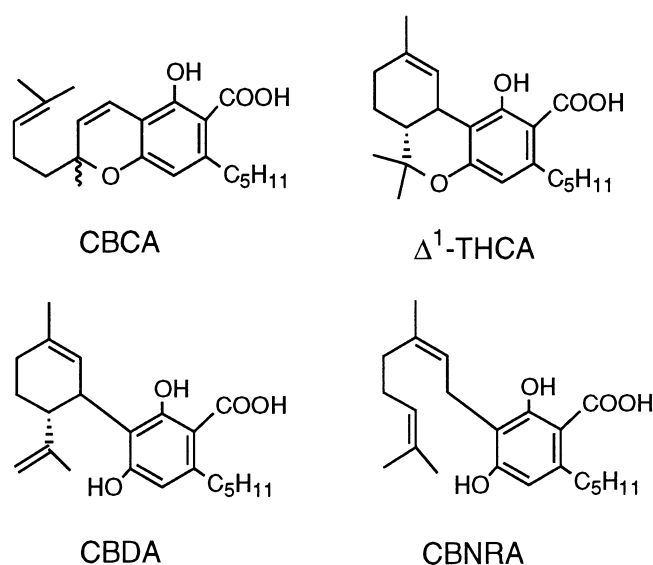*Author to whom correspondence should be addressed.

Fig. 1. Structures of cannabinoids.

activity precipitated between 30 and 75% saturation of ammonium sulfate, resulting in a 3-fold purification. The CBCA-synthase-active fraction was applied to a DEAE-cellulose column. The enzyme activity did not bind to the matrix, and most of the activity was recovered in the void volume. The void volume fractions still displayed weak CBDA synthase activity, but this chromatography yielded a 28-fold purification. CBCA synthase was further purified by applying the DEAE-cellulose eluate onto a phenyl-Sepharose CL-4B hydrophobic interaction column. Elution of the bound enzyme by a descending gradient of ammonium sulfate led to a 226-fold purification. The CBCA-synthase-active fractions showed no CBDA synthase activity, although they were still contaminated with several proteins (Fig. 2). Finally, the enzyme was purified to homogeneity by hydroxylapatite chromatography using an increasing gradient of NaCl. At individual steps of the purification procedure, the intensity of the 71 kDa band increased on SDS-PAGE as the preparation was enriched in activity, and the hydroxylapatite fraction migrated as a single band (Fig. 2). CBCA synthase was the first enzyme involved in CBCA biosynthesis to be purified.

Subunit mass, native mass and isoelectric point of CBCA synthase

SDS-PAGE of the purified CBCA synthase showed a subunit molecular mass of 71 kDa (Fig. 2). The native molecular mass was estimated from the elution volume of CBCA synthase on Sephacryl S-200 HR chromatography, where the enzyme eluted as a single molecular species with a molecular mass of 136 kDa. These results suggested that CBCA synthase exists as a homodimer composed of two identical 71 kDa subunits. On the other hand, the pI for CBCA synthase was determined to be 7.3 by comparison with marker proteins of known pI on isoelectrofocusing gels.

Substrate specificity

When the purified CBCA synthase was tested with CBGA and cannabinerolic acid (CBNRA), the Z-isomer of CBGA, it became clear that the enzyme catalyses the formation of CBCA from both substrates. Since these reactions followed Michaelis-Menten kinetics in response to changes in the concentrations of CBGA and CBNRA, the V_{max} and K_m values were determined by Lineweaver-Burk double reciprocal plots (Fig. 3). The V_{max} value

Table 1. Purification of CBCA synthase from *Cannabis sativa* (CBDA strain)*

Step	Protein (mg)	Total activity (pkat/mg)	Specific activity (pkat/mg)	Recovery (%)	Purification (fold)
100 000 g Supernatant	267	508	1.9	100	1
30–75% $(NH_4)_2SO_4$	77.6	435	5.6	86	3
DEAE-cellulose (DE-52)	2.1	113	54	22	28
Pheny-Sepharose CL-4B	0.09	38	430	5.6	226
Hydroxylapatite	0.02	13	607	2.6	319

*Data are based on the extraction of 30 g of rapidly expanding leaves.
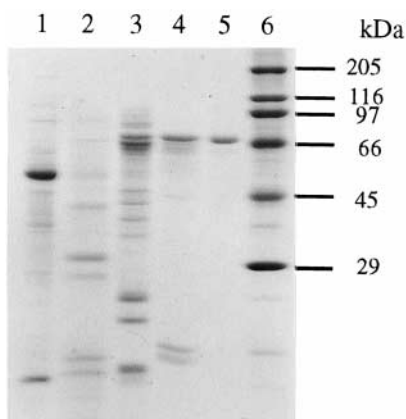
Fig. 2. SDS-PAGE analysis of CBCA synthase. Samples from each purification step were resolved by electrophoresis on a 12.5% acrylamide gel. Proteins were stained with Coomassie Brilliant Blue. Lane 1, soluble fraction; lane 2, ammonium sulfate precipitation; lane 3, DE-52 column; lane 4, phenyl-Sepharose CL-4B column; lane 5, hydroxylapatite column; lane 6, molecular standards with the indicated molecular masses.

(0.63 nkat/mg) for CBGA was higher than that (0.14 nkat/mg) for CBNRA, suggesting that CBCA is predominantly biosynthesized from CBGA rather than CBNRA. On the other hand, the K_m values for CBGA (23 μM) and CBNRA (33 μM) were similar to each other, but lower than those of CBDA synthase (134 μM for CBGA and 254 μM for CBNRA) and Δ^1-tetrahydrocannabinolic acid (Δ^1-THCA) synthase (137 μM for CBGA and 206 μM for CBNRA) that catalyse the formation of CBDA and Δ^1-THCA, respectively [5, 6]. These
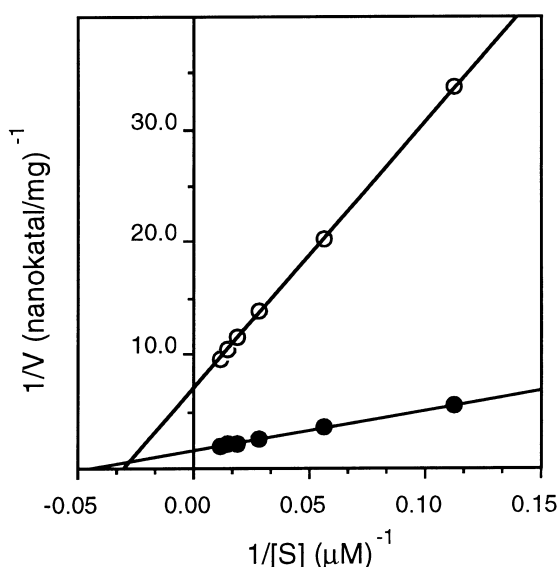

Fig. 3. Kinetic analyses of CBCA synthase. CBCA synthase activity was measured using various concentrations of CBGA (●) and CBNRA (○). Double-reciprocal plots of the velocity data are shown. The velocity data were fitted by the nonlinear least squares test to determine the apparent K_m and V_{max} for both substrates.

kinetic data indicate that CBCA synthase has higher affinity for CBGA than CBDA synthase and Δ^1-THCA synthase. In contrast, the turnover number ($k_{cat} = 0.04$ s^{-1}) was lower than those reported for Δ^1-THCA synthase ($k_{cat} = 0.20$ s^{-1}) and CBDA synthase ($k_{cat} = 0.19$ s^{-1}) [5, 6]. However, judging from the k_{cat}/K_m values, we concluded that CBGA is an efficient substrate for CBCA biosynthesis because CBCA synthase displays a higher value (1717 M^{-1}S^{-1}) for CBGA than CBDA (1492 M^{-1}S^{-1}) and Δ^1-THCA synthases (1382 M^{-1}S^{-1}).

Biochemical properties of CBCA synthase

CBCA synthase is an oxidoreductase that catalyses the cyclization of CBGA. Hence the properties of CBCA synthase were investigated using cofactors, coenzymes and inhibitors of oxidoreductases. As shown in Table 2, hydrogen peroxide apparently inhibits the enzyme activity, indicating that CBCA synthase is not a peroxidase. On the other hand, CBCA synthase did not require molecular oxygen for the oxidocyclization of CBGA, suggesting that it is not an oxygenase like P-450. This was further confirmed by the fact that P-450 inhibitors such as CO, KSCN [7] and triazole little affect CBCA synthase activity. From these results, it became evident that the oxidocyclization of CBGA to CBCA is not accompanied by hydroxylation. In addition, radical formation was not observed during the enzymatic oxidation, because the radical scavenger, 2-methyl-2-nitrosopropane did not significantly affect the enzyme activity [8]. Thus, the enzymatic reaction was considered to proceed through direct dehydrogenation without hydroxylation and radical formation, although CBCA synthase did not require the typical coenzymes (NAD, NADP, FAD and FMN) of dehydrogenases. CBCA biosynthesis has

Table 2. CBCA synthase activity under various conditions*

Conditions	Relative activity (%)
Standard	100
1 mM H_2O_2	54
N_2†	98
CO‡	103
2 mM Triazole	97
2 mM KSCN	108
2 mM 2-Methyl-2-nitrosopropane	103
1 mM NADPH	99
1 mM NAD	94
1 mM NADP	97
1 mM FAD	99
1 mM FMN	32

* All assay mixtures contained 0.11 μg of purified CBCA synthase and 200 μM CBGA. The relative activity of 100% is 0.6 nkat/mg, and the SD was always within 5% of the mean of triplicate determinations.
† Molecular oxygen in the assay mixture was removed by nitrogen purge for 5 min.
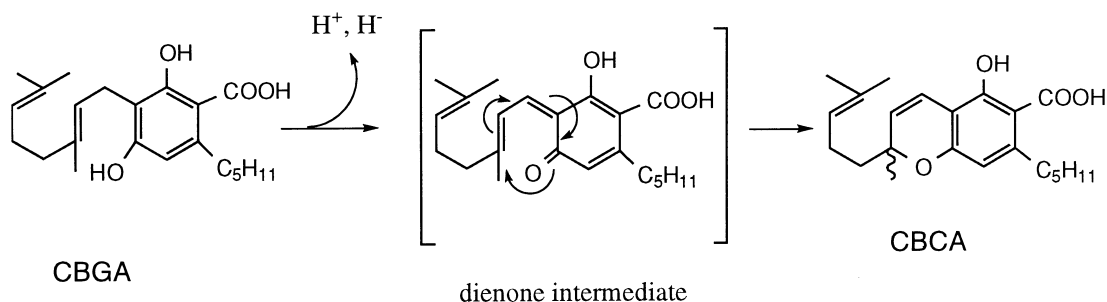‡ Assay solution was saturated with CO gas (purged for 5 min).

Fig. 4. Mechanism of CBCA biosynthesis by CBCA synthase.

hitherto been assumed to proceed via a hydroxy or a dienone intermediate [9], but the properties of CBCA synthase apparently indicate that CBGA does not cyclise to CBCA via the hydroxy intermediate. Therefore, it seems likely that the conversion of CBGA to CBCA involves the dienone intermediate (Fig. 4). The formation of a chromen ring by dienone cyclisation has also been reported for the biosynthesis of the rotenoid deguelin, and it was demonstrated that deguelin cyclase catalyses the formation of deguelin from rot-2′-enoic acid via a dienone intermediate [10]. It is notable that like CBCA synthase deguelin cyclase is assumed not to be a P450-type enzyme [8].

The metal ion requirement of CBCA synthase was also examined. However, none of the metal ions tested (Mg^{2+}, Zn^{2+}, Ca^{2+} and Cu^{2+}, 2 mM each) stimulated the enzyme activity, and Hg^{2+} (1 mM) completely inhibited the reaction. The chelating agent EDTA slightly increased the enzyme activity (10% stimulation), suggesting that the enzymatic reaction does not require metal ions.

Oxidoreductases that require neither cofactors nor coenzymes are rarely found in plants, but these biochemical properties of CBCA synthase resemble those of CBDA and Δ^1-THCA synthases [5, 6]. Thus, these cannabinoid synthases are unique oxidoreductases catalysing the cyclization of terpene moiety. Although various monoterpene cyclases that catalyse the cyclization of geranyl pyrophosphate have been confirmed in several plants [11–15], they are not oxidoreductases.

EXPERIMENTAL

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

Assays of enzyme activities. CBCA synthase activity was assessed by incubating enzyme (50 μl) in substrate buffer (0.4 ml) containing 0.2 mM CBGA, 0.05% (w/v) SDS and 0.1 M Na-Pi (pH 6.5) at 30° for 2 hr [4]. After terminating the reaction with 450 μl of MeOH, a 50-μl aliquot was applied to

analytical HPLC under the same conditions as described previously [4]. The effects of metal ions and EDTA were determined by dialysing an enzyme preparation against buffer A (10 mM Na-Pi buffer (pH 7.0), 3 mM 2-mercaptoethanol) containing 2 mM EDTA (12 hr), then against buffer A alone (12 hr). CBDA synthase activity was measured as described previously [5].

Extraction and purification of CBCA synthase. Unless otherwise indicated, all extraction and purification procedures were performed at 4°. Young leaves (30 g) of CBDA strain were homogenized in a Waring blender at high speed together with 300 ml of extraction buffer (10 mM Na-Pi buffer (pH 7), 1 M NaCl, 10 mM 2-mercaptoethanol). After the homogenate was filtered through a Nylon screen, the filtrate was centrifuged at 100 000 g for 1 hr. The supernatant was then fractionated with $(NH_4)_2SO_4$. Proteins precipitating at 30–75% sat were collected by centrifugation at 20 000 g for 15 min, re-suspended in *ca* 30 ml of buffer A and dialysed overnight against three changes of the same buffer. Insoluble materials were removed by centrifugation at 20 000 g for 15 min. The supernatant was applied at a flow rate of 1 ml/min to a 1.5 × 15 cm column of DE-52 cellulose equilibrated with buffer A. After washing with 150 ml of buffer A, the column was developed with buffer A containing 0.5 M NaCl. Elution was monitored photometrically at 280 nm, and the eluent was collected in 15 ml fractions. Most of CBCA synthase activity was found in the fractions eluted with buffer A. The active fractions (fractions 2–3) from the DE-52 column were concd to 6 ml and brought to 0.7 M $(NH_4)_2SO_4$ by adding 6 ml of buffer A containing 1.4 M $(NH_4)_2SO_4$. The sample was passed over a 1 × 10 cm column containing phenyl-Sepharose CL-4B equilibrated with buffer B (buffer A containing 0.7 M $(NH_4)_2SO_4$). The column was rinsed with 3 column vols of the same buffer, and bound proteins were then eluted by a 300 ml linear gradient of buffer B to buffer A at a flow rate of 1 ml/min. The most active fractions (fractions 10–12, each 15 ml) were pooled, concd and dialysed overnight against three changes of buffer A. The dialysed sample was applied to a 1 × 10 cm column containing hydroxyl-

apatite equilibrated with buffer A. After washing with 3 column vols 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 ml/min. The most active fractions (fractions 14–16, each 8 ml) were pooled, concd and dialysed overnight against two changes of buffer A. The purity of CBCA synthase was confirmed by SDS-PAGE analysis of the dialysate.

Analysis of proteins by SDS-PAGE and isoelectric focusing. SDS-PAGE was carried out with the system of ref. [16] in a 12.5% acrylamide gel of 0.75 mm thickness. Samples were dialysed against H_2O, lyophilized, resuspended in SDS sample buffer and denatured in a steam bath (95°) for 2 min before loading. Gels were run at a constant current of 30 mA for about 2 hr. Proteins were visualized by Coomassie Brilliant Blue R-250 and silver staining. The subunit molecular mass of the enzyme was determined by comparison with high molecular mass protein standards. Isoelectric focusing was conducted according to ref. [17] using 7.5 mm glass tubes. The ampholyte gradient ranged from pH 4.0 to pH 7.0. After electrophoresis at 750 V for 2 hr, proteins were visualized by Coomassie Brilliant Blue R-250 staining. The pI of the purified enzyme was determined by comparison with marker proteins.

Determination of native molecular mass. The native molecular mass of CBCA synthase was determined by gel filtration chromatography on a 1.5×75 cm column of Sephacryl S-200 HR equilibrated with buffer A at a flow rate of 0.3 ml/min. Fractions of 15 ml were collected. Molecular mass markers from 29 to 700 kDa were resolved under the same conditions prior to running the fraction containing CBCA synthase activity.

Substrate specificity and kinetic parameters. CBGA and CBNRA were tested as potential substrates of CBCA 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 CBCA-producing reaction with an increasing concn of substrates.

Protein assay. Protein concentrations were measured according to ref. [18] using bovine serum albumin as the standard.

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