Prenylation of olivetolate by a hemp transferase yields cannabigerolic acid, the precursor of tetrahydrocannabinol

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Abstract A new enzyme, geranylpyrophosphate:olivetolate geranyltransferase (GOT), the first enzyme in the biosynthesis of cannabinoids could be detected in extracts of young leaves of *Cannabis sativa*. The enzyme accepts geranylpyrophosphate (GPP) and to a lesser degree also nerylpyrophosphate (NPP) as a cosubstrate. It is, however, specific for olivetolic acid; its decarboxylation product olivetol is inactive as a prenyl acceptor.
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Key words: Cannabis; Cannabigerolic acid biosynthesis; Geranylpyrophosphate:olivetolate geranyltransferase

1. Introduction

Cannabinoids are terpenophenolics found in Indian hemp, *Cannabis sativa*. The principal psychoactive component of this plant is tetrahydrocannabinol (THC), which has also valuable antiemetic properties that help to reduce the side-effects of nausea and vomiting caused by cancer chemotherapeutic agents [1]. The phenolic moiety of the cannabinoids is most likely formed via the polyketide pathway whereby a triketo-acid may be postulated as an intermediate; its cyclization leads to olivetolic acid (OA) [2]. C-alkylation by geranylpyrophosphate (GPP) could yield further on cannabigerolic acid [3] which in turn is a known substrate for Δ^1-tetrahydrocannabinolic acid synthase [4] thus furnishing Δ^1-tetrahydrocannabinolic acid (Δ^1-THCA) [5] which yields THC after decarboxylation (Scheme 1). This important oxidative cyclization step has been clarified at the enzyme level by the excellent work of Y. Shoyama and his associates [4]. This latter enzyme, Δ^1-THCA synthase, has been purified to homogeneity and the N-terminal sequence has been reported [4]. The final step, the decarboxylation of THCA to THC, might be a non-enzymatic reaction and can be caused by external factors such as temperature and light in the course of drying and storage [5].

One of the crucial enzymatic reactions left in the biosynthesis of cannabinoids is the postulated condensation of olivetolic acid with GPP. This enzyme reaction finds analogies in the biosynthesis of shikonin [6,7] and other pharmacologically active compounds [8]. We therefore attempted to detect the enzyme which by alkylation of olivetolic acid with GPP should yield cannabigerolic acid. This enzyme is herewith detected in crude homogenates from hemp leaves and its properties studied. It was named geranylpyrophosphate:olivetolate geranyltransferase.

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2. Materials and methods

2.1. Plant material

Seeds of *Cannabis sativa* var. Skunk were purchased in a Dutch 'coffee shop' and grown in a protected greenhouse with legal permission. The enzyme in question was extracted from young rapidly expanding leaves of about 20 week old plants.

2.2. Preparation of crude extracts

Leaves (10 g) were homogenized with mortar and pestle with Tris-HCl buffer (50 ml, 100 mM, pH 7.5) containing DTT (5 mM), Na_2EDTA (1 mM), KF (20 mM), glycerol (10%) and PVP (1 g) at 4°C. The homogenate was filtered through cotton and centrifuged at $50\,000 \times g$ for 30 min. The supernatant was fractionated with ammonium sulfate and the cut between 40–75% saturation was collected by centrifugation and applied to a PD-10 column equilibrated with Tris-HCl buffer (50 mM, pH 7.0) containing DTT (5 mM), Na_2EDTA (1 mM), KF (20 mM) and glycerol (10%) and used as an enzyme source if not otherwise stated. Protein concentration was measured according to [9].

2.3. Enzyme assay

The transferase was assayed unless otherwise stated as follows. The incubation mixture consisted of a total volume of 300 µl of olivetolic acid (60 nmol); GPP (600 nmol); $MgCl_2$ (1.5 µmol); KF (3 µmol); ATP (3 µmol); Tris-HCl, pH 7.0 (50 µmol) and protein (60 µg). After incubation for the indicated time at 30°C, the reaction was stopped by adding 1 µl of HCl to the reaction mixture. 4-Pentylbenzoic acid (20 nmol) was added as internal standard and the reaction mixture was extracted with 500 µl ethyl acetate. The organic layer was taken to dryness and dissolved in 200 µl of methanol and exactly 1/4 was used for HPLC. Recovery of the cannabinoids was under these conditions in the range of 85–90% as determined by the internal standard.

2.4. HPLC

The column used was Eurosphere 100-C18, 5 µm, 250×4 mm. The solvent system consisted of solvent A: $H_2O/CH_3CN = 98:2$ containing 0.01% H_3PO_4 and solvent B: $CH_3CN/H_2O = 98:2$ containing 0.01% H_3PO_4. A linear gradient from 50–100% of B in A was applied for 10 min, followed by an isocratic step at 100% of B in A for another 10 min. The flow rate was 1 ml/min with detection at 225 nm (diode array).

2.5. Chemicals

Olivetolic acid was synthesized according to the literature [2] and GPP and nerylpyrophosphate (NPP) according to [10]. GPP was free of NPP and vice versa. Cannabigerolic acid was synthesized according to [11]. The structures of the aromatic compounds were verified by MS and ^{1}H- as well as ^{13}C-NMR.
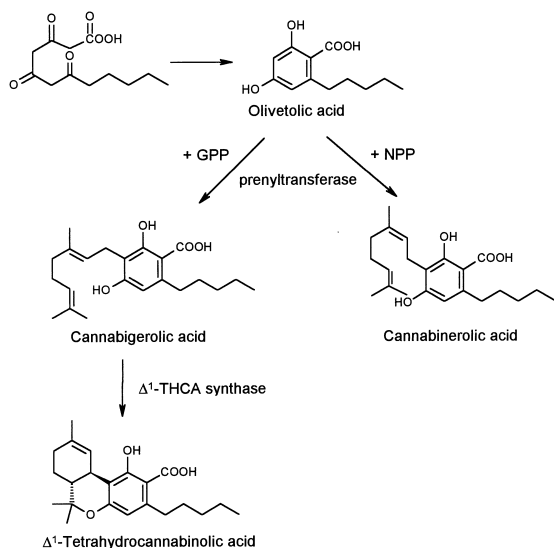
2.6. Mass spectra

The enzymatically generated cannabinoids after HPLC fractionation were transformed into methyl esters by diazomethane in ether. Mass spectra were performed with a Finnigan MAT SSQ 700 instrument. Analysis was done by direct insertion mode. CI was at 70 eV with isobutane as reactant gas and EI ionisation was done without reactant gas at 70 eV.

3. Results

Young expanding hemp leaves were used as an enzyme

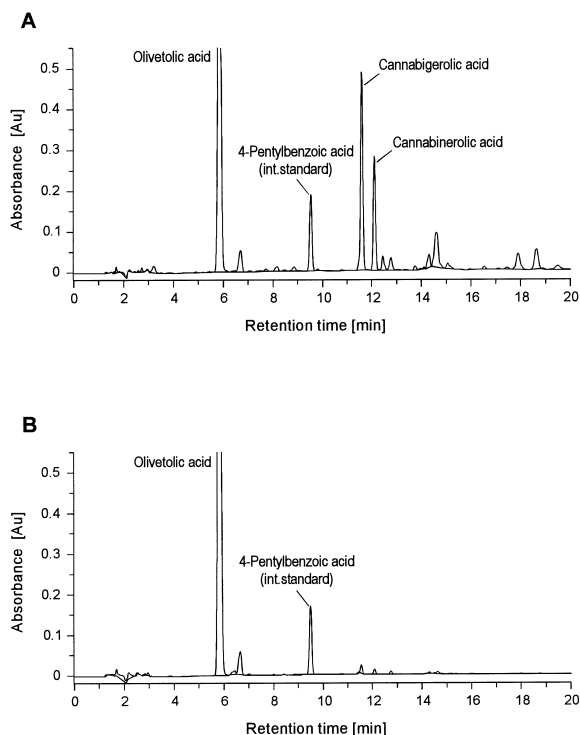Scheme 1. Biosynthesis of Δ^1-tetrahydrocannabinolic acid.

Fig. 1. HPLC identification of the products of the geranylpyrophosphate:olivetolate geranyltransferase reaction. A: Incubation under standard assay conditions with the major products CBGA ($R_t = 11.5$ min) and CBNA ($R_t = 12.2$ min). B: Control incubation mixture with heat denatured protein. HPLC: Eurosphere 100-C18, 5 μm, 250×4 mm; solvent system: solvent A, $H_2O/CH_3CN = 98:2$ containing 0.01% H_3PO_4 and solvent B, $CH_3CN/H_2O = 98:2$ containing 0.01% H_3PO_4; linear gradient from 50–100% of B in A during 10 min, followed by an isocratic step of 100% B for another 10 min; flow rate 1 ml/min; detection: absorption at 225 nm (diode array).

source since it had been previously shown that these contain the later enzymes of the THCA biosynthetic pathway [4,12,13]. A considerable body of evidence points to the fact that prenyltransferases are membrane bound (e.g. [14,15]). Therefore, we first carefully tested particulate fractions obtained by density gradient centrifugation and incubated these fractions under standard assay conditions. There was, however, a complete lack of enzyme activity with regard to the formation of CBGA. It cannot, however, be excluded that some transferase may be present in the particulate fraction but masked by endogenously present enzyme inhibitors. Since the enzyme in question could not be detected, we used the soluble fraction of the crude extract after centrifugation and collected proteins precipitating between 40–75% $(NH_4)_2SO_4$ saturation. Incubation of this fraction under standard assay conditions yielded two new major products with R_t 11.5 and 12.2 min upon HPLC resolution (Fig. 1A) that were not seen in the control incubation mixture using heat denatured protein (Fig. 1B).

The major compound ($R_t = 11.5$ min, Fig. 1A) was isolated from the incubation mixture by HPLC, subjected to MS after transformation into its methyl ester and the CI and EI mass spectra recorded. The mass spectrum of the enzymatically formed product shows a M^{+1} peak of 375 and the spectrum corresponds in all details to synthetic CBGA after transformation to its methyl ester. The R_t upon HPLC analysis was identical for the enzymatic product and the synthetic CBGA. The minor compound (which had an almost identical UV spectrum as CBGA) formed ($R_t = 12.2$ min, Fig. 1A) was analyzed in the same way as above and also shown to have a M^{+1} peak of 375. This second product seems to be the *cis*-isomer of CBGA, cannabinerolic acid (CBNA) [16]. The co-formation of both CBGA and CBNA indicates that in the crude enzyme preparation there may be a geranylpyrophosphate isomerase present, yielding nerylpyrophosphate which in turn yields CBNA possibly by action of the same prenyltransferase. CBNA is also a known constituent of hemp [16]. In Fig. 2 the time course is shown of GBGA formation under standard incubation conditions. It is clearly shown that there is no transfer of the prenyl moiety onto olivetolic acid in the

absence of enzyme. The reaction comes to an almost complete stop after 120 min. We have observed that even in the presence of KF, a known phosphatase inhibitor, and an excess of ATP, which is solely used as a phosphatase substrate in the incubation mixture to minimize GPP degradation, GPP and NPP, each in 2 mM concentration, are hydrolyzed to a large degree. This indicates that the reaction kinetics observed in Fig. 2 can be explained by a rapid loss of GPP as a cosub-
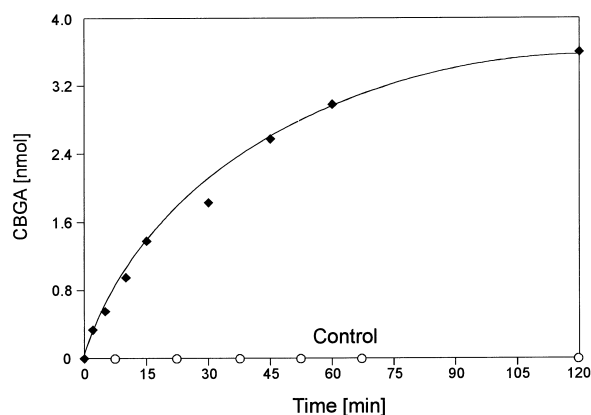

Fig. 2. Time-course of geranylpyrophosphate:olivetolate geranyltransferase in an extract of *C. sativa* measured under standard assay conditions. Controls with heat denatured enzyme.

Table 1
Influence of substrates, cofactors and inhibitors on the activity of geranylpyrophosphate:olivetolate geranyltransferase

	Product formation	
	(nmol)	% of control
Standard assay	9.1	100[a]
minus MgCl$_2$	0	0
minus GPP+NPP	1.8	19.8[b]
minus GPP	0	0
minus olivetolic acid	0	0
minus olivetolic acid plus olivetol	0	0
with heat denatured enzyme	0	0

[a]Ratio CBGA/CBNA = 2:1; [b]ratio CBGA/CBNA = 1:1.
The standard assay contained olivetolic acid (60 nmol), GPP (600 nmol), MgCl$_2$ (1.5 μmol), KF (3 μmol), ATP (3 μmol), Tris-HCl pH 7.0 (50 μmol) and protein (60 μg).

strate for this reaction. The pH optimum for this transferase is pH 7 (Tris-HCl) and the optimum temperature is 30°C. Omissions in the standard incubation mixture are shown in Table 1. The enzyme reaction is clearly shown to be Mg^{2+} dependent. Omission of GPP or olivetolic acid leads to no reaction products, as does use of a heat denatured enzyme. In the presence of GPP, the reaction yields a ratio of CBGA/CBNA = 2:1. Substitution of GPP by NPP changes the ratio to 1:1 at only 20% of the velocity observed for GPP. The enzyme exhibited normal Michaelis-Menten kinetics with GPP as substrate. The apparent K_m value was calculated to be 2 mM for GPP.

This enzyme catalyzes the first step in cannabinoid formation in hemp, the prenylation of olivetolic acid. It appears to be a soluble enzyme but membrane bound activity cannot yet be excluded. With this discovery of the enzyme responsible for CBGA synthesis and the Δ^1-THCA synthase having previously been purified to homogeneity [4], a formal enzymatic synthesis of Δ^1-THCA from readily available precursors is possible.

Furthermore, cloning of the specific prenyltransferase and silencing of this gene in commercial hemp varieties should lead to plants without drug abuse potential.

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