Biocatalysis and Agricultural Biotechnology 17 (2019) 525–528

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Cytochrome P450 mediated hydroxylation of ibuprofen using *Pichia pastoris* as biocatalyst

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ARTICLE INFO

Keywords:
Cytochrome P450
Ibuprofen
Hydroxylation
API Metabolism

ABSTRACT

Class VIII self-sufficient CYP from *Aspergillus fumigatus* (CYP505X) and a variant with five mutations were heterologously expressed in *Pichia pastoris*. Ibuprofen – a widely used non-steroidal anti-inflammatory drug (NSAID) – was oxidized most efficiently in a whole cell mediated biotransformation. The two major metabolites were isolated by preparative HPLC and their structure elucidated by NMR. Specifically, ibuprofen was hydroxylated at the tertiary carbon atom and the benzylic position on the lipophilic side chain.

1. Introduction

In drug development, not only the drug as such, but also the impact of its metabolites on the organism are of great importance to avoid adverse drug reactions (ADRs). Authentic standards of human drug metabolites are needed for structure elucidation and toxicological tests. One of the challenges is the identification of the molecular structure of these metabolites. Another is to prepare sufficient amounts of each compound. Metabolite structures are typically elucidated by LC-MS/MS analysis of samples from incubations with disrupted tissue samples (e.g. human liver homogenate) or microsomal preparations thereof (Guengerich, 2015). Also bactosomes (Kajbaf et al., 2011) or supersomes (Chang et al., 2011) that contain recombinant drug metabolizing enzymes are used for this purpose. The application of recombinant human enzymes in preparative syntheses has been shown for several API metabolites (Winkler et al., 2018), but human enzymes are typically more complex in comparison to their microbial counterparts. Consequently, they are less well accessible from microbial host systems, and they tend to be less active and stable. Human cytochrome P450 enzymes (CYPs), for example, are membrane associated. An electron transport system provides the electrons to CYPs for oxygen activation and substrate oxidation (Nebert et al., 2013) and human CYPs require accessory proteins for this purpose. Microbial CYPs partly mimic the action of human CYPs (Worsch et al., 2018). We recently investigated CYP505X from *Aspergillus fumigatus* (*Af*CYP505X) (Migglautsch et al., 2018). *Af*CYP505X is a self-sufficient class VIII CYP, which is characterized by the fusion of the reductase domain to the CYP domain (Weis et al., 2009). From the overall structure, *Af*CYP505X resembles the well investigated CYP102 from *Bacillus megaterium* (CYP BM3) (Whitehouse et al., 2012) and the protein from *Fusarium oxysporum* (CYPfoxy) (Ichinose, 2014). The advantage of self-sufficient CYPs is on the one hand their intrinsically balanced ratio of CYP to redox partner of 1:1 and on the other hand their soluble nature, which typically leads to higher expression levels as compared to membrane associated CYPs.

Ibuprofen is widely used as non-steroidal anti-inflammatory drug (NSAID), mostly for the treatment of inflammation, pain and fever. Prostaglandin is associated to inflammation and fever and ibuprofen inhibits the prostaglandin producing enzymes cyclo-oxygenases COX1 and COX2 (de Martino et al., 2017). Based on its good tolerability profile, ibuprofen is the only NSAID approved for use in children from the age of 3 months on and also a preferred analgesic or anti-inflammatory drug for nursing mothers (de Martino et al., 2017; Drugs and Lactation Database, 2006). Ibuprofen is marketed as an oral suspension for paediatric use and avoiding the risk of Reye's syndrome has replaced acetylsalicylic acid for the treatment of inflammation. However, associated with its increased consumption, the Paediatric Working Group of the Italian Drugs Agency (AIFA) noted an increase of suspected ADRs, including gastrointestinal bleeding or renal damage (Baiardi et al., 1982; de Martino et al., 2017). The main enzymes in metabolism of ibuprofen in the human liver are CYP2C8 and CYP2C9, which are responsible for its stereoselective hydroxylation in phase I drug metabolism (Martínez et al., 2012).

Within the last two decades the methylotrophic yeast *Pichia pastoris*, recently re-classified as *Komagataella phaffii* (Kurtzman, 2005), has

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https://doi.org/10.1016/j.bcab.2018.12.022
Received 9 November 2018; Accepted 19 December 2018
Available online 20 December 2018

become an important alternative to *E. coli* or mammalian cell lines for the production of recombinant proteins (Ahmad et al., 2014). Easy handling, strong promoters (such as for example the methanol inducible *AOX1* promoter) and high cell density cultivations as well as the capability of post-translational modifications are some of the major benefits of this yeast. Recent sequencing approaches further promoted the development of a versatile *P. pastoris* expression toolbox (Sturmberger et al., 2016; Vogl et al., 2014). In contrast to *E. coli*, *P. pastoris* contains endogenous CYPs. Yet, this is not considered as a complication since endogenous P450s appear in insignificant levels and are hardly detectable by CO-difference spectroscopy (Lee et al., 2010). Herein, we investigated *P. pastoris* strains expressing wild-type *Af*-CYP505X and a variant harboring five mutations (5xmut) (Migglautsch et al., 2018).

2. Materials and methods

2.1. Expression and bioconversion

For the expression of *Af*CYP505X (EAL92660.1) the *P. pastoris* host strain BG11 (*aox1*Δ, Mut^S), which originates from strain BG08 (Sturmberger et al., 2016), was used. *P. pastoris* strain BG11 was transformed with plasmid pPpT4 (Näätsaari et al., 2012) harboring the *Af*CYP505X gene under control of the *AOX1* promoter. Cultivation of *P. pastoris* whole cell biocatalysts was done according to Weis et al. (Weis et al., 2004), however, in 2 L baffled shake flasks and with methanol supplementation to 0.5% every 24 h. Cells were harvested, washed twice in ice cold 100 mM KPi buffer (pH7.4) and resuspended in approximately 10 ml of reaction buffer (PS buffer, 100 mM KPi, pH7.4, 8.5% sucrose) per gram wet cell weight. Cells were lyophilized and/or frozen and stored at −80 °C until further use.

2.2. Analytics

Determination of oxidative activity was performed with HPLC-MS as described previously (Migglautsch et al., 2018). For analysis of ibuprofen and its metabolites, a stepwise gradient was used at 25 °C: 20% ACN (0–1 min), 20–100% ACN (1–3 min), 100% ACN (3–5 min) and 20% ACN (5–6 min) re-equilibration. The compounds were detected at 239 nm (VWD). The mass spectrometer was operated in positive electrospray ionization mode. The drying gas flow was 12 L/min at 350 °C, and the capillary voltage was 3 kV. Selected-ion monitoring or full-scan product ion spectra were collected. MS spectra supported data evaluation. For calculations, peak areas of the UV chromatograms recorded at 239 nm were used. Retention times were 2.88 min for 2-OH ibuprofen, 3.01 min for an unidentified mono-oxidized ibuprofen metabolite, 3.13 min for 1-OH ibuprofen and 3.76 min for ibuprofen.

2.3. Preparative scale reaction

A total reaction volume of 500 ml (optical density at 600 nm (OD_{600}) corresponding to 100) was used for preparative scale oxidation of 502 mg ibuprofen (dissolved in 25 ml of DMSO) as described previously for capsaicin (Migglautsch et al., 2018). Unreacted ibuprofen and its metabolites were extracted with EtOAc. The solvent of combined organic layers was removed under reduced pressure. Compounds were separated via reverse phase HPLC on and Agilent Technologies Series 1100. Detection was accomplished with a Variable Wavelength Detector. The separations were carried out on a Zorbax SB-C18 (250 × 21 mm, 5.0 µm) column. As eluents acetonitrile and water with 0. 1% HCOOH were used in a stepwise gradient: 0–1 min linear increase 0–25% ACN, 1–15 min linear increase to 25–100% ACN, 15–17 min 100% ACN isocratic, 17–20 min linear decrease to 25% ACN, 20–30 min linear decrease to 0% ACN, 4 ml/min, 25 °C.

2.4. NMR

^{1}H and ^{13}C NMR spectra of lyophilized pooled fractions were recorded on a Varian 500 spectrometer (^{1}H: 499.86 MHz; ^{13}C: 125.69 MHz) and chemical shifts are referenced to residual protonated solvent signals as internal standard.

2-Hydroxyibuprofen (NMR signals identical to those of commercial standard LGC DRE-C14278160)

^{1}H NMR (499.86 MHz; DMSO-d_6) δ = 7.17 (m, 4 H, Ar-H), 4.23 (bs, 1 H, OH), 3.62 (q, $^3J_{HH}$ = 7.1 Hz, 1 H, **CH**-CH_3), 2.63 (s, 2 H, CH_2), 1.38 (d, $^3J_{HH}$ = 7.1 Hz, 3 H, **CH_3**-CH), 1.03 (s, 6 H, 2xCH_3)

^{13}C NMR (125.69 MHz; DMSO-d_6) δ = 178.5, 140.2, 139.8, 130.7, 128.2, 51.8, 44.2, 29.2, 18.9.

1-Hydroxyibuprofen

^{1}H NMR (499.86 MHz; DMSO-d_6) δ = 7.20 (m, 4 H, Ar-H), 4.18 (d, $^3J_{HH}$ = 6.4 Hz, 1 H, **CH**-OH), 3.49 (q, $^3J_{HH}$ = 7.1 Hz, 1 H, **CH**-CH_3), 1.88 (m, 1 H, CH), 1.36 (d, $^3J_{HH}$ = 7.1 Hz, 3 H, **CH_3**-CH), 0.86 (d, $^3J_{HH}$ = 6.4 Hz, 3 H, CH_3), 0.76 (d, $^3J_{HH}$ = 6.4 Hz, 3 H, CH_3).

^{13}C NMR (125.69 MHz; DMSO-d_6) δ = 177.5, 142.8, 141.8, 128.7, 128.2, 77.9, 47.9, 35.6, 19.0, 18.9, 17.9.

3. Results and discussion

Owing to varying substrate transport phenomena in different microbial hosts, a substrate profile was established for lyophilized cells of a wild type (A15) and 5xmut *Af*CYP505X (A21) expressing *P. pastoris* strain using more than 30 active pharmaceutical ingredients (APIs) and (hetero)cyclic compounds as substrates. Of all tested compounds, 9 were oxidized by CYP505X wild-type and 13 by the 5x mutant (Table 1). Using *P. pastoris* as biocatalyst, capsaicin was oxidized most efficiently by wild-type and also by the variant to similar metabolites as described previously for *E. coli* (Migglautsch et al., 2018). Conversions of other compounds, however, were significantly higher as compared to those obtained when using *E. coli* as whole cell biocatalyst. Intrigued by promising conversion levels of ibuprofen to three mono-oxidized metabolites as judged by mass spectroscopy, we hypothesized that CYP505X – being similar to fatty acid hydroxylases – should preferably oxidize the isobutyl moiety.

Ibuprofen oxidation by CYP505X wild type and 5xmut were studied further using a resting cell approach with intact frozen cells and citrate and $NADP^+$ to support cofactor regeneration (Hanlon et al., 2012, 2007). Three possible products were detected by HPLC-MS in both, the CYP505X wild-type and the CYP505X 5xmut biooxidation series. Conversion rates for wild type were low, reaching a maximum of about 15% of total product after 16 h of reaction time (data not shown). In

Table 1
Screening of CYP505X and variant expressed in *P. pastoris* for their ability to oxidize APIs. Conversions were calculated by area normalization and represent the average of at least two separate measurements. 5 mM of substrate was used each.

entry	Substrate	CYP505X wild type Conversion (%)	CYP505 × 5x mutant
1	Capsaicin	96[a]	61[a]
2	Chlorzoxazone	36	32
3	Clopidogrel	0	24
4	Ethionamide	19	39
5	Famciclovir	0	< 1
6	Harmine	0	< 1
7	Ibuprofen	26[a]	52[a]
8	Metoprolol	< 1	3
9	Naphthol	11	21
10	Nebivolol	7	6
11	Phenacetin	3	5
12	Progesterone	7	19[a]
13	Tolbutamide	0	6[a]

[a] HPLC-MS indicated the formation of 2–3 products.

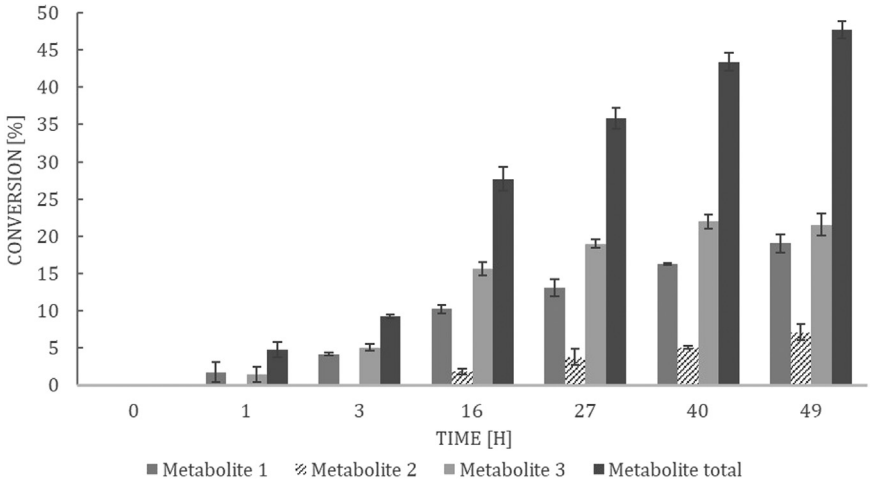


Fig. 1. Whole cell ibuprofen biooxidation over 49 h by CYP505X 5xmut (A21) expressed in *P. pastoris*. Conversions were calculated by area normalization and gave three monooxygenation products (metabolite 1–3) according to HPLC/MS; M total is the sum of metabolite 1–3 and represents the overall total conversion.

contrast, the CYP505X quintuple mutant (A21) oxidized approximately 50% of the added ibuprofen within 49 h (Fig. 1). One of the three oxidized products (metabolite 3) seemed to be favorized by this mutant, whereas the WT produced metabolite 1 and metabolite 3 in equal amounts, and only traces of metabolite 2. The reaction rate for ibuprofen oxidation of strain CYP505X 5xmut (A21) was determined in parallel end-point measurements and was 0.147 mM/h. The specific reaction rate *s* was 0.12 $U/mg_{(DCW)}$.

*Af*CYP505X 5xmut (A21) was studied in a preparative scale reaction using 500 mg of substrate. Ibuprofen biooxidation was monitored for 30.5 h. Several samples were withdrawn and analyzed by HPLC/MS to determine conversion. After 22 h about 81% of substrate were converted and the analytical yield did not further increase until the reaction was stopped (Fig. 2). Consistent with analytical scale experiments, three metabolites were formed to an extent of 30% (metabolite 1), 16% (metabolite 2) and 37% (metabolite 3), respectively.

According to HPLC/MS, the three metabolites had a mass of 223 g/mol, respectively, and are in agreement with Neunzig et al. (2012) most likely 1-OH-ibuprofen, 2-OH-ibuprofen or 3-OH-ibuprofen. Isolation and characterization of metabolites of ibuprofen was performed according to Kepp et al. (1997). The metabolites were isolated by preparative reversed phase chromatography followed by lyophilization. Neunzig et al. (2012) published MSD spectra of 2-OH- and 3-OH-ibuprofen. We compared our chromatograms and MSD spectra (unpublished data of ibuprofen conversion in negative mode) with their data, but were not able to identify any of our products unambiguously. Since we presumed, that one of these metabolites would be 2-OH-ibuprofen, we compared our data to a commercial standard (LGC, item no.: DRE-C14278160). Comparison of the NMR spectra revealed, that metabolite 1 was indeed 2-hydroxyibuprofen. HPLC fractions containing metabolite 3 were analyzed additionally by 2D NMR and could clearly be identified as 1-hydroxyibuprofen. The reaction scheme of ibuprofen oxidation is summarized in (Scheme 1).

4. Conclusion

Self-sufficient CYP505X from *Aspergillus fumigatus* AF293 expressed in *P. pastoris* was evaluated as a biocatalyst for the biooxidation of APIs and heterocyclic compounds. A variant with five mutations in the amino acid sequence was able to oxidize 13 xenobiotics and was most efficient for the metabolization of ibuprofen using a resting cell biotransformation approach. This reaction was studied in detail and upscaled to 500 ml preparative scale. The products were isolated, purified by preparative HPLC and their chemical structures determined by NMR. Two metabolites were identified as 1- and 2-hydroxyibuprofen.

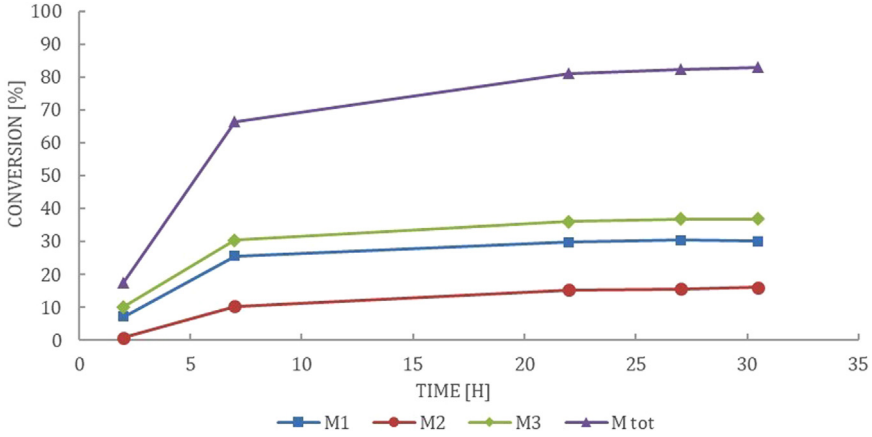
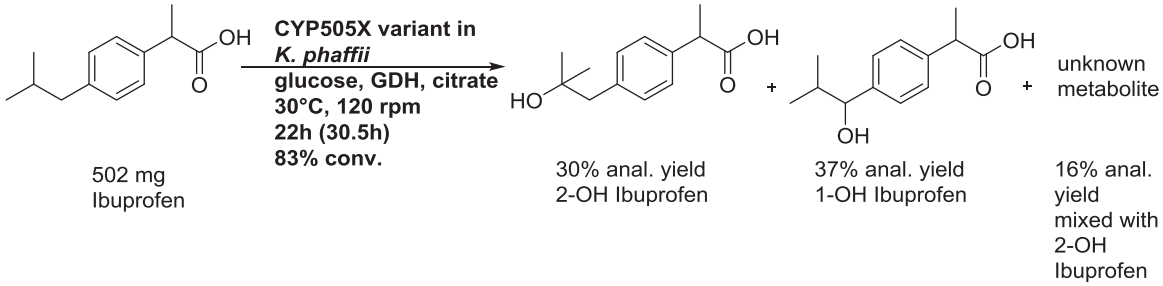


Fig. 2. Ibuprofen biooxidation of CYP505X 5xmut expressing *P. pastoris* cells in a preparative scale reaction using 500 mg of substrate. metabolite 1–3: mass of 223 g/mol, metabolite 1 = 2-hydroxyibuprofen (2-OH-Ibu), metabolite 2 = unidentified, Metabolite 3 = 1-hydroxyibuprofen (1-OH-Ibu).



Scheme 1. Biooxidation of ibuprofen.

CRediT authorship contribution statement

Claudia Rinnofner: Supervision, Validation, Writing - original draft. **Bianca Kerschbaumer:** Investigation, Methodology. **Hansjörg Weber:** Investigation, Formal analysis. **Anton Glieder:** Conceptualization, Resources. **Margit Winkler:** Conceptualization, Data curation, Funding acquisition, Project administration, Supervision, Writing - original draft, Writing - review & editing.

Acknowledgements

We would like to acknowledge Jörg-Patrick R. Eichmann and Anna Alkofer for their skillful support in experimental work. This work received funding from the EU project ROBOX (grant agreement no 635734) under EU's H2020 Programme Research and Innovation actions H2020-LEIT BIO-2014-1 Disclaimer: This publication reflects the author's view and the *Agency* is not responsible for any use that may be made of the information it contains. This work has also been supported by bisy e.U. and the Austrian BMWD, BMVIT, SFG, Standortagentur Tirol, Government of Lower Austria and Business Agency Vienna through the Austrian FFG-COMET- Funding Program.

Declarations of interest

bisy has a commercial interest in biocatalysts for drug metabolite synthesis.

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