Review

Studies on tetrahydrocannabinolic acid synthase that produces the acidic precursor of tetrahydrocannabinol, the pharmacologically active cannabinoid in marijuana

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ABSTRACT: Tetrahydrocannabinol (THC), the psychoactive component of marijuana, is now regarded as a promising medicine because this cannabinoid has been shown to exert a variety of therapeutic activities. It has been demonstrated that THC is generated from the acidic precursor, tetrahydrocannabinolic acid (THCA) by non-enzymatic decarboxylation, and that THCA is biosynthesized by THCA synthase, which catalyzes a unique biosynthetic reaction, the stereospecific oxidative cyclization of the geranyl group of the substrate cannabigerolic acid. Molecular characterization of THCA synthase has revealed its structural characteristics and reaction mechanism. THCA synthase is the first cannabinoid synthase to be studied and is potentially attractive target for various biotechnological applications as it produces the direct precursor of THC. This review describes the research history of this enzyme, *i.e.*, purification, molecular cloning, biochemical characterization, and possible biotechnological application of THCA synthase.

Keywords: Cannabinoid, *Cannabis sativa*, marijuana, tetrahydrocannabinol, tetrahydrocannabinolic acid synthase

1. Introduction

To date, more than 60 cannabinoids have been isolated from marijuana or fresh *Cannabis sativa* (*C. sativa*) plants (*1*). Among them, tetrahydrocannabinol (THC) is the well-known psychoactive cannabinoid (*2*). Recent studies have demonstrated that this cannabinoid exerts a variety of therapeutic activities, and therefore, THC has attracted a great deal of attention as a promising medicine for treating various diseases (*3*). In some countries, THC has been approved as a medicine for suppressing nausea and vomiting caused by cancer chemotherapy, and more recently, Sativex, a *Cannabis*-based preparation containing THC, was licensed in Canada as a neuropathic pain reliever for adult patients with multiple sclerosis (*4*). The demand for THC has been increasing; however, asymmetric synthesis of this cannabinoid requires very intricate procedures (*5*). In addition, it is not easy to isolate THC because marijuana contains a complicated mixture of various cannabinoids.

Cannabinoids are classified into two types, neutral cannabinoids and cannabinoid acids, based on whether they contain a carboxyl group or not. In fresh *Cannabis* plants, cannabinoids are biosynthesized and accumulated as cannabinoid acids, and non-enzymatically decarboxylized into their neutral forms during storage and smoking (*6,7*). Likewise, THC is generated from an acidic precursor, tetrahydrocannabinolic acid (THCA) (Figure 1). With respect to the biosynthesis of THCA,

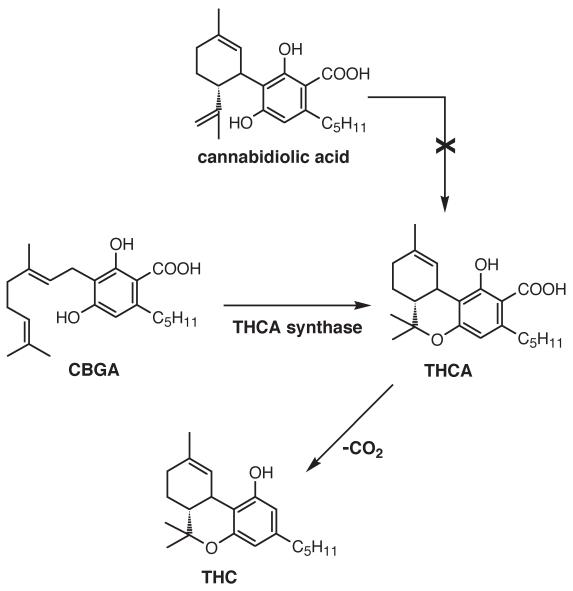

Figure 1. Biogenesis of THC. THCA synthase catalyzes the oxidative cyclization of CBGA to form THCA. THC is generated from THCA by non-enzymatic decarboxylation.

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it has been demonstrated that THCA is biosynthesized by an oxidoreductase named THCA synthase, which converts cannabigerolic acid (CBGA) into THCA, contrary to the generally postulated scheme that THCA is derived from cannabidiolic acid (*8*) (Figure 1).

THCA synthase was identified in the young leaves of *C. sativa*, and then purified and characterized (*8*). However, the molecular structure and detailed reaction mechanism could not be determined by the studies on the native enzyme. Thus, the cDNA encoding THCA synthase was cloned, and the structural and biochemical characterization was conducted in detail subsequently (*9*). In addition, cost- and fermentation-friendly expression systems for recombinant THCA synthase were established as a first step to develop a novel biotechnological production system of THC (*9,10*).

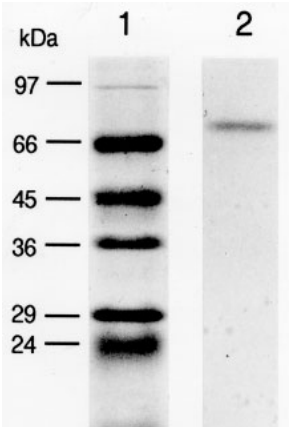

Figure 2. SDS-PAGE analysis of THCA synthase purified from *C. sativa*. Lane 1, molecular mass standards; Lane 2, purified THCA synthase.

2. Purification and characterization of THCA synthase from *C. sativa*

THCA, the acidic precursor of THC, is one of the major constituents of *C. sativa*. With regard to the biosynthesis of THCA, it had long been postulated that THCA is formed by ring closure of cannabidiolic acid, an isomer of THCA. However, the isomerase activity, which converts cannabidiolic acid into THCA, could not be detected in any enzyme assays using crude enzyme extracts prepared from various parts of *C. sativa*. In contrast, a potent THCA producing activity was confirmed in the soluble fraction from young leaves when CBGA was included as a substrate. Therefore, it appeared evident that THCA is actually biosynthesized from CBGA *via* oxidative cyclization of the geranyl group by the action of a novel enzyme named THCA synthase (*8*) (Figure 1).

To evaluate its biochemical properties, THCA synthase was purified to homogeneity by various column chromatography steps using DE-52, phenyl Sepharose CL-4B, and hydroxylapatite. The purified THCA synthase was detected as a single band with a molecular mass of ~75 kDa on SDS-PAGE analysis (Figure 2). The native molecular mass was estimated to be ~76 kDa by gel filtration chromatography, indicating that THCA synthase is a monomeric protein. Concerning the stereoselectivity of the enzyme reaction, the CD spectrum of THCA produced by the purified enzyme was identical to that of authentic (-)-THCA, confirming that THCA synthase stereoselectively produces (-)-THCA.

THCA synthase catalyzes a unique monoterpene cyclase-like reaction coupled with a two-electron oxidation. As most monoterpene cyclases require divalent ions such as Mg^{2+} or Mn^{2+} for their activity (*11*), the effects of metal ions on THCA synthase activity were tested. In addition, to obtain information on the enzymatic oxidation mechanism, the effects of a variety of cofactors and coenzymes, including NAD, NADP, FAD, and FMN, were also investigated. As a result, purified THCA synthase was found not to require any metal ions, cofactors, or coenzymes. These properties indicated that this enzyme can complete the oxidative cyclization reaction by itself. As described above, THCA synthase is the first cannabinoid synthase to be purified and characterized.

3. Molecular cloning and heterologous expression

Because characterization of the native enzyme did not provide detailed functional and structural information, cDNA cloning and molecular characterization of THCA synthase was attempted (*9*). The molecular cloning was carried out by reverse transcription and polymerase chain reaction techniques using degenerate and gene specific primers. The THCA synthase cDNA consisted of a 1,635-nucleotide open reading frame, encoding a 545-amino acid polypeptide, of which the first 28 amino acids constituted the signal peptide. THCA synthase was the first enzyme involved in cannabinoid biosynthesis to be cloned.

Surprisingly, the primary structure deduced from the cDNA exhibited high homology to berberine bridge enzyme from *Eschscholtzia californica*, which is involved in alkaloid biosynthesis (*12*). It is of great interest that homologous enzymes work in apparently distinct secondary pathways, namely the 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 (*13*). The structural similarity implied the possibility that THCA synthase is also a FAD dependent oxidase type enzyme.

For detailed characterization, recombinant THCA synthase was overexpressed using a baculovirus-insect expression system. The purified recombinant enzyme obtained from the insect culture medium gave yellow coloration suggesting flavin binding.

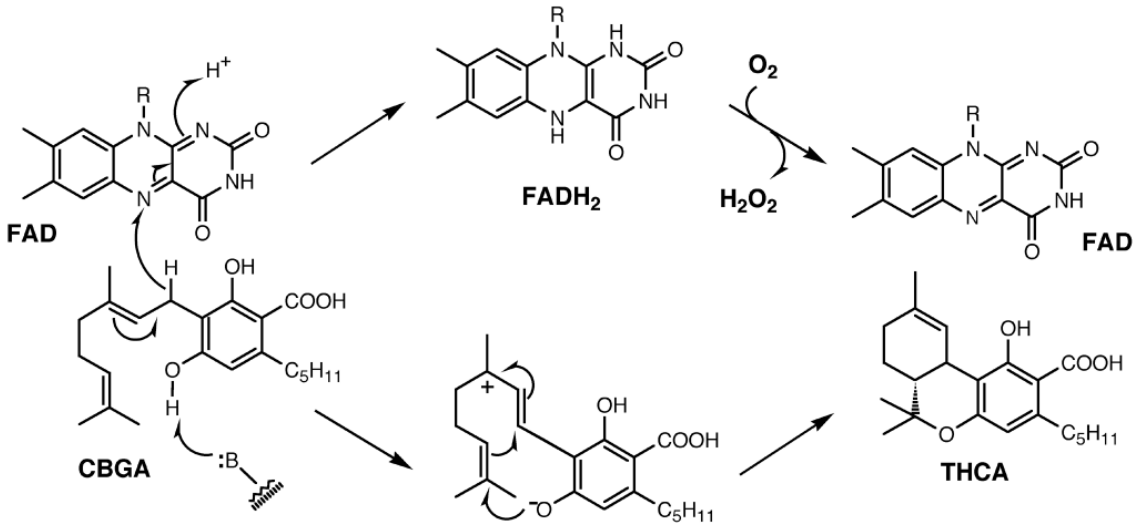

Figure 3. The reaction mechanism of THCA synthase. R is the rest of FAD molecule and B is the proposed basic residue of the enzyme.

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 because the site-directed mutant enzyme at this position exhibited neither absorption characteristics of flavoproteins nor THCA synthase activity. In addition, it was also confirmed that THCA synthase requires molecular oxygen and releases hydrogen peroxide stoichiometrically with THCA. Based on the biochemical properties of THCA synthase, the reaction mechanism was proposed as shown in Figure 3. Subsequent studies, such as X-ray crystallographic analysis (*14*), may demonstrate the structure-function relationship of the enzyme active site and may provide rational strategies for controlling the oxidative cyclization reaction.

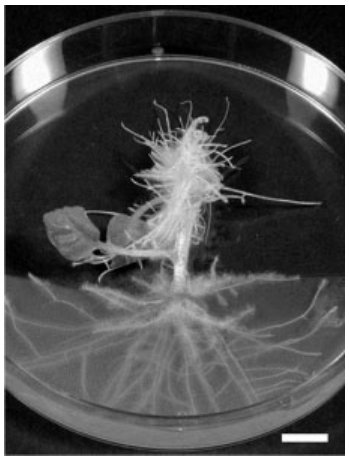

Figure 4. Transgenic tobacco hairy roots harboring THCA synthase. Bar, 1 cm.

4. Production of THCA by recombinant THCA synthase

As described above, THCA synthase stereoselectively synthesizes THCA from CBGA. Because CBGA is easy to synthesize (*9*), and THCA is readily decarboxylized into THC by heating (*6*), it was considered that THCA synthase could contribute to the biotechnological production of THC once a suitable expression system was developed. The insect cell system expression afforded a large amount of enzyme (~1 mg/L culture); however, the system required an expensive complex medium as well as elaborate viral infection and amplification procedures. Therefore, two different cost- and fermentation-friendly expression systems have been established, *i.e.*, THCA synthase was expressed in transgenic tobacco hairy roots (*9*) and methylotrophic yeast *Pichia pastoris* (*10*). The bioconversion of CBGA into THCA by recombinant THCA synthase was attempted as a first step toward the biotechnological production of THC (*9*,*10*).

4.1. *THCA production by THCA synthase in transgenic tobacco hairy roots*

For expression in plants, the THCA synthase cDNA was cloned into a pBI121 vector with a cauliflower mosaic virus 35S promoter (*15*). The resulting construct was introduced into the tobacco (*Nicotiana tabacum* cv Xanthi) genome using *Agrobacterium rhizogenes* (16). The transformants appeared as rapidly growing hairy roots from tobacco stems infected by *Agrobacterium* (Figure 4). The transgenic hairy roots could produce THCA upon feeding of CBGA. When the hairy roots were cultured in liquid medium (30 mL) supplemented with 1 mg of CBGA, the maximum level of THCA (82 μg, 8.2% conversion from CBGA) was produced 2 days after the addition of CBGA. Although the

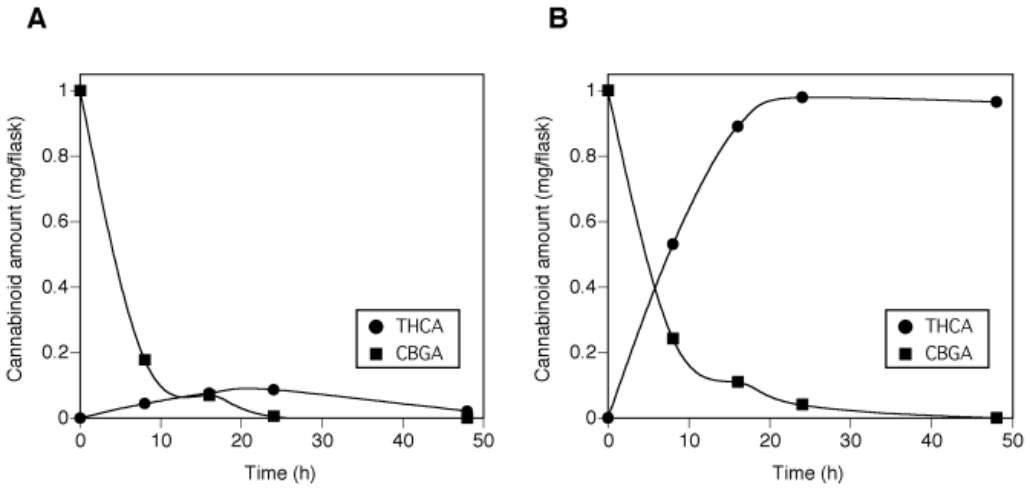

Figure 5. Conversion of CBGA into THCA by recombinant THCA synthase. A, Bioconversion by *P. pastoris* cultures. CBGA (1 mg) was added to the cultures (30 mL), and the amounts of CBGA and THCA were monitored by HPLC as described (*10*). B, Enzymatic conversion by the culture supernatant. CBGA (1 mg) was added to the culture supernatant (30 mL), and the cannabinoid amounts were measured.

conversion rate was limited, this result provided direct evidence for the *in vivo* functionality of the recombinant THCA synthase, and suggested a possibility that the THCA synthase gene can control THCA production not only in *C. sativa* but also in other plants such as tobacco.

4.2. *THCA production by THCA synthase secreted from transgenic P. pastoris*

The coding region of the THCA synthase cDNA was introduced into the genome of SMD1168h, a proteinase-deficient *P. pastoris*. The transformed cells cultured in liquid medium could secrete a catalytically active THCA synthase. Under optimized culture conditions, the bioconversion of CBGA into THCA was attempted. When CBGA (1 mg) was added directly to the culture (30 mL), THCA production was not more than 10% in 24 h, and prolonged incubation metabolized the THCA (Figure 5A). Although the level of production was very low, the CBGA concentration decreased quickly and was almost fully removed after 24 h incubation (Figure 5A). These results implied that enzymes other than THCA synthase metabolized both the substrate and the product. On the other hand, the culture supernatant, from which the cells were removed, could effectively convert CBGA into THCA with a maximum conversion rate of ~98% in 24 h (Figure 5B). The yield of THCA was 0.98 mg/flask, equal to 32.6 mg per liter of medium, which was much more than that obtained by bioconversion using transgenic tobacco root cultures. The THCA produced was stable in the solution as it was not metabolized at a significant rate by a further 24 h of incubation (Figure 5B). Therefore, cannabinoid-metabolizing enzymes produced by *Pichia* cells were not secreted into the medium. This was the first effective production of THCA using a recombinant biosynthetic enzyme.

However, it was not possible to further improve the yield of THCA by added larger amounts of CBGA because of the low solubility of this substrate in the culture medium. Therefore, to obtain more THCA, a feeding method of the substrate should be investigated. For example, an immobilized-enzyme-based method may be a possible way to produce THCA more efficiently. In the near future, recombinant THCA synthase may contribute to the development of a practical system for producing THC with the combined application of a previously established procedure for heat decarboxylation of THCA (*6*).

5. Conclusion and Perspective

THCA synthase, the key enzyme in the biogenesis of THC, was identified, cloned, and characterized. The culture medium of transgenic *P. pastoris* harboring THCA synthase gene could produce THCA upon feeding the substrate CBGA, suggesting a potential biotechnological production system for THC. Further molecular studies on cannabinoid biosynthesis may develop a biomimetic de novo production system without the need for feeding precursors. In addition, THCA synthase gene could also contribute to the artificial control of THCA production in *C. sativa*. For example, the overexpression of THCA synthase could produce THCA-rich plants with increased therapeutic potential. Conversely, THCA-free plants without abuse potential may be produced by silencing of the gene. THCA synthase is an attractive target for various biotechnological applications.

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