Catalytic activity of cannabinoid synthases in organic phase

Paweł Rodziewicz[1], Stefan Loroch[2], Łukasz Marczak[3], Albert Sickmann[2], and Oliver Kayser[1]

[1]Technical University Dortmund, Emil-Figge-Str. 66, 44227 Dortmund, Germany
[2]Leibniz-Institut für Analytische Wissenschaften - ISAS – e.V., Bunsen-Kirchhoff-Str. 11, 44139 Dortmund, Germany
[3]European Centre for Bioinformatics and Genomics, Institute of Bioorganic Chemistry PAS, Piotrowo 2, 60-965 Poznan, Poland

INTRODUCTION

Cannabinoid biosynthesis takes place in glandular trichomes of *Cannabis sativa* L. The capitate-stalked glandular trichomes, which are abundantly present on *Cannabis* female inflorescences, comprise of one basal cell, several stalk cells and secretory cells surrounded by large sub-cuticular storage cavity, and contain the largest quantities of cannabinoids. Nowadays, these compounds have become promising therapeutic agents for treating cancer, inflammations, appetite disorders, and also neurological diseases. Most of the known medicinal effects are associated with decarboxylated forms of two major cannabinoids found in the plant – the psychoactive tetrahydrocannabinol (THC) and its non-psychoactive isomer cannabidiol (CBD) [1]. The increasing demand for pharmaceutical grade cannabinoids as well as complicated law status of *Cannabis* made scientists to look for new biotechnology-based strategies of their synthesis.

Tetrahydrocannabinolic acid synthase (THCA synthase) and cannabidiolic acid synthase (CBDA synthase) catalyse the oxidative cyclization of cannabigerolic acid (CBGA), which results in the production of THCA and CBDA, respectively (Fig. 1). It was suggested that cannabinoid synthases follow the secretory pathway and that the final products may be biosynthesized in the hydrophobic exudates of glandular trichomes [2, 3]. In this study we tested the activity of cannabinoid synthases accumulated in the exudates of glandular trichomes in the organic phase.

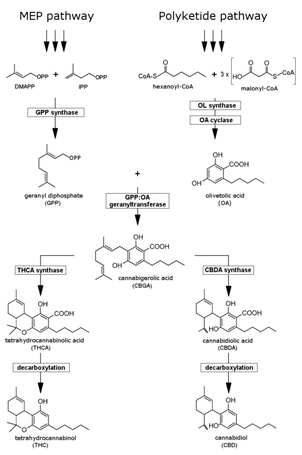

Fig. 1. The biosynthetic pathway of major cannabinoids. The precursors of cannabinoids originate from two distinct biosynthetic pathways: the polyketide pathway, giving rise to olivetolic acid (OA) and the plastidal 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, leading to the synthesis of geranyl diphosphate (GPP). DMAPP - dimethylallyl pyrophosphate, IPP - isopentenyl pyrophosphate.

Fig. 2. Female cannabis plant at 7th week of flowering stage. Floral leaves containing glandular trichomes (marked in squares) were used for experiments.

METHODS

Floral leaves containing glandular trichomes (Fig. 2) of drug type *Cannabis sativa* L. were used for experiments. Secretions from storage cavities were collected using thin capillary attached to cell micromanipulator TransferMan 2 (Eppendorf, Germany). For bioassay in organic phase the exudates were collected into 100 µl vials containing 20 µl of ice-cold hexane. Each of the acquired sample was divided into two, each containing 9 µl of collected material. Subsequently, 1 µl of hexane (control) or 100 µM CBGA in hexane (assay) was added; 10 µl of 10 µM CBGA in hexane was used as a negative control. The samples were incubated in 37 °C for 30 min followed by centrifugation (16,000 g, 1 min) and vacuum concentration. The samples were subjected to GC-MS analysis.

To identify the enzymatic component in the exudates the material was collected into vials containing ice-cold acetone. The exudates obtained from week 5th and 8th were digested in-solution. Briefly, samples were centrifuged (20,000g, 4 °C, 15 min) and the precipitate was re-solubilized in 2 µl of 2 M guanidinium hydrochloride and 10 mM DTT. Cysteines were reduced by incubation at 56°C for 30 min and alkylated by adding 1 µl 60 mM IAA and incubation for 30 min in the dark. Twelve µl 50 mM ABC/1mM $CaCl_2$ and 1 µl of 10 ng/µl trypsin (sequencing grade, Promega, USA) were added. The samples were incubated for 14 h at 37°C. One µl of 1 % TFA was added to stop the reaction. The samples were subjected to nanoLC-MS/MS analysis.

RESULTS

The drug-type *Cannabis* used in our experiments is a medicinal strain with moderate amounts of THCA and CBDA (1:1 ratio). Due to low amount of the collected material, isomeric and hydrophobic nature of the analyzed compounds, we chose GC-MS to evaluate the samples after the bioassay. However, high temperatures operating during GC analysis cause decarboxylation of the analyzed cannabinoids, and, therefore, we identified their neutral forms (Fig.3). After incubating trichome exudates diluted in hexane with CBGA we noted 2.7 and 2.3-fold change in CBD and THC concentrations, respectively (Fig. 4).

We also identified the enzymatic component of the exudates - THCA synthase and CBDA synthase (Tab. 1). The accumulation profile of both cannabinoid synthases increased substantially from early-mid (5th week) to late (8th week) flowering stages (Fig. 5).

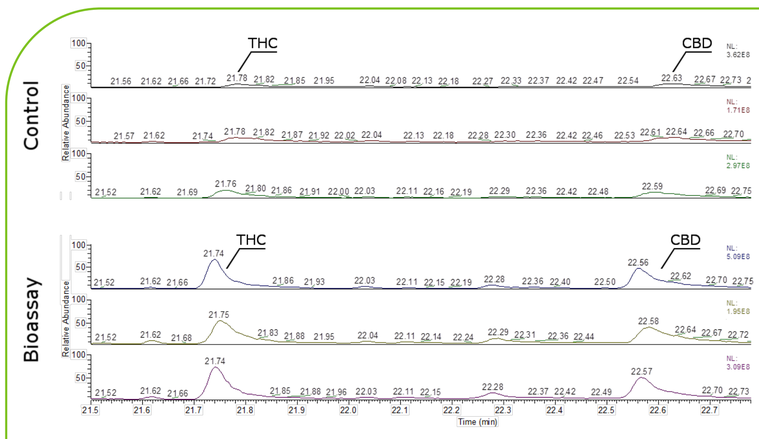

Fig. 3. Total ion chromatograms showing peaks identified as CBD (1) and THC (2) of the exudates isolated from glandular trichomes of the drug-type *Cannabis* plants after incubation in hexane without CBGA (**a**), and with CBGA.

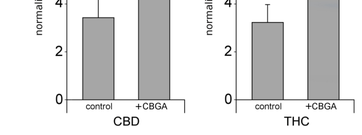

Fig. 4. The normalized intensity of THC and CBD in control samples and after incubation with CBGA; n=3; values represent mean ± SD.

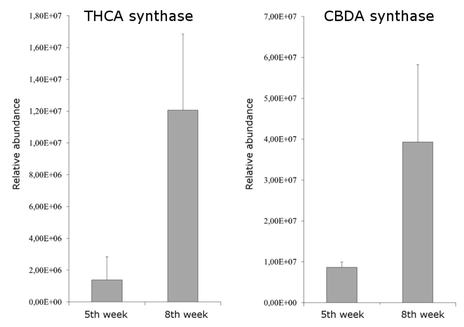

Fig. 5. Accumulation profiles of THCA synthase and CBDA synthase in the exudates of glandular trichomes isolated from the drug-type *Cannabis* plants at the 5th and 8th week of the flowering period; n = 3; values represent mean ± SD. The proteins were quantified on the basis of identified unique peptides.

Tab. 1. MASCOT search results for THCA synthase and CBDA synthase identified in the exudates of *Cannabis* glandular trichomes.

Accession	Description	Organism	Score	Coverage	Proteins	Unique Peptides	Peptides	PSMs	AAs	MW [kDa]	calc. pI
A6P6V9	Cannabidiolic acid synthase	*Cannabis sativa*	398	5,88	4	4	5	20	544	62,2	8,72
Q8GTB6	Tetrahydrocannabinolic acid synthase	*Cannabis sativa*	1007	3,85	4	1	2	29	545	61,9	8,95

CONCLUSIONS

In this study we demonstrated that cannabinoid synthases accumulated in the exudates of *Cannabis sativa* glandular trichomes are catalytically active in the organic phase. Our results support the hypothesis that the oxidative cyclization of CBGA may occur extracellularly in the exudates of glandular trichomes. However, a more detailed studies are needed to elucidate the exact mechanism of cannabinoid biosynthesis in glandular trichomes. Further analyses of trichome exudates from other plant species may reveal more enzymes, which are biosynthetically active in hydrophobic conditions. Understanding the mechanisms of biocatalysis in hydrophobic conditions may contribute to the development of trichomes as natural biosynthetic factories, and also advance the field of biosynthesis of secondary metabolites in organic solvents.

REFERENCES

1. Andre, C.M., Hausman, J.-F., and Guerriero, G. (2016). Cannabis sativa: The Plant of the Thousand and One Molecules. Frontiers in Plant Science 7.
2. Mahlberg, P.G, Kim, E.S. (2004). Accumulation of Cannabinoids in Glandular Trichomes of Cannabis (Cannabaceae). Journal of Industrial Hemp 9: 15–36.
3. Sirikantaramas, S. (2005). Tetrahydrocannabinolic Acid Synthase, the Enzyme Controlling Marijuana Psychoactivity, is Secreted into the Storage Cavity of the Glandular Trichomes. Plant Cell Physiol. 46, 1578–1582.