ORIGINAL RESEARCH PAPER

Biocatalysed halogenation of nucleobase analogues

Rosario Médici · Juan Ignacio Garaycoechea ·
Lucas Andrés Dettorre · Adolfo Marcelo Iribarren ·
Elizabeth Sandra Lewkowicz

Abstract The synthesis of halogenated nucleosides
and nucleobases is of interest due to their chemical
and pharmacological applications. Herein, the enzy-
matic halogenation of nucleobases and analogues
catalysed by microorganisms and by chloroperoxi-
dase from *Caldariomyces fumago* has been studied.
This latter enzyme catalysed the chlorination and
bromination of indoline and uracil. *Pseudomonas*,
Citrobacter, *Aeromonas*, *Streptomyces*, *Xanthomo-
nas*, and *Bacillus* genera catalysed the chlorination
and/or bromination of indole and indoline. Different
products were obtained depending on the substrate,
the biocatalyst and the halide used. In particular, 85%
conversion from indole to 5-bromoindole was
achieved using *Streptomyces cetonii*.

Introduction

Nucleobase and nucleoside analogues have applica-
tions in several areas, including their use as antibac-
terial, antiviral and anticancer chemotherapeutic
agents. Particularly, halogenated benzimidazole
nucleosides have received much attention due to the
potent and selective inhibition of HCMV replication
displayed by ribosylated poly-halogenated benzimi-
dazoles. Halogenated indole nucleosides are proposed
as alternative to benzimidazole ones but both are
rapidly degraded in vivo due to the instability of their
glycosidic bonds (Williams et al. 2004). In this sense,
a bio-catalysed methodology that could afford sugar
modified nucleosides of theses nucleobases may
provide an appropriate solution (Li et al. 2010).

To date, the number of known halogenating is
small (Murphy 2003). They belong to four main
groups: haloperoxidases, perhydrolases, methyl trans-
ferases, and halogenases. Haloperoxidases (HPO)
catalyze the halogen–carbon bond formation of many
organic compounds in the presence of halide ions and
peroxides such as H_2O_2. Depending on the prosthetic
group required, they can be subdivided into heme and
vanadium-dependent (Littlechild 1999). Due to their
reaction mechanism, HPO produces free diffusible

R. Médici · J. I. Garaycoechea · L. A. Dettorre ·
A. M. Iribarren · E. S. Lewkowicz (✉)
Biotransformation Laboratory, Universidad Nacional de
Quilmes, R. S. Peña 352 Bernal, 1876 Buenos Aires,
Argentina
e-mail: elewko@unq.edu.ar

A. M. Iribarren
INGEBI, CONICET, Vuelta de Obligado 2490,
1428 Buenos Aires, Argentina

hypohalous acids that act as halogenating reagents. The most extensively studied heme-containing peroxidase is the chloroperoxidase (CPO) from the fungus *Caldariomyces fumago* (Yazbik and Ansorge-Schumacher 2010).

Perhydrolases, previously named bacterial HPO, are mainly isolated from *Streptomyces* and *Pseudomonas*. Unlike HPOs, these enzymes have no prosthetic group, do not require metal ions and follow a different reaction mechanism (van Pée 2001). The crystal structure of halogenating enzymes from *Streptomyces lividans* and *Pseudomonas fluorescens* revealed that they belong to a larger group of hydrolases that contain a α/β-fold and the serine-histidine-aspartate catalytic triad (Hofmann et al. 1998). In presence of short-chain carboxylic acids and H_2O_2, perhydrolases catalyse the formation of peracids, which oxidise the halide ion resulting in the formation of hypohalous acid as the halogenating agent.

Cytosine, uracil, cytidine, and pyrazole as well as iodination of uracil and pyrazole can be brominated by a vanadium-dependent haloperoxidase (Itoh et al. 1987). The perhydrolase from *Pseudomonas pyrrocinia* also catalysed the regioselective chlorination of indole at carbon seven (Wiesner et al. 1988) while *C. fumago* CPO produced only oxindole (Wiesner et al. 1986). Spite of the fact that HPOs and perhydrolases generate hypohalous acid, these reports suggest that they may show different catalytic behaviour.

In this study, we screened our cell collection for halogenating activity and compared the performance of the selected strains with that of the commercial CPO from *C. fumago* with the final goal of preparing halogenated nitrogen heterocycles and nucleobases.

Materials and methods

Chemicals and microorganisms

Reagents and substrates were purchased from Sigma-Aldrich, Riedel de Häen or Fluka. The culture media components were obtained from Merck, Difco or Britania. Solvents for qualitative and quantitative analyses were from J. T. Baker, Sintorgan or Biopack. CPO from *Caldariomyces fumago* (aq, 25,000 U/ml) was from Sigma. Most of the microorganisms were kindly supplied by the *Colección*

Española de Cultivos Tipo (CECT), *Universidad de Valencia* (Spain).

Biocatalyst preparation

The strains were cultured in liquid media at the below detailed optimum temperature (T) and time (t), according to the American Type Culture Collection (ATCC): *Aeromonas* (T: 30°C, t: 1 day), *Pseudomonas* (T: 26°C, t: 1 day), *Bacillus* (T: 30°C, t: 1 day), *Achromobacter* (T: 30°C, t: 2 days), *Citrobacter* (T: 37°C, t: 1 day), *Enterobacter* (T: 37°C, t: 1 day), *Klebsiella* (T: 37°C, t: 2 days), *Escherichia* (T: 37°C, t: 1 day), *Proteus* (T: 37°C, t: 1 day), *Xanthomonas* (T: 26°C, t: 1 day), *Cellulomona* (T: 30°C, t: 1 day), *Staphylococcus* (T: 37°C, t: 1 day), *Micrococcus* (T: 30°C, t: 1 day), *Agrobacterium* (T: 26°C, t: 2 days) and *Serratia* (T: 26°C, t: 5 days) were grown in Luria Broth medium; *Erwinia* (T: 30°C, t: 1 day) and *Arthrobacter* (T: 26°C, t: 2 days) in Agar II; *Corynebacterium* (T: 30°C, t: 2 days) and *Brevibacterium* (T: 30°C, t: 2 days) in *Corynebacterium* medium; *Lactobacillus* (T: 37°C, t: 1 day) in MRS broth (oxoid CM359); *Streptomyces* (T: 28°C, t: 5 days) in *Streptomyces* medium; *Alicyclobacillus acidocaldarius* (T: 55°C, t: 1 day) in *Alicyclobacillus acidocaldarius* medium while *Nocardia* (T: 30°C, t: 1 day) in YEME (Bennett's agar) medium.

The saturated culture broths were centrifuged at $12,000 \times g$ for 10 min and the pellets, washed with 0.1 M sodium acetate buffer pH 4 and recentrifuged, were directly used as the biocatalysts.

Enzymatic biotransformation

The optimised assay consisted of 0.1 M sodium phosphate buffer pH 2.75, 20 mM KCl or KBr, 10 mM nitrogen heterocyclic compound and 4 U CPO/ml. The reaction was initiated by the addition of H_2O_2 (0.1 mmol pulses every 30 min for 3 h) to afford a final concentration of 20 mM. The mixture was further stirred for 3 h at 30°C. Aliquots taken at different times were analysed by TLC and HPLC. All assays were performed in triplicate.

Microbial biotransformation

The optimised assay consisted of 0.1 M sodium acetate buffer pH 4, 14 mM KCl or KBr, 10 mM

nitrogen heterocyclic and 200 mg wet wt biocatalyst/ml. The reaction was initiated by the addition of 20 mM H_2O_2 and the mixture was stirred at 35°C for 24 h. Samples were centrifuged at $\sim 8{,}000 \times g$ for 30 s and the supernatants were analysed by HPLC and TLC. All assays were performed in triplicate.

Analysis of reaction mixtures

TLC analyses were performed on silica gel plates using the following mobile phases: indole:CHCl$_3$/methanol (90:10 v/v); uracil and purine: CHCl$_3$/methanol (85:15 v/v); indoline: CH$_2$Cl$_2$; benzimidazole: petroleum ether: ethyl acetate (80:20 v/v).

HPLC analyses were performed using a C-18 column (150×4 mm). The UV detector was set at 254 nm and the column was operated at room temperature. Elution at 1 ml/min was as follows: 5 min water/methanol (50:50 v/v), 8 min gradient to water/methanol (25:75 v/v), and 2 min water/methanol (25:75 v/v).

GC-MS analyses were performed on a Shimadzu GC17A and QP-5000 equipment operating at ionization potential 70 eV, using Ultra-2 column (30 m $\times$ 0.20 mm $\times$ 0.33 μm) and the following temperature program: 60°C (1 min), 10°C/min, 280°C (10 min).

Results and discussion

The most widely used assay to screen HPO activity employs monochlorodimedone (MCD) as a synthetic substrate for the halogenating enzyme (Hager et al. 1966). The method is based on the conversion of MCD to the dihalogenated derivative (Fig. 1), which can be easily monitored by the decrease in UV absorption that is directly proportional to the enzymatic activity.

Applying this methodology, a primary screening of our cell collection was performed. The selected microorganisms that showed chlorinating activity were *Streptomyces griseus*, *Streptomyces baldacii*, and *Alicyclobacillus acidocaldarius* while those that showed brominating activity were *Aeromonas salmonicida* and *Sthaphilococcus auricularis*. On the other hand, *Pseudomonas putida*, *Citrobacter koseri*, *Aeromonas hydrophila*, *Streptomyces cetonii*, *Xanthomonas traslucens*, *Streptomyces baldaccii*, *Bacillus*
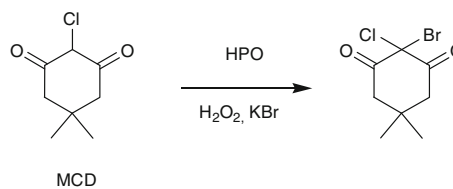

Fig. 1 Monochlorodimedone (MCD) bromination by haloperoxidase (HPO). MCD assay: The reaction mixture consisted of 0.1 M sodium acetate buffer pH 4, 200 mM potassium chloride or bromide, 0.1 mM MCD, and 5×10^9 dehydrated cells/ml. The reaction was initiated by the addition of 2 mM hydrogen peroxide and the mixture was stirred at 35°C for 2 h. The conversion of MCD to dichlorodimedone or bromochlorodimedone was measured in a UV spectrophotometer as the loss of absorbance at 278 nm (Hager et al. 1966). Aliquots were also analysed by TLC employing as mobile phase hexane: ethyl acetate/acetic acid (15:50:5 v/v) for the chlorination reaction and 40:60:2 v/v in case of bromination. All assays were performed in triplicate

cereus, *Bacillus thermoglucosidasius*, *Bacillus stearothermophilus* exhibited both activities.

Since the biocatalysed halogenation of indole had already been reported (Wiesner et al. 1986), this substrate was employed in a secondary screening using the biocatalysts previously selected. After 24 h, almost all strains consumed ca. 50% of the original indole in both reactions. The main product obtained in all cases was identified as 2-oxindole.

In order to improve the halogenation yields, the following variables were analysed: (a) biocatalyst preparation: dehydrated cell or wet cell paste; (b) biocatalyst amount: up to 200 mg/ml; (c) substrate concentration: 0.1–10 mM; (d) oxidant concentration: 2–20 mM. Using the optimised conditions described in "Materials and methods" section, only *Streptomyces cetonii* catalysed the formation of 7-chloroindole, while 5-bromoindole was produced using *Streptomyces*, *Pseudomonas*, *Aeromonas* and *Citrobacter* strains (Table 1; Fig. 2). In particular, *Streptomyces cetonii* afforded 85% conversion to 5-bromoindole (w/w, based on indole), being 2-oxindole the unique minor product, determined by GC-MS using commercial reference samples.

The activity of CPO from *Caldariomyces fumago* was assayed in similar conditions to those employed for the microbial transformation. After 3 h, the mixture was analysed by TLC and HPLC, showing that 2-oxindole was the principal product obtained. Therefore, different experimental conditions were analysed to improve halogenation: (a) buffer: sodium

Table 1 Halogenated products obtained by *C. fumago* CPO and selected bacteria

Substrate	Product	Conversion (%)[a]	Biocatalyst source[b]
Indole	7-chloroindole	<5	*Streptomyces cetonii*
	5-bromoindole	85	*Streptomyces cetonii*
		16	*Pseudomonas putida*
		6	*Aeromonas hydrophila*
		28	*Citrobacter koseri*
Indoline	5-chloroindoline	<5	CPO, *Xanthomonas traslucens*
	7-chloroindoline	<5	*Pseudomonas putida, Citrobacter koseri, Aeromonas hydrophila, Alicyclobacillus acidocaldarius*
	5-bromoindoline	5	CPO
	7-bromoindoline	<5	*Pseudomonas putida, Citrobacter koseri, Aeromonas hydrophila, Bacillus cereus*
Uracil	5-chlorouracil	<5	CPO
	5-bromouracil	15	CPO

[a] % conversion = [product]$_{obtained}$ × 100/[substrate]$_{initial}$

[b] 4 U CPO/ml or 200 mg wet weight bacteria/ml (28.9 ± 1.0 mg dry weight *Streptomyces cetonii*/ml; 17.7 ± 0.4 mg dry weight *Citrobacter koser*/ml; 8.8 ± 0.2 mg dry weight *Alicyclobacillus acidocaldarius*/ml; 8.1 ± 0.9 mg dry weight *Pseudomonas putida*/ml; 11.5 ± 1.0 mg dry weight *Bacillus cereus*/ml; 9.4 ± 0.3 mg dry weight *Aeromonas hydrophila*/ml; 8.6 ± 0.5 mg dry weight *Xanthomonas traslucens*/ml)
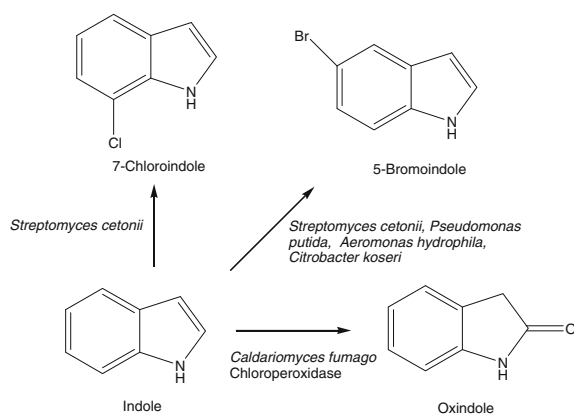
Fig. 2 Indole conversion by halogenating enzymes

phosphate pH 2.75 or sodium acetate pH 4; (b) halide concentration: 14 or 20 mM; (c) indole concentration: 0.1, 1, 2 or 10 mM; (d) CPO units: 3, 4, 6, 10 or 12 U/ml; (e) oxidant: H_2O_2 or terbutyl hydroperoxide; (f) oxidant adding strategy: at the beginning or in pulses; (g) H_2O_2 concentration: 2–20 mM; (h) temperature: 25, 30 or 35°C. In every tested condition, 2-oxindole was the only product observed.

Similar experiments with other substrates (benzimidazole, uracil, indoline, purine, hypoxanthine and adenine) were performed employing the selected microorganisms and free CPO. Table 1 summarizes the results, showing that only indole, indoline and uracil reacted under the optimised conditions and that the reaction products depended on the biocatalyst, the substrate and the halide used. However, the conversion for the majority of the reactions was low, with the exception of 5-bromoindole.

In addition, reactions of indole, indoline and uracil performed by the direct addition of HClO produced the same products than the reaction catalysed by CPO, what confirms that this enzyme generates HClO and then diffuse out to chlorinate the substrates in free solution (Manoj 2006).

In the reaction catalysed by bacterial perhydrolases, as in heme-containing HPO, a specific halide binding site does not seem to be present. However, the hydrophobic environment and the size of the perhydrolase active site pocket could influence a preferred substrate orientation and may therefore enhance the efficiency and/or selectivity (Hofmann et al. 1998). These considerations may account for the differential selectivity observed for halogenation depending on the used biocatalysts (Fig. 2).

The enzymatic synthesis of nucleoside analogues could be carried out by transglycosylation through a two step mechanism biocatalysed by two intracellular

nucleoside phosphorylases (NPs) (Lewkowicz and Iribarren 2006). Alternatively, a one step reaction can be catalysed by one NP employing the corresponding nitrogen heterocycle and α-furanose 1-phosphate, the transglycosylation intermediate. The last approach has been carried out using 5-bromouracil, 2′-deoxyribose 5-phosphate, thymidine phosphorylase and phospho-pentomutase (Taverna-Porro et al. 2008). The enzymatic synthesis of nucleoside analogues carrying different sugars and the halogenated heterocycles obtained in this work will be assessed employing the biocatalysed approaches mentioned above.

Conclusions

Some bacteria from our cell collection that contain halogenating activity were selected and the experimental reaction conditions were optimised. The halogenation by *C. fumago* CPO was also studied. CPO and different bacterial perhydrolases afforded different products depending on the substrate, the biocatalyst and the halide used, although the conversion for most of the reactions was low, with the exception of 5-bromoindole.

References

Hager LP, Morris DR, Brown FS, Eberwein H (1966) Chloroperoxidase II. Utilization of halogen anions. J Biol Chem 241:1769–1777

Hofmann B, Tölzer S, Pelletier I, Altenbuchner J, van Pee KH, Hecht HJ (1998) Structural investigation of the cofactor-free chloroperoxidases. J Mol Biol 279:889–900

Itoh N, Izumi Y, Yamada H (1987) Haloperoxidase-catalyzed halogenation of nitrogen-containing aromatic heterocycles represented by nucleic bases. Biochemistry 26:282–289

Lewkowicz E, Iribarren AM (2006) Nucleoside phosphorylases. Curr Org Chem 10:1197–1215

Li N, Smith TJ, Zong MH (2010) Biocatalytic transformation of nucleoside derivatives. Biotechnol Adv 28:348–366

Littlechild J (1999) Haloperoxidases and their role in biotransformations. Curr Opin Chem Biol 3:28–34

Manoj MK (2006) Chlorinations catalyzed by chloroperoxidase occur via diffusible intermediate(s) and the reaction components play multiple roles in the overall process. Biochem Biophys Acta 1764:1325–1339

Murphy CD (2003) New frontiers in biological halogenation. J Appl Microbiol 94:539–548

Taverna-Porro M, Bouvier LA, Pereira CA, Montserrat JM, Iribarren AM (2008) Chemoenzymatic preparation of nucleosides from furanoses. Tetrahedron Lett 49: 2642–2645

van Pée KH (2001) Microbial biosynthesis of halometabolites. Arch Microbiol 175:250–258

Wiesner W, van Peé KH, Lingens F (1986) Detection of a new chloroperoxidase in *Pseudomonas pyrrocinia*. FEBS Lett 209:321–324

Wiesner W, van Pée KH, Lingens F (1988) Purification and characterization of a novel bacterial non-heme chloroperoxidase from *Pseudomonas pyrrocinia*. J Biol Chem 263:13725–13732

Williams S, Ptak RG, Drach J, Townsend L (2004) Synthesis, antiviral activity, and mode of action of some 3-substituted 2, 5, 6-trichloroindole 2′- and 5′-deoxyribonucleosides. J Med Chem 47:5773–5782

Yazbik V, Ansorge-Schumacher M (2010) Fast and efficient purification of chloroperoxidase from *C. fumago*. Process Biochem 45:279–283