

REVIEW**Phytocannabinoids in *Cannabis sativa*: Recent Studies on Biosynthetic Enzymes**

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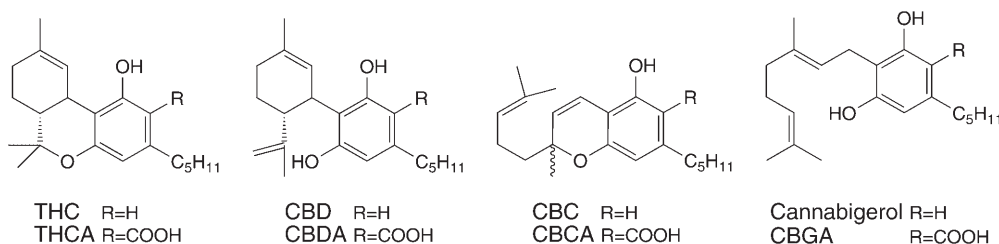
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1. Introduction. – Cannabinoids, which are found only in *Cannabis sativa*, are secondary metabolites featuring alkylresorcinol and monoterpene moieties in their molecules. More than 60 cannabinoids have been isolated from marijuana or fresh *Cannabis* leaves, and their pharmacological properties have been extensively investigated [1]. Among them, Δ^9 -tetrahydrocannabinol (THC) is the well-known psychoactive component of marijuana [2]. In addition, this cannabinoid is shown to exert a variety of therapeutic activities such as the relief of nausea caused by cancer chemotherapy [3] and the suppression of spasticity associated with multiple sclerosis [4]. Furthermore, recent studies have demonstrated that THC activates two types of cannabinoid receptors (termed CB1 and CB2), which are expressed in the mammalian brain and immune cells, respectively [5][6]. Thus, THC has attracted a great deal of attention [7].

Besides THC, several cannabinoids also show various interesting activities, although they do not activate cannabinoid receptors. For example, cannabidiol (CBD), the isomer of THC, is a potent antioxidative and anti-inflammatory agent to provide neuroprotection in acute and chronic neurodegeneration [8][9]. This



cannabinoid is effective against the toxicity caused by 6-hydroxydopamine, which may be relevant to *Parkinson's* disease [9]. Cannabichromene (CBC) also shows various activities including anti-inflammatory, antifungal, and antibacterial activity [10]. The co-action of CBC with THC was investigated, as marijuana contains a considerable amount of CBC [11]. In addition, tetrahydrocannabivarin, the propyl homologue of THC in specific types of marijuana, is a cannabinoid CB1 and CB2 receptor antagonist [12]. Since, in the 1990s, endogenous cannabinoid receptor ligands (endocannabinoids, e.g., anandamide and 2-arachidonoylglycerol) have been identified in mammalian tissues [13–15], cannabinoids from *Cannabis sativa* are now often called phytocannabinoids to distinguish them from endocannabinoids.

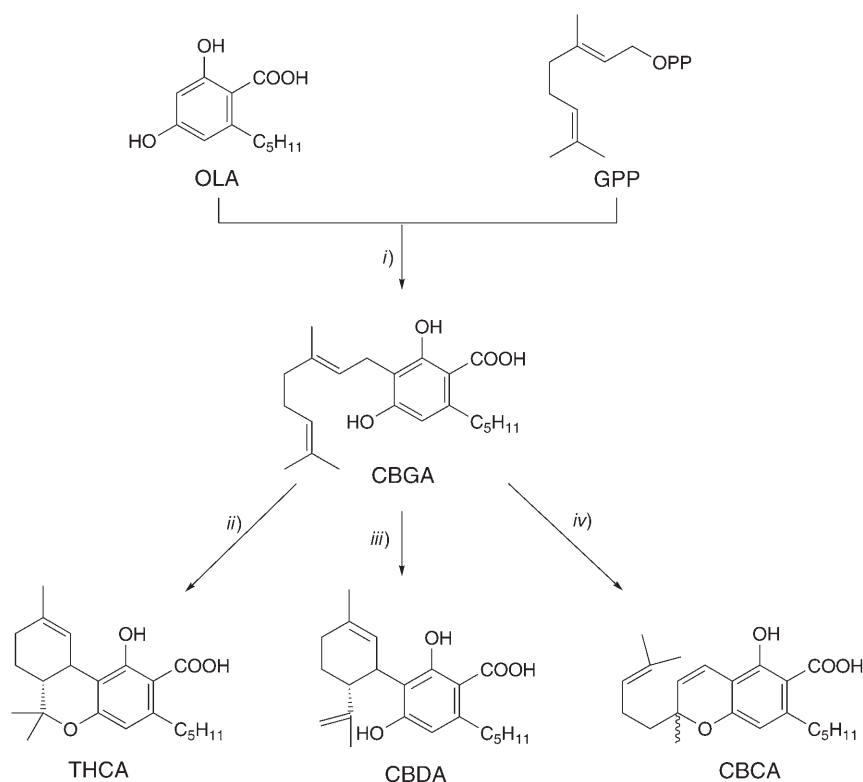
Phytocannabinoids are classified into two types, *i.e.*, neutral cannabinoids and cannabinoid acids, based on whether they contain a carboxy group or not. In the fresh *Cannabis* plants, cannabinoids are biosynthesized and accumulated as cannabinoid acids, and nonenzymatically decarboxylized into their neutral forms during storage and smoking [16][17]. Several plausible hypotheses had been proposed for cannabinoid biosynthesis, and most of them were based on the presumption that tetrahydrocannabinolic acid (THCA) is biosynthesized by isomerization of cannabidiolic acid (CBDA), whereas CBDA and cannabichromenic acid (CBCA) are formed by oxidative cyclization of cannabigerolic acid (CBGA) [18]. To confirm the biosynthetic pathway, feeding experiments of radiolabeled precursors were attempted, but no clear results could be obtained due to low incorporation of radioactivity into cannabinoids [19][20].

To overcome these difficulties, from the middle of 1990s, several research groups, including our laboratory, started to investigate the enzymes involved in cannabinoid biosynthesis, and successfully established the biosynthetic pathway of major phytocannabinoids as illustrated in *Scheme 1*. In the pathway, THCA, CBDA, and CBCA are biosynthesized from the common precursor CBGA by the action of unique oxidoreductases, *i.e.*, THCA synthase, CBDA synthase, and CBCA synthase, respectively [21–23]. On the other hand, CBGA is synthesized by alkylation of olivetolic acid (OLA) with geranylpyrophosphate (GPP) by a novel prenyltransferase called geranylpyrophosphate:olivetolate geranyltransferase (GOT) [24]. This article reviews individual biosynthetic enzymes and related topics.

2. Tetrahydrocannabinolic Acid Synthase. – 2.1. Purification and Characterization.

THCA, the acidic precursor of THC, is the major constituent in the drug type *Cannabis sativa* (*Fig. 1*). Since it had been postulated that this cannabinoid is formed by isomerization of CBDA, we first attempted to detect the enzyme that catalyzes this

Scheme 1. *Biosynthesis of Phytocannabinoids*. The biosynthetic enzymes are GOT (i), THCA synthase (ii), CBDA synthase (iii), and CBCA synthase (iv).



reaction, using the crude enzyme extract prepared from a drug type (Mexican strain). However, we could not identify the isomerase despite testing various extraction and assay conditions. In contrast, a potent THCA-producing activity was confirmed in the soluble fraction from leaf bud tissues, when CBGA is incubated as substrate. Therefore, we concluded that THCA is actually biosynthesized from CBGA *via* oxidative cyclization of the geranyl group by the action of a novel enzyme named THCA synthase [21].

To evaluate the biochemical properties, we purified THCA synthase to homogeneity by various column chromatographies on *DE-52*, phenyl *Sepharose CL-4B*, and hydroxylapatite. The purified THCA synthase showed a single band with a molecular mass of *ca.* 75 kDa on SDS-PAGE analysis (Fig. 2). The native molecular mass was estimated to be *ca.* 76 kDa by a gel-filtration chromatography, indicating that THCA synthase is a monomeric protein. The circular dichroism (CD) spectrum of THCA produced by the purified enzyme was identical to that of authentic (–)-THCA. Therefore, THCA synthase reaction is stereoselective.

THCA Synthase catalyzes a unique monoterpene cyclase-like reaction coupled with a two-electron oxidation. As most monoterpene cyclases require divalent ions

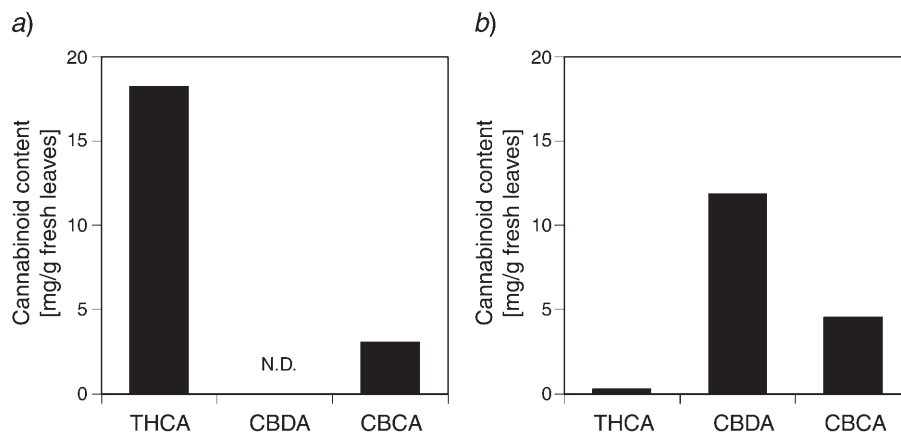


Fig. 1. Cannabinoid content in fresh leaves of a drug type (Mexican strain (a)) and a fiber type (CBDA strain (b)) *Cannabis sativa*. N.D., not detected.

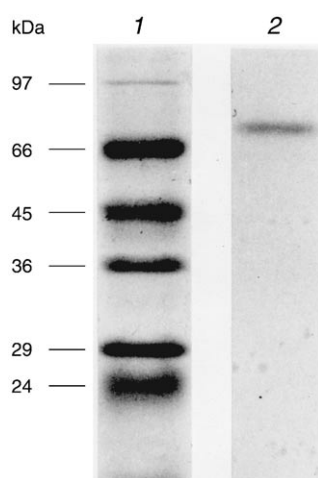
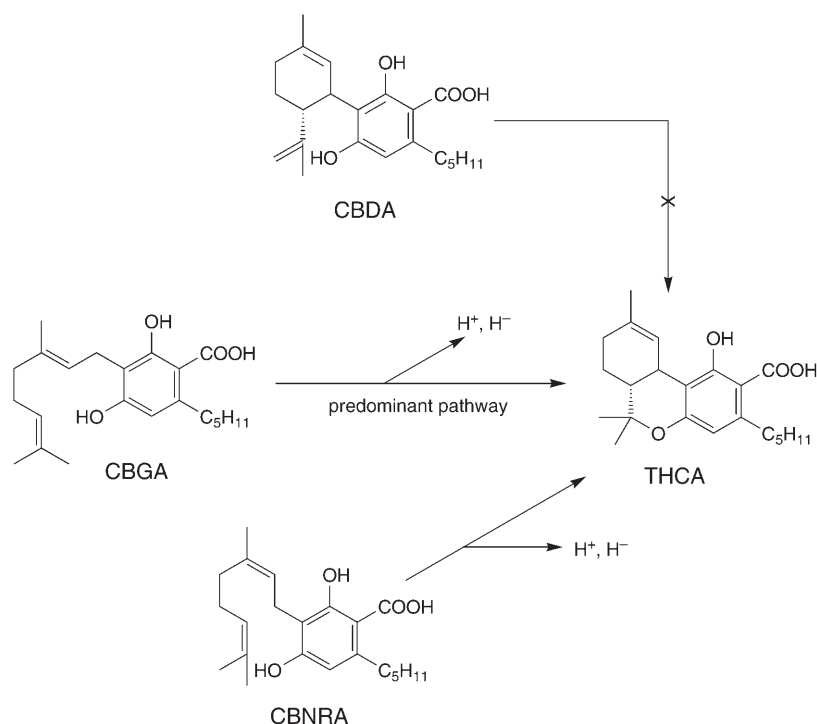


Fig. 2. SDS-PAGE Analysis of THCA synthase purified from *Cannabis sativa*. Lane 1: molecular mass standards; Lane 2: purified THCA synthase.

such as Mg^{2+} or Mn^{2+} for their activity [25], we tested whether metal ions activate THCA synthase. In addition, to reveal the oxidation mechanism, effects of a variety of cofactors and coenzymes, such as NAD, NADP, FAD, and FMN, were also investigated. As a result, purified THCA synthase did not require any metal ions, cofactors, and coenzymes. These properties indicated that this enzyme can complete the oxidocyclization reaction by itself.

It is of interest that THCA synthase could convert cannabimerolic acid (CBNRA), the (*Z*)-isomer of CBGA, into THCA. However, lower specificity of THCA synthase for CBNRA compared with that for CBGA indicated that THCA is predominantly biosynthesized from CBGA (Scheme 2). It is noteworthy that THCA synthase could not accept neutral cannabinoids such as cannabigerol as substrates. Therefore, the

Scheme 2. Biosynthesis of THCA by THCA Synthase



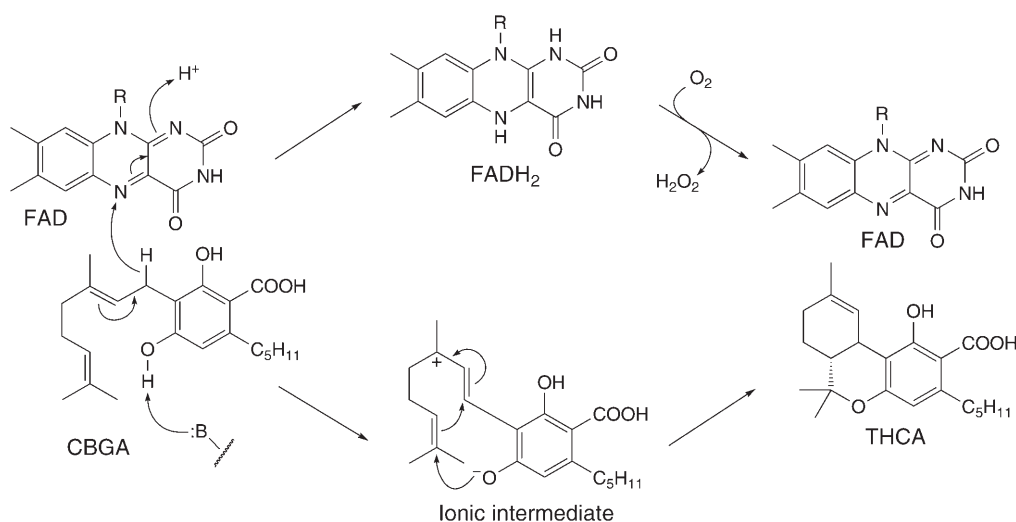
carboxy group is essential for substrate recognition by this enzyme. As described, THCA synthase is the first cannabinoid synthase to be purified and characterized.

2.2. Molecular Cloning and Heterologous Expression. Since characterization of the native enzyme did not provide the detailed functional and structural information, we next attempted to clone the gene encoding THCA synthase. The molecular cloning was carried out by reverse transcription and polymerase chain reaction (PCR) techniques using degenerate gene-specific primers. Consequently, we cloned a THCA synthase cDNA (*THCAS*), which consists of a 1635-nucleotide open reading frame, encoding a 545-amino acid polypeptide of which the first 28 amino acids constitute the signal peptide. *THCAS* is the first gene involved in cannabinoid biosynthesis to be cloned [26].

Surprisingly, the primary structure deduced from cDNA exhibited high homology to berberine-bridge enzyme from *Eschscholtzia californica*, which is involved in alkaloid biosynthesis [27]. Of great interest is that homologous enzymes work in apparently distinct secondary pathways such as cannabinoid and alkaloid biosynthetic pathways. Berberine-bridge enzyme is a well-characterized covalently flavinylated oxidase that catalyzes FAD-dependent oxidation of (*S*)-reticuline to form (*S*)-scoulerine [28]. The structural similarity implied the possibility that THCA synthase is also a FAD-dependent oxidase type enzyme.

For detailed characterization, we overexpressed the recombinant THCA synthase using a baculovirus-insect expression system. The purified recombinant enzyme obtained from the insect culture medium gave yellow coloration, suggesting flavin binding. Furthermore, various spectroscopic analyses of the enzyme demonstrated that THCA synthase contains covalently attached FAD cofactor at a molar ratio of FAD to protein of 1:1. The FAD-binding residue was determined to be His-114 since the site-directed mutant enzyme at this position exhibited neither absorption characteristics of flavoproteins nor THCA synthase activity. In addition, we also confirmed that THCA synthase requires molecular oxygen and release hydrogen peroxide equal to THCA. Based on the biochemical properties of THCA synthase, we proposed the reaction mechanism as shown in *Scheme 3*.

Scheme 3. *The Reaction Mechanism of THCA Synthase*. R is the rest of FAD molecule, and B is the proposed basic residue of enzyme.



Recently, we started the X-ray crystallographic analysis of THCA synthase to unequivocally determine the reaction mechanism at the atomic level. We have already obtained the crystals of the recombinant THCA synthase by a hanging-drop vapor-diffusion method (*Fig. 3*), and reported the preliminary characterization of the crystals [29]. Subsequent studies would demonstrate the structure–function relationship of the enzyme active site, and provide the rational strategy for controlling the oxidocyclization reaction.

In the course of molecular study on THCA synthase, we also established a heterologous plant expression system for THCA synthase; *i.e.*, the transgenic tobacco hairy roots harboring *THCAS* were developed by an *Agrobacterium rhizogenes* mediated gene transfer (*Fig. 4*) [26]. Notably, the liquid culture of the roots could produce THCA upon feeding of CBGA. This result provided not only direct evidence for the *in vivo* functionality of *THCAS* but also a potential biotechnological production system for THC, since CBGA is easy to synthesize [30][31], and THCA is readily

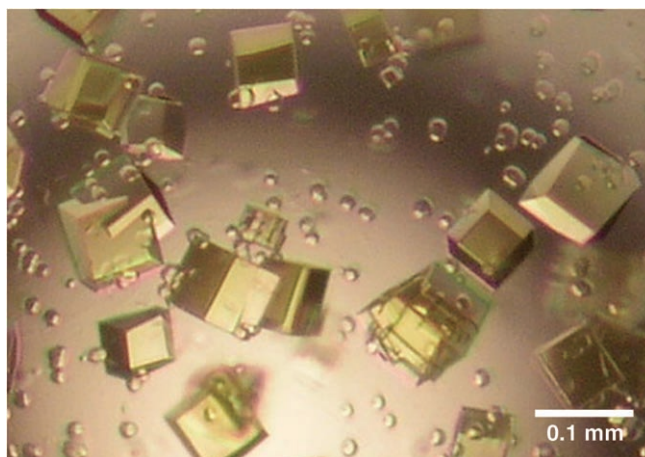


Fig. 3. Crystals of the recombinant *THCA synthase*

decarboxylized to THC by heating [17]. Further molecular studies on cannabinoid biosynthesis may develop a biomimetic production system for THC without the need for feeding precursors.



Fig. 4. The transgenic tobacco hairy roots harboring *THCA synthase*

2.3. Localization and Possible Physiological Function. With regard to the localization of cannabinoids in *Cannabis sativa*, it has been reported that THC is accumulated only in the secretory cavity of the glandular trichomes (Fig. 5) [32][33]. In addition, we have reported that the leaf bud tissue, which is rich in glandular trichomes, contains a potent THCA synthase activity [21]. Thus, it appears possible that THCA is biosynthesized in the glandular trichomes of *Cannabis* plants. To investigate this possibility, we have studied the cell-specific expression and localization of THCA synthase, and demonstrated that THCA is biosynthesized in the storage cavity of the glandular trichomes based on the following observations [34]. 1) The exclusive expression of THCA synthase was confirmed in the secretory cells of glandular trichomes by RT-PCR analysis. 2) THCA Synthase activity was detected in the storage cavity content. 3) Transgenic tobacco expressing THCA synthase fused to GFP showed fluorescence in the trichome head corresponding to the storage cavity. These results also showed that secretory cells of the glandular trichomes secrete not only metabolites but also biosynthetic enzyme. To our knowledge, THCA synthase is the first enzyme that is sorted into the secretory cavity.

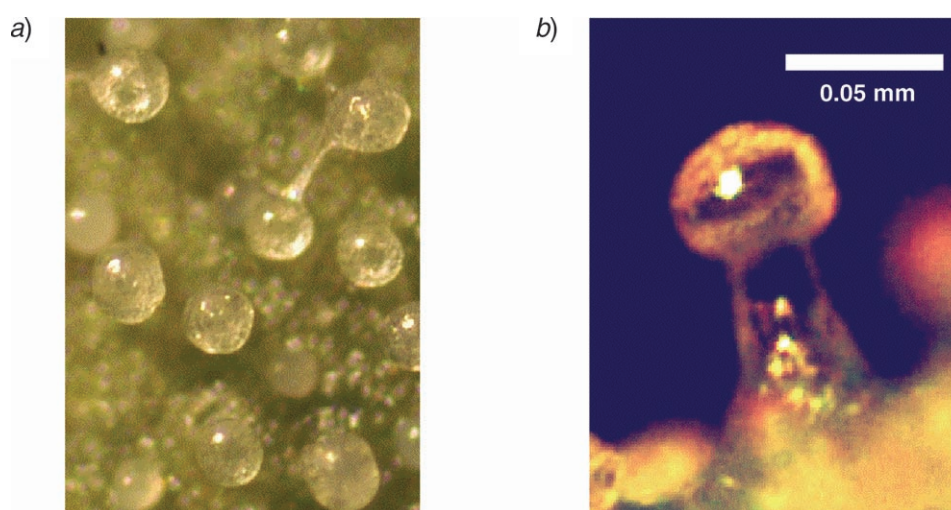


Fig. 5. Glandular trichomes on the seed coat surface (a) and side view of a glandular trichome (b). The trichome head contains a secretory cavity.

Concerning the reason why this enzyme must be secreted for THCA production, we have reported the novel fact that THCA is a very toxic substance to plant cells including *Cannabis* cells [34]. Therefore, it seems reasonable that THCA is synthesized in the extracellular compartment such as secretory cavity to avoid cellular damage. In addition, we also confirmed that THCA induces cell death not only in plant cells but also in insect cells. This result suggested that THCA acts as a plant-defense compound. Since cannabinoid-producing glandular trichomes are distributed in physically fragile young tissues of *Cannabis* plants, THCA would protect these tissues from predators such as insects. Furthermore, THCA synthase reaction produces H_2O_2 as well as THCA

during the oxidation of CBGA [26]. Since the *Cannabis sativa* produces a very large amount of THCA [16], a toxic amount of H_2O_2 might also be accumulated in the storage cavity as a result of THCA synthase reaction. Accordingly, THCA synthase might contribute to the self-defense of *Cannabis* plants by producing both THCA and H_2O_2 .

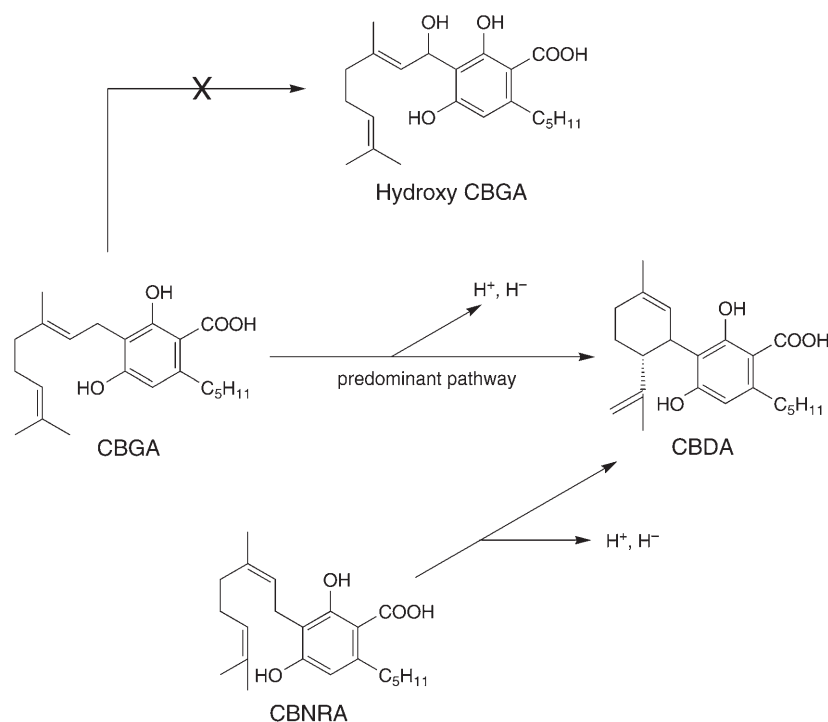
3. Cannabidiolic Acid Synthase. – CBDA is the dominant constituent of the fiber type *Cannabis sativa*. The CBDA chemotype produces a large amount of CBDA, but only a small amount of THCA in contrast to the drug type (*Fig. 1*). Using a fiber type (CBDA strain), we have identified a novel enzyme termed CBDA synthase that catalyzes oxidative cyclization of CBGA to form CBDA [22].

CBDA Synthase was purified from leaf buds of CBDA strain by a three-step column-chromatographic procedure. The purified CBDA synthase was a monomeric enzyme with a molecular mass of *ca.* 74 kDa judging from SDS-PAGE and gel-filtration analysis. Notably, the NH_2 -terminal amino acid sequence was very similar to that of THCA synthase (13 out of 15 residues were identical).

It is confirmed that CBDA synthase stereoselectively synthesizes (–)-CBDA, based on CD analysis. Like THCA synthase reaction, the CBDA synthase reaction did not require any metal ions, cofactors, and coenzymes. Although hydroxy CBGA was proposed as an intermediate during the reaction from CBGA to CBDA [18], we could not identify such intermediate in the assay solution. In addition, monooxygenase inhibitors such as triazole and KSCN did not inhibit CBDA synthase. Therefore, we concluded that CBDA is biosynthesized without oxygenation of CBGA. As described for THCA synthase, CBDA synthase also exhibited higher specificity for CBGA than that for CBNRA, indicating that CBDA is predominantly biosynthesized from CBGA (*Scheme 4*). We are now attempting further biochemical characterization of CBDA synthase with the recombinant enzyme to establish the structure and reaction mechanism in detail.

CBDA Synthase and THCA synthase are also of interest from a genetic point of view, as these enzymes determine the well-known chemical phenotypes of *Cannabis sativa*; drug type (THCA-rich) and fiber type (CBDA-rich) chemotypes. Some research groups have attempted the cross-breeding of the two chemotypes, and obtained similar results as follows [35][36]. When pure THCA and CBDA chemotypes were crossed, all of the F_1 plants showed mixed chemotype producing both THCA and CBDA. In addition, subsequent inbred of the F_1 plants resulted in a segregation of the three chemotypes (pure THCA, mixed THCA-CBDA, and pure CBDA) of F_2 plants fitting a 1:2:1 proportion. Based on such codominant inheritance of two chemotypes, *Mandolino* and co-workers have proposed a hypothesis that THCA synthase and CBDA synthase, the chemotype-determinantal enzymes, are encoded by two alleles at the cannabinoid synthase locus in the genome of *Cannabis* plants [36].

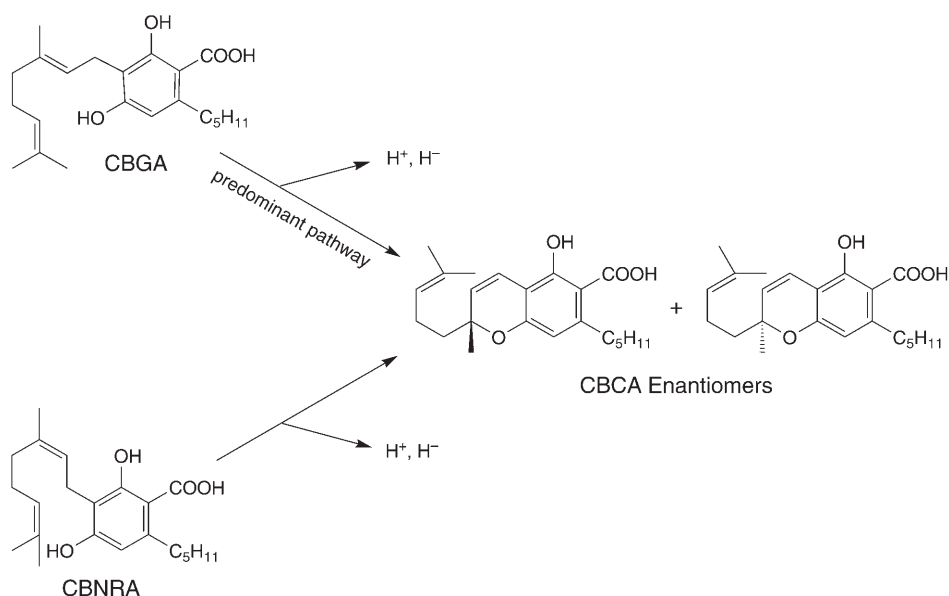
4. Cannabichromenic Acid Synthase. – CBCA is one of the major constituents in both of the drug type and fiber type *Cannabis sativa* (*Fig. 1*). It has been proposed that CBCA is derived from CBGA, and that this biosynthetic reaction proceeds non-stereospecifically, unlike the biosynthesis of THCA and CBDA, based on the observation that CBCA shows little optical rotation [18].

Scheme 4. *CBDA Biosynthesis by CBDA Synthase*

To reveal the precise mechanism of CBCA biosynthesis, we have identified and partially purified CBCA synthase, which catalyzes oxidative cyclization of CBGA to form CBCA, from young leaves of *Cannabis sativa* (CBDA strain) [23]. The structural characterization of enzymatically synthesized CBCA has demonstrated that the reaction is stereoselective, because an apparent *Cotton* effect was observed in the CD spectrum. In addition, optical purity of CBCA was investigated by chiral HPLC analysis of enzyme-derived and synthetic (racemic) CBC samples. Consequently, the elution profiles of HPLC analysis suggested that CBCA is biosynthesized as a mixture of enantiomers with a ratio of 5 : 1 (*Scheme 5*). The stereospecificity of CBCA synthase reaction is relatively lower than those of THCA synthase and CBDA synthase, as the latter two enzymes synthesize (–)-THCA and (–)-CBDA with > 95% optical purity. Thus, the reaction intermediates might be released in part from the active site of CBCA synthase before completion of the reaction.

The general properties of CBCA synthase were quite similar to those of THCA synthase and CBDA synthase [37]. CBCA Synthase did not require any of metal ions, cofactors, and coenzymes for oxidocyclization of CBGA. This enzyme also could accept CBNRA as an alternative substrate with less specificity (*Scheme 5*). It is of interest that cannabinoids having different ring systems are biosynthesized from the common substrate CBGA by similar reaction mechanisms.

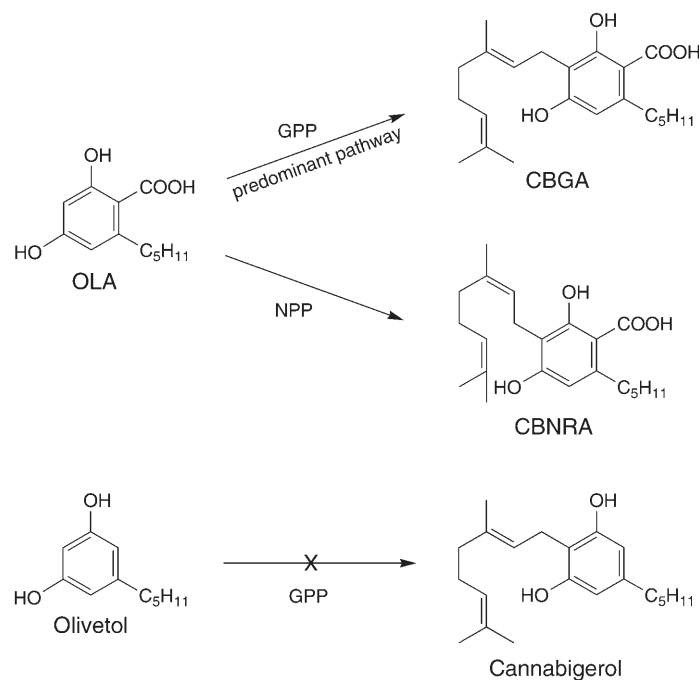
Scheme 5. *CBCA Biosynthesis by CBCA Synthase*. The enzyme produces both enantiomers with a molar ratio of 5:1, but it is unknown which one is the major product.



5. Biosynthetic Mechanism Leading to Cannabigerolic Acid. – 5.1. *Geranylpyrophosphate:Olivetolate Geranyltransferase*. As described above, CBGA is an important cannabinoid as the central precursor of various cannabinoids in the biosynthetic pathway. This cannabinoid is formed by condensation between OLA and GPP. The excellent enzymological study conducted by *Fellermeier* and *Zenk* has led to the identification of a novel prenyltransferase that catalyzes the biosynthesis of CBGA in the expanding leaves of *Cannabis sativa* [24]. Notably, this enzyme called GOT is a soluble protein contrary to the fact that most of the reported plant aromatic prenyltransferases are membrane-bound [38–42]. A similar exception is found in hop plant (*Humulus lupulus*) where the soluble prenyltransferase is involved in the bitter acid biosynthesis [43].

As shown in *Scheme 6*, GOT reaction showed interesting substrate specificity. The enzyme accepted not only GPP but also nerylpyrophosphate (NPP) to yield CBNRA, suggesting that CBGA and CBNRA are synthesized by the same enzymatic reaction. Observed velocity of GOT for GPP higher than that for NPP is consistent with the report that *Cannabis sativa* contains a higher amount of CBGA than CBNRA [44]. With respect to prenyl acceptor, GOT is specific to OLA; *i.e.*, olivetol, the decarboxylation product of OLA, was not a substrate for this enzyme. Therefore, the carboxy group is crucial for the GOT reaction. This result is reasonable because all of the downstream cannabinoid synthases, THCA synthase, CBDA synthase, and CBCA synthase, could not accept cannabigerol as a substrate.

Needless to say, the identification of GOT was a great progress; however, subsequent studies such as purification and molecular cloning of this enzyme have not

Scheme 6. *Reactions Catalyzed by GOT*

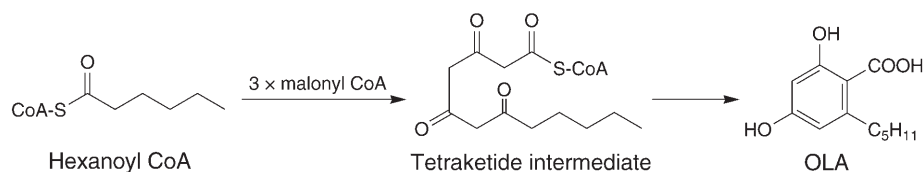
been reported to date. Since plant aromatic prenyltransferases have rarely been studied at molecular level, it is of great interest to know the structure–function relationship of GOT, which would provide novel insights into the enzymology of prenyltransferase.

5.2. Origin of the Monoterpene Moiety. Prior to 1990, all of the plant terpenoid precursors, such as isopentenyl pyrophosphate and dimethylallyl pyrophosphate, had been assumed to be biosynthesized *via* the mevalonate pathway. However, recent studies have shown that many plant terpenoids are biosynthesized *via* the recently discovered deoxyxylulose phosphate pathway [45][46]. Thus, to determine which pathway contributes to biosynthesis of cannabinoid precursors, *Fellermeier et al.* conducted feeding experiments using ^{13}C -labeled glucose. In their study, labeled glucose was fed to sprouts of *Cannabis sativa*, and then THCA and CBCA were analyzed by quantitative NMR spectroscopy. Consequently, the ^{13}C incorporation and coupling patterns clearly provided the evidence that the terpenoid moiety of cannabinoids is predominantly biosynthesized *via* the deoxyxylulose phosphate pathway [47].

Because the deoxyxylulose phosphate pathway operates in the plastids of higher plants, GOT as well as GPP synthase would function in the plastids of *Cannabis sativa*. If so, CBGA would be transported from plastids into the secretory cavity of trichomes for the biosynthesis of THCA. Further investigations are needed for more precise knowledge on subcellular localization and regulation of the cannabinoid biosynthetic pathway. These studies would provide essential information for the metabolic engineering of the cannabinoid biosynthesis.

5.3. Origin of the Alkylresorcinol Moiety. Based on the substrate specificity of GOT, it became evident that OLA is a precursor of cannabinoids. OLA is a kind of alkylresorcinol and has been assumed to be biosynthesized *via* the polyketide pathway. In fact, ^{13}C -incorporation experiments conducted by *Fellermeier et al.* clearly demonstrated that the alkylresorcinol moiety of cannabinoids is derived from acetate units [47]. Thus, it is proposed that a polyketide synthase catalyzes the biosynthesis of OLA. In the proposed reaction mechanism (*Scheme 7*), OLA is synthesized from hexanoyl CoA and three molecules of malonyl CoA *via* an aldol condensation of the tetraketide intermediate to form a resorcinol carboxylic acid structure [48].

Scheme 7. Proposed Biosynthetic Mechanism of OLA



Raharjo et al. attempted to detect the polyketide synthase activity in the crude extract from various parts of *Cannabis sativa* [49]. As reported, chalcone synthase activity was detected in the enzyme from flower when incubated with *p*-coumaroyl CoA and malonyl CoA. However, OLA formation was not confirmed in all enzyme assays with hexanoyl CoA as a starter substrate, whereas olivetol-forming activity was unexpectedly detected in the enzyme from flower. These results were mysterious, because olivetol is not a prenyl acceptor in the GOT reaction. One possible explanation is that olivetol was synthesized by a stilbene synthase that catalyzes aldol condensation with decarboxylation. However, stilbene synthase activity, which produces resveratrol from *p*-coumaroyl CoA, was not detected in the same enzyme extract. These results implied the difficulty to identify OLA synthase with plant extract as enzyme source.

Recent advances in the study on plant polyketide synthases have demonstrated that various kinds of enzymes belong to chalcone synthase superfamily having high sequence identity to each other [50]. Thus, it is now possible to perform homology-based molecular cloning of genes encoding novel polyketide synthases. To obtain OLA synthase cDNA, *Raharjo et al.* conducted polymerase chain reactions (PCRs) with primers designed from the primary structures of chalcone synthase family enzymes [51]. As a result, they cloned a novel cDNA from *Cannabis sativa*, and prepared the recombinant enzyme by a bacterial expression. However, the recombinant enzyme did not synthesize OLA when incubated with hexanoyl CoA, whereas it produced naringenin chalcone from *p*-coumaroyl CoA, indicating that the cDNA encoded a chalcone synthase. Thus, as OLA synthase has been neither identified nor cloned, the biosynthetic mechanism of OLA remained largely unclear. Since homology-based strategy did not work, an enzyme having lower similarity to chalcone synthase might be responsible for the reaction.

6. Conclusions. – The biosynthetic mechanism of phytocannabinoids had long been uncertain mostly due to the lack of experimental evidence. Especially, identification of

biosynthetic enzymes was essential to clarify the pathway. Hence, in the last decade, various enzymological studies have been conducted, and identified a geranyltransferase (GOT) to form CBGA and three oxidoreductases, THCA synthase, CBDA synthase, and CBCA synthase. Studies on these enzymes led to a considerable progress to understand the cannabinoid pathway.

However, there are still many problems to solve for the comprehensive understanding of biosynthetic enzymes. For example, molecular cloning, structure determination, and biochemical characterization should be conducted for each enzyme to unequivocally establish the biosynthetic mechanism. In addition, physiological studies are also needed to understand the regulation of the pathway. These studies will open the way to various biotechnological applications of biosynthetic enzymes and genes such as 1) effective biomimetic production of cannabinoids in heterologous hosts, 2) metabolic engineering to control the cannabinoid content in *Cannabis* plants for medicinal and industrial purposes, and 3) rational design of enzyme active site to improve or modify the catalytic functions. Thus, we have to keep on studying.

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