Antimicrobial compounds as side products from the agricultural processing industry

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Chapter 6

Anthranilate synthase inhibition

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Abstract

Anthranilate synthase (AS) is an enzyme in the biosynthetic pathway of tryptophan, and is used as a target enzyme for the study of the inhibitory growth of microorganisms. An HPLC assay was used to measure inhibition of a plant AS that was produced by a transgenic *Escherichia coli* strain. *Cannabis sativa* flower extracts showed the highest inhibition of AS, compared with two kinds of *Humulus lupulus* flower extracts. Among cannabinoids, cannabigerolic acid (CBGA) showed the highest inhibition followed by tetrahydrocannabinolic acid (THCA). Also hop bitter acids inhibited AS. The strongest AS inhibition was shown by adhumulone followed by *β*-acids and humulone. Iso-*trans*-adhumulone showed the highest inhibition compared with other iso-*α*-acids and iso-*cis*-adhumulone.

6.1 Introduction

Anthranilate synthase (AS) is a key enzyme in the biosynthesis of the amino acid tryptophan. This pathway occurs in microorganisms, plants and some parasites, but not in mammals. It is thus an interesting target for developing anti-microbial compounds. Anthranilate synthase catalyses the first committed step in the sequence of reactions which lead to the biosynthesis of tryptophan from chorismate. Tryptophan is an essential amino acid which is utilized in microorganisms and plants as a substrate for protein biosynthesis. In almost all microbial species, AS consists of nonidentical subunits designated as the AS α–subunit (ASI or component I) and AS β-subunit (ASII or component II) [Kawamura et al., 1978; Romero et al., 1994; Poulsen et al., 1993]. The plant anthranilate synthase has two subunits, the enzyme complex does not contain other functionalities. The gene for these subunits has been isolated from *Arabidobsis thaliana* [Niyogi and Fink, 1992; Niyoki et al., 1993]. Poulsen et al. [1993] were the first to purify and characterize anthranilate synthase (EC 4.1.3.27). They used cell cultures of *Catharanthus roseus* as a source.

In this study we used transgenic *Escherichia coli* (M15) which overexpressed the anthranilate synthase gene (EC 4.1.3.27) from *C. roseus* [Bongaerts, 1998]. *Cannabis sativa* and *Humulus lupulus* extracts and isolated compounds were tested for their inhibition of AS using the only known inhibitor, tryptophan [Robinson and Levy, 1976] as a control.

6.2 Materials and Methods

6.2.1 Plant extracts and compounds

Cannabis sativa and *Humulus lupulus* chloroform-methanol ($CHCl_3$-MeOH, 1:1) extracts are described in Chapter 3. The supercritical carbon dioxide extract of *H. lupulus* flowers was received from Botanix (Paddock Wood, Kent, UK).

A total of seven pure cannabinoid compounds, Δ^9–tetrahydrocannabinol (Δ^9–THC), tetrahydrocannabinolic acid (THCA), cannabidiol (CBD), cannabidiolic acid (CBDA), cannabigerol (CBG), cannabigerolic acid (CBGA) and cannabinol (CBN) were obtained from the *C. sativa* flower extract by centrifugal partition chromatography (CPC type LLB-M Sanki Engineering Limited Kyoto, Japan) according to a previous method [Hazekamp et al., 2004]. The bitter acids from *H. lupulus* supercritical carbon dioxide extracts were separated by CPC (type LLN, Sanki Engineering Limited Kyoto, Japan) following the method of Hermans-Lokkerbol et al. [1994]. The α-acids (humulone, cohumulone, adhumulone), β-acids, iso-α-acids and rest fractions of α-acids [patent pending, Wilson et al., 2006] were also used for this assay.

6.2.2 Enzyme preparation

Anthranilate synthase (EC 4.1.3.27) from transgenic *Escherichia coli* (M15) with Clone 4 (plasmid pQE-30-AS_4) was used in this study. Bacterial cell stock suspension from -80 °C was grown in LB broth (Difco, Le Pont de Claix, France) with antibiotics overnight and then streaked on LB agar plates containing antibiotics. The antibiotics used in this experiment were 25 mg/mL Kanamycin and 100 mg/mL Ampicillin (Duchefa, Haarlem, The Netherlands). These plates were incubated overnight before inoculating a single colony in 2 mL of LB broth with antibiotics for 16 hours. Bacterial cell suspensions were then mixed in 500 mL of LB broth containing antibiotics and incubated for 1 hour. One mM isopropyl-*β*-D-thiogalactopyranoside (IPTG, Eurogentec, Seraing, Belgium) was added to this culture 6 hours before incubation for the induction of gene expression. All incubations were done in a 37 °C incubator shaker (New Brunswick Scientific, New Jersey, USA) at 250 rpm. Cell suspensions were transferred to 50 mL tubes and centrifuged at 4 °C at 3,000 rpm for 20 minutes to precipitate the cells. The supernatants were discarded before mixing the cells with lysis buffer. The mixtures were centrifuged at 4 °C at 3,000 rpm for 20 minutes after swayed for 1 hour. Crude enzymes from the supernatants were transferred to Eppendorf tubes and put directly in liquid nitrogen and stored at -80 °C.

6.2.3 Incubation mixture

The incubation mixtures contained 250 mM $MgCl_2 \cdot (6\ H_2O)$ (Merck, Darmstadt, Germany), 0.1 M L-Glutamine (Merck Darmstadt, Germany), 0.1 M Tris-hydrochloric acid (pH 7.5) (Invitrogen, Scotland, UK), crude enzyme, Inhibitor and 10 mM Chorismate (Sigma, St. Louis, USA). The negative control did not contain an inhibitor, while the positive control contained L-tryptophan (Merck, Darmstadt, Germany) as an inhibitor. The mixtures were incubated in a water bath at 37 °C for 6 hours. The reaction was stopped by the addition of 1M phosphoric acid (H_3PO_4) (Merck, Darmstadt, Germany) before detecting the product of AS activity. These assays were done in triplicate.

6.2.4 Enzyme activity detection

HPLC with fluorescence detector (Shimadzu, Japan) was used to detect the AS at 340 nm excitation wavelength and 400 nm emission wavelength. The separations were done using a C_{18} reverse phase column (4.6 X 250 mm, 5 μM particle diameter, Vydac, USA). The mobile phase was a mixture of water-MeOH (4:1) with 50 mM H_3PO_4 that was adjusted with 6 M sodium hydroxide (NaOH) (Merck, Darmstadt, Germany) to the final pH 2.6-2.8. The flow rate was 1

mL/minute and the retention time of AS was 15 minutes. This method was adapted from Poulsen et al. [1991]. A twofold dilution series of AS (Fluka, Steinheim, Germany) from 0.078 to 5 µM were used as standard reference. From the peak areas of anthranilate the percentage of AS inhibition and the IC_{50} values were determined. All data were analyzed by SPSS 12.0 statistic analysis software (Chicago, USA) using one way analysis of variance (ANOVA) and least-significance difference (LSD), at 95% confidence.

6.3 Results and Discussion

Cannabis and hop flower $CHCl_3$-MeOH (1:1) extracts (2 mg/mL) inhibited anthranilate synthase enzyme activity. Cannabis flower extracts gave the highest inhibition followed by hop extracts and supercritical carbon dioxide extracts (Figure 6.1).

The seven cannabinoids (0.1 mM) were also tested for inhibition of AS. CBGA showed the highest percentage of enzyme inhibition followed by THCA. Weak inhibition was found for Δ^9-THC and CBN, no activity was found on CBD or CBDA (Figure 6.2). At a higher concentration of the seven cannabinoids (1.0 mM), CBGA again showed the highest percentage of enzyme inhibition, followed by THCA and CBDA. Weak inhibition was found for CBG, but no activity was found for Δ^9-THC, CBN or CBD (Figure 6.3).

The α-acids (cohumulone, humulone, adhumulone) and the β-acids, including two unknown contaminants of α-acids and iso-α-acids (1.0 mM), were tested for inhibition of AS. Adhumulone, β-acids and humulone showed the highest enzyme inhibition. The contamination of the α-acids also showed inhibition (Figure 6.4). At the lower test concentration of α-acids (0.1 mM), humulone and adhumulone still showed inhibition of anthranilate synthase but no inhibition was observed with cohumulone (data not shown).

The iso-α-acids (0.1 mM) were also tested for inhibition of AS. Iso-*trans*-adhumulone alone showed the highest percentage of enzyme inhibition, followed by the iso-mixture. Weak inhibition was found for iso-*cis*-cohumulone, iso-*cis*-adhumulone and iso-*trans*-cohumulone, no activity was found for iso-*cis*-humulone or iso-*trans*-humulone (data not shown).

For the AS enzyme feedback inhibitor, L-tryptophan, and the most active inhibitors, THCA and CBGA, the IC_{50} values were determined by twofold dilution to the concentrations of 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.0312 mM. The IC_{50} values of L-tryptophan, THCA and CBGA are 63.1, 313.8 and 398.2 µM, respectively (Figure 6.5).

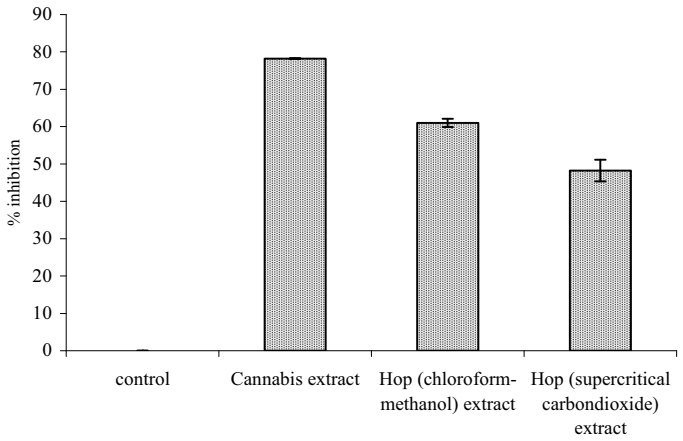

Figure 6.1 Percentage of anthranilate synthase enzyme inhibition from 2 mg/mL cannabis and hop flower extracts. Data shown as mean (n=3) with standard deviation as error bars; significant difference *($p<0.05$)*.

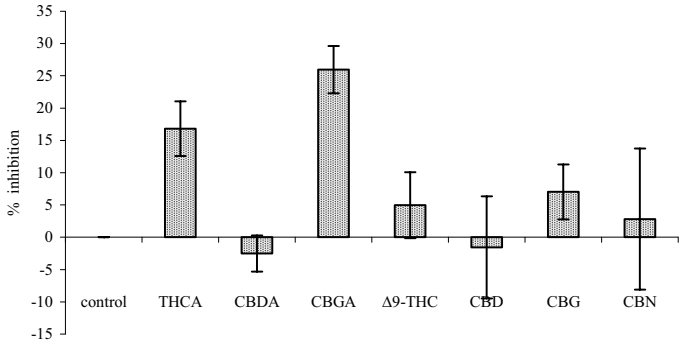

Figure 6.2 Percentage of anthranilate synthase enzyme inhibition by 0.1 mM cannabinoid. Data shown as mean (n=3) with standard deviation as error bars; significant difference (*$p<0.05$*).

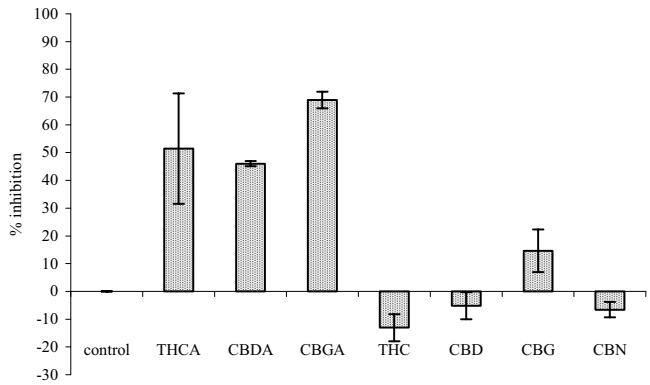

Figure 6.3 Percentage of anthranilate synthase enzyme inhibition by 1.0 mM cannabinoid. Data shown as mean (n=3) with standard deviation as error bars; significant difference ($p<0.05$).

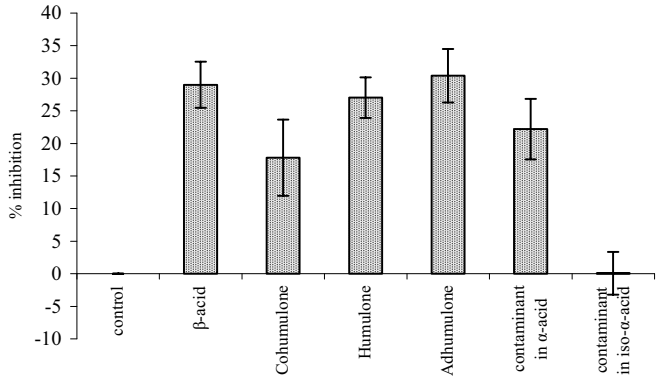

Figure 6.4 Percentage of anthranilate synthase enzyme inhibition by 1.0 mM α-acid (adhumulone, humulone or cohumulone), β-acids and two contamination compounds of α-acids and iso-α-acids. Data shown as mean (n=3) with standard deviation as error bars; significant difference ($p<0.05$).

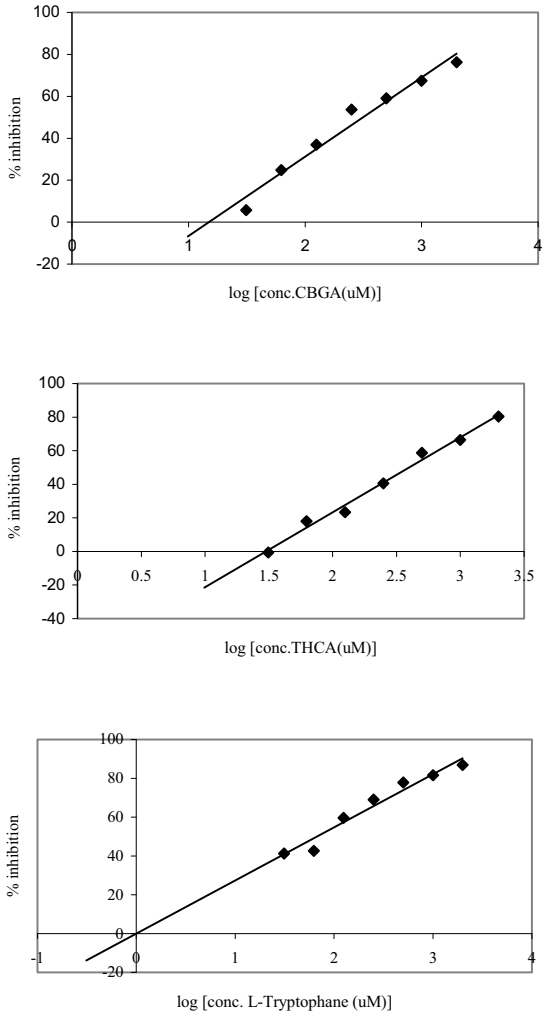

Figure 6.5 Percentage of anthranilate synthase enzyme inhibition by CBGA, THCA and L-tryptophan at the concentrations of 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.0312 mM. Linear equations of CBGA, THCA and L-tryptophan are $y = 37.828x - 44.446$ ($r^2 = 0.967$), $y = 44.546x - 65.825$ ($r^2 = 0.9891$) and $y = 27.364x$ ($r^2 = 0.958$), respectively.

6.4 Conclusion

Anthranilate synthase is an interesting target enzyme for antimicrobial activity due to its presence in microorganisms for the synthesis of the essential amino acid tryptophan. In the tests of the seven cannabinoids and hop α- and β-acids, CBGA showed the highest inhibition followed by THCA. For the bitter acids, the highest AS inhibition was found for adhumulone, followed by the β-acids and humulone. Although some inhibitory compounds were found, their activity was not as strong as tryptophan. This study shows that AS can be used as a target enzyme to investigate the mode of action of plant compounds in microorganisms.