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Critical view on the monochlorodimedone assay utilized to detect haloperoxidase activity

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Abstract

The current study aimed to identify the halogenating enzymes involved in the biosynthesis of the ambigols A, B, C and tjipanazole D, isolated from the cyanobacterium *Fischerella ambigua.* Haloperoxidase (HPO) activity within *F. ambigua* was therefore assayed spectrophotometrically by using monochlorodimedone (MCD) during protein purification. This strategy revealed the isolation of a protein positive in the MCD-assay, but an involvement in halogenating processes could not be verified. N-terminal sequencing rather demonstrated homology to cytochrome c_6 from other cyanobacteria and green algae. From our findings it thus has to be concluded that the spectrophotometrical MCD-assay routinely used to detect HPO activity may yield false positive results, mainly since the assay focuses on the decline of the educt and not on the formation of the product. Our data indicate that the reaction of MCD with proteins of the cytochrome *c*– family leads to unspecific products.

Keywords: Biohalogenation; Biosynthesis; Cyanobacteria; Cytochrome c_6; Haloperoxidase; Monochlorodimedone; Organohalogens; Protein purification

1. Introduction

Natural products are a significant source for innovative therapeutics and play an essential role in ecological processes. In the area of cancer and infective disease treatment up to 75% of the available drugs originate from natural sources (Gullo et al., 2006). Ecologically, natural chemicals are important in interactions within and between organisms (Keller and Surette, 2006).

The current study aimed at identifying the halogenating enzymes involved in the biosynthesis of the cyanobacterial metabolites ambigol A, B, C and tjipanazole D from *Fischerella ambigua* (Fig. 1), for which antimicrobial activities have been reported (Falch et al., 1993; Wright et al., 2005). A precise understanding of biological halogenation will give rise to new enzymatic methods for generating halogenated analogues of biologically active compounds, which might have altered bioactivities (Hofrichter and Ullrich, 2006; Kling et al., 2005; Murphy, 2006; van Pee, 1996).

In nature halogenated metabolites are widely spread and show varied biological activities. Around 4000 organohalogen compounds are known to be produced by living organisms (Anderson and Chapman, 2006; Gribble, 2003). Most of these biogenic organohalogens are marine-derived and proposed to serve as antifeedant or antibacterial defense mechanisms. Besides simple haloalkanes like chloroform, bromoform, CH_2Br_2 or $CHBr_2CI$, particularly released by Phaeophytae, complex halogenated metabolites are known, such as terpenes and acetogenins from e.g., *Laurencia* species (Gross and König, 2006). Metabolites found in marine sponges, including halogenated fatty acids, indoles and phenolic derivatives, are often ascribable to associated microorganisms, including cyanobacteria (König et al., 2006) (Fig. 2). In particular chlorinated natural products (Fig. 1) such as the anticancer compound cryptophycin A from *Nostoc* sp. strain GSV 224 (Trimurtulu et al., 1994), different chlorinated indole

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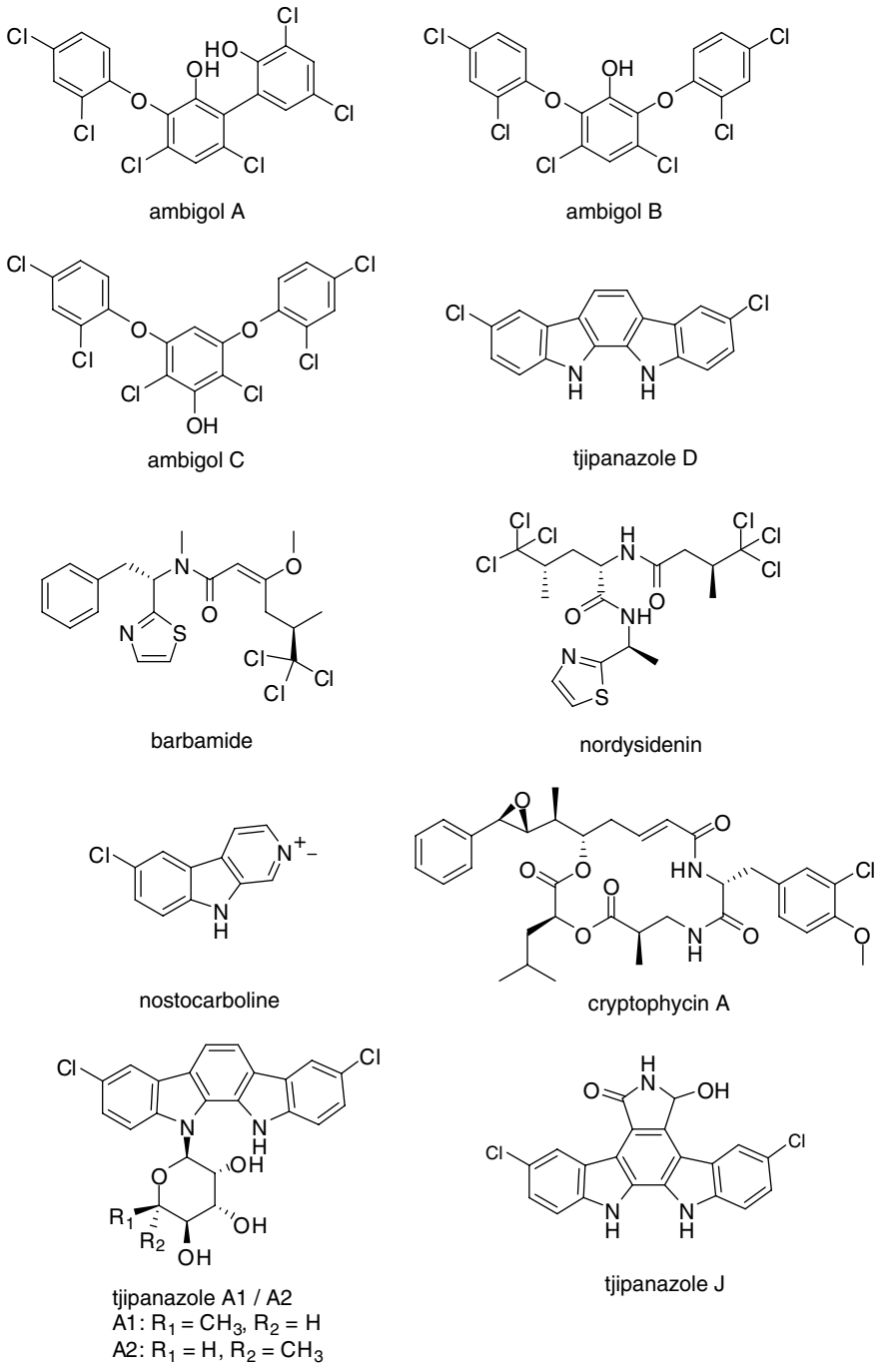


Fig. 1. Selected halogenated metabolites isolated from cyanobacteria: ambigol A, B, C and tjipanazole D originate from *Fischerella ambigua*, barbamide and nordysidenin from *Lyngbya majuscula* strains. Nostocarboline and cryptophycin A have been isolated from *Nostoc* sp. and tjipanazole A1, A2 and J from *Tolypothrix tjipanensis*.

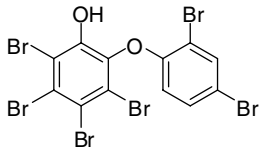
derivatives (Becher et al., 2005; Bonjouklian et al., 1991; Falch et al., 1995) as well as the aliphatically chlorinated compounds barbamide (Orjala and Gerwick, 1996) or nordysidin (Jimenez and Scheuer, 2001) have been found in cyanobacteria.

Due to the wide distribution of halogenated natural compounds, enzymes involved in biohalogenation were discovered from a broad range of organisms, including fungi, algae and bacteria. Halogenating enzymes are mainly grouped into two classes: haloperoxidases (HPO) and halogenases (Kling et al., 2005). HPOs were shown to be less specific, whereas halogenases could be demonstrated to be highly substrate specific (Kling et al., 2005; van Pee and Patallo, 2006). More recently, the group of α-ketoglutarate-O_2-dependent halogenases were shown to be involved in the biosynthesis of halogen incorporation in aliphatic moieties, as in syringomycin E or barbamide (Fig. 1) (Flatt et al., 2006; Murphy, 2006; Vaillancourt

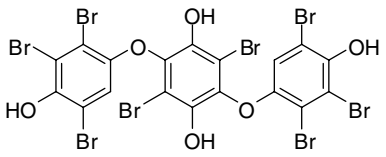
et al., 2005). Furthermore, methyltransferases have been described to be involved in the formation of carbon halogen bonds of CH_3Cl, CH_3Br and CH_3I (Ballschmiter, 2003; van Pee et al., 2006).

HPOs generate hypohalous acid which in most cases is released from the enzyme to halogenate organic substrates. The enzymes are found to contain either heme or vanadate as cofactor in the active site. The first halogenating enzyme was described from the caldariomycin-producing fungus *Caldariomyces fumago* as a heme containing HPO (Morris and Hager, 1966) and later several heme-HPOs were discovered from diverse fungi, algae and microorganisms. Furthermore other heme-proteins were found to have halogenation activity, i.e. an unusual lignin peroxidase from *Agrocybe aegerita* (Ullrich et al., 2004), mammalian myeloperoxidase and eosinophil peroxidase (Henderson and Heineche, 2003), the catalase-peroxidase KatG of *Synechocystis* PCC 6803 (Jakopitsch et al., 2001) and the nitrate reductase from *Thioalkalivibrio nitratireducens* (Antipov et al., 2003).

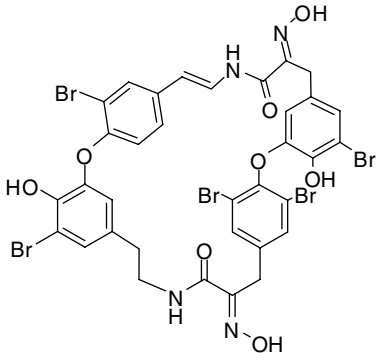
Vanadate ion containing bromoperoxidases (Va-BrPO) have been isolated mainly from marine algae. This group of enzymes seems to play a role in the formation of marine natural products, e.g. halogenation and cyclization of



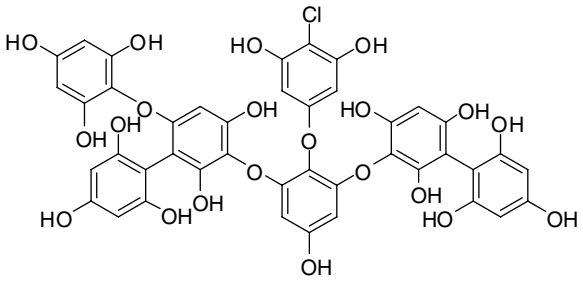
diphenylether



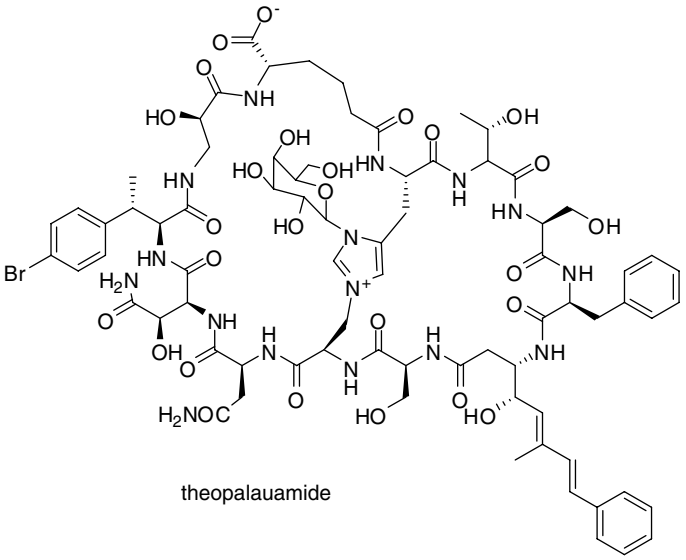
triphenylether



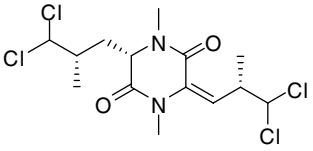
bastadin derivative



fucophlorethol



theopalauamide



didechlorodihydrodysamide C

Fig. 2. Selected marine derived halometabolites: brominated di- and triphenylethers are known from *Dysidea* sp. as well as from the hemicorate *Ptychodera flava laysanica* respectively. Brominated bastadin derivatives have been isolated from *Ianthella quadrangulata* and chlorinated fucophlorethol was isolated from *Cystophora retroflexa.* Didechlorodihydrodysamide C is ascribed to *Dysidea herbacea* associated cyanobacterial symbiont *Oscillatoria spongeliae*, as well as theopalauamide to *Theonella swinhoei* associated δ-proteobacteria.

terpenes (Carter-Franklin et al., 2003). The substrate channel leading to the vanadium binding site is proposed to influence substrate specificity. In absence of the suitable substrate however, HOBr released from the enzyme carries out unselective bromination as observed for aqueous bromine. Asymmetric bromination and cyclization of the terpenoid precursor (E)-(+)-nerolidol could be demonstrated for Va-BrPO, producing single diastereomers of the marine natural products α- and β-snyderol, as isolated from *Laurencia obtusa* (Butler and Carter-Franklin, 2004; Carter-Franklin and Butler, 2004). The first Va-BrPO to be characterized was the one of the brown seaweed *Ascophyllum nodosum* (Vilter, 1984). However, with the isolation of a Va-BrPO from the lichen *Xanthoria parietina* it became obvious, that this group of enzymes not only occurs in marine environments, but can also be found in terrestrial eukaryotic organisms (Plat et al., 1987).

Va-chloroperoxidases on the other hand have been found essentially in fungi. To date it has not been proven that chloroperoxidases (CPO), heme as well as vanadate containing, are involved in the biosynthesis of halogenated secondary metabolites. Excreted by terrestrial fungi, their physiological function is rather discussed to be important for the degradation of plant cell walls. The produced hypohalorous acid facilitates penetration of the fungal hyphen into the host (Wever and Hemrika, 2001).

HPOs have been isolated by classical protein purification in combination with functional assays. The most applied assay uses monochlorodimedone (MCD) as a synthetic substrate for the potential halogenating enzyme. MCD is discussed as a mimic of the natural precursor of caldariomycin, the chlorinated compound isolated from *C. fumago* (Hager et al., 1966). The method is based on the conversion of the monohalogenated diketone MCD to a dihalogenated product (Fig. 3). This can be easily monitored spectrophotometrically by the decrease in UV absorption, which is directly proportional to the enzymatic activity.

The MCD-assay became the routinely used analytical method for identifying HPOs and for characterizing their halogenating activity (van Pee et al., 2006; Wever and Hemrika, 2001). In this enzymatically initiated reaction the actual halogenation is catalyzed unspecifically via free diffusible hypohalous acid and therefore, on substrates generally susceptible to halogenation, similar patterns of halogenation are observable as reported for non-enzymatic reactions with hypohalous acid in free solution.

The intention of the current study was to use the MCD-assay in order to detect enzymes responsible for the biohalogenation of ambigol A, B, C and tjipanazole D precursors. To date little is known concerning biohalogenation processes in cyanobacteria (Welker and von Döhren, 2006). HPO activity has only been shown for the multifunctional enzyme KatG from *Synechocystis* PCC 6803 (Jakopitsch et al., 2001), while no Va-BrPO could be identified from cyanobacterial species yet (Welker and von Döhren, 2006). A $FADH_2$-dependent halogenase (ApdC) has been characterized from *Anabaena* strain 90, producing the cyclic depsipeptides anabaenopeptilides (Rouhiainen et al., 2000), and another putative $FADH_2$-dependent halogenase in the cyanopeptolin-cluster of *Microcystis* cf. *wesenbergii* N-C 172/5 (Tooming-Klunderud et al., 2007). Two α-ketoglutarate-O_2-dependent halogenases (BarB1, BarB2) have been identified to act in a tandem to incorporate the halogen atoms during barbamide biosynthesis in *Lyngbya majuscula* (Flatt et al., 2006; Galonic et al., 2006).

The MCD-assay-guided protein purification from *F. ambigua* however, proved to be difficult and led to false positive results, making a critical review of this widely used assay for HPO activity necessary.

2. Results

The terrestrial cyanobacterium *F. ambigua* is a filamentous growing organism of the family Stigonemataceae and originates from a sample obtained from a shallow depression at Mellingen, Switzerland. The polyhalogenated aromatic metabolites ambigol A, B, C and tjipanazole D (Fig. 1) have been isolated from this strain using different culture media (Falch et al., 1993; Wright et al., 2005). Antimicrobial activity could be demonstrated for all of them,

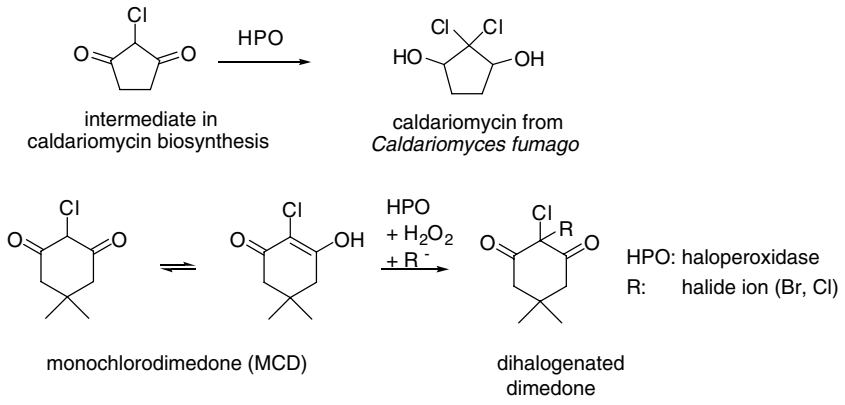


Fig. 3. Monochlorodimedone assay.

while antiplasmodial and trypanocidal effects were additionally observed for ambigol C.

The MCD-assay was regarded as a suitable method to guide protein purification from *F. ambigua* in order to detect enzymes with HPO activity, probably involved in ambigol and tjipanazole D formation. As a positive control CPO of the fungus *C. fumago* was used and yielded declining UV absorptions at 290 nm dependant on the CPO amounts (units/ml) used.

Purification of cell contents such as DNA or proteins from the investigated cyanobacterium proved to be most difficult due to an interfering thick polysaccharide sheath surrounding the cell filaments. Therefore, intensive homogenization of the biomass, which originated from large-scale cultivation, was needed to achieve sufficient cell breakage by sonication. After removal of all cell debris by centrifugation and an ammonium sulfate precipitation a crude protein extract was obtained.

No halogenating activity could be observed in the crude extract which is frequently reported to be due to interfering catalases (van Pee and Lingens, 1985). Subsequent protein purification was hence achieved by fast protein liquid chromatography (FPLC) and directly after the first FPLC purification step on Butyl Sepharose 4FF a decreasing UV absorbance was monitored within the MCD-assay. This activity within the protein fractions became more conspicuous with each step of purification. The effectiveness of sonication treatment and of all purification steps was traced by polyacrylamide gel electrophoresis. The purified protein gave a single band between 6 and 8 kDa on a silver nitrate stained gel (Fig. 4) while the fraction eluted from the Superdex 200 column indicated a molecular weight of approximately 17 kDa for the native protein. The results of the purification procedure are summarized in Table 1.

During the purification procedure the functional assay was initially performed using potassium bromide, since bromination reactions are known to occur less specific than chlorinations (van Pee, 1990). Subsequently the purified protein was assayed for chlorination activity using potassium chloride and found to be active even though to a lower extent.

The UV/Vis absorption spectrum of the purified protein displayed a Soret band at 417 nm along with two small bands at 522 nm and 552 nm as is typical for proteins with a heme prosthetic group (Fig. 5) (Mäntele, 1998; Reuter and Wiegand, 2001). The involvement of a heme moiety was confirmed by the observation that the MCD-assay reaction could be inhibited by addition of sodium azide.

MALDI-TOF analysis yielded a molecular mass of 9.7 kDa for the monomer (Fig. 6, Supplementary data) which corresponded with the data determined by denaturizing polyacrylamide gel electrophoresis. In gel filtration chromatography the native protein eluted as a dimer for

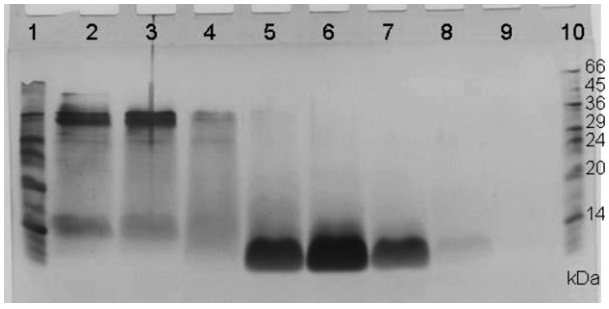


Fig. 4. Silver stained SDS–PAGE of Superdex 200 gel chromatography fractions: Lane 5–7 display the active fractions 31–33, whereby the most prominent activity within the MCD-assay was observed for fraction 32 (lane 6). Lane 2–4, 8 and 9 show the non-active side fractions 28, 29, 30, 34 and 35. Protein marker was applied to lane 1 and 10.

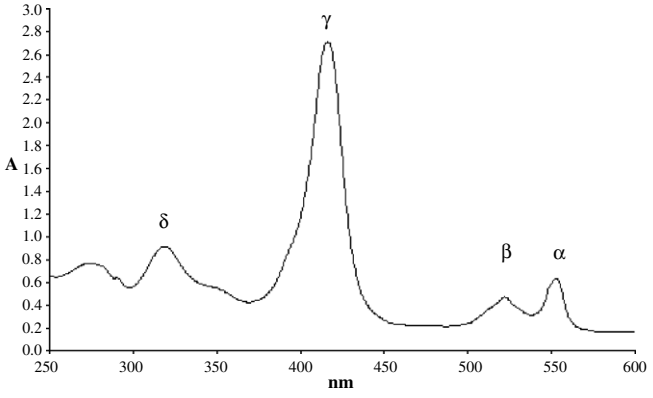


Fig. 5. UV/Vis-spectrum of the purified protein from *F. ambigua* displaying (α-, β-, γ-, δ-) absorbance maxima typical for reduced heme moieties. The spectrum was recorded in Tris buffer (20 mM, pH 7.2, 150 mM NaCl) at a concentration of 0.055 mg/ml.

Table 1
Summary of the MCD-assay guided protein purification procedure from *F. ambigua*. Specific protein-activity was expressed as μmol MCD decrease per minute per mg protein

	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery %	Purification (*n*-fold)
Crude extract from 850 g wet biomass	1400	3450	n.d.[a]	n.d.	n.d.	n.d.
40% ammonium sulfate supernatant	240	600	n.d.	n.d.	n.d.	n.d.
Butyl Sepharose 4FF	50	260	0.044	0.0002	100	1
DEAE-Sepharose FF	40	42.3	0.025	0.0006	57	3
Mono Q HR 5/5	3	0.975	0.028	0.029	64	145
Superdex 200 gel filtration	3.5	0.193	0.025	0.128	57	640

[a] Not determinable.

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G-Mobr   -EADLAL-GKAVFDGNCAACHAGGGNNVIPDHTLQKAAIEQFLDGGFNIEAIVYQIENGK 58
C-Fiam   --ADLAL-XKQVFDGNXAAXHSGGGNSVI------------------------------ 26
G-Scob   -SADLAL-GKQTFEANCAACHAGGNNSVIPDHTLRKAAMEQFLQGGFNLEAITYQVENGK 58
G-Clgl   VDAELLADGKKVFAGNCAACHLGGNNSVLADKTLKKDAIEKYLEGGLTLEAIKYQVNNGK 60
G-Brma   -GGDLEI-GADVFTGNCAACHAGGANSVEPLKTLNKEDVTKYLDGGLSIEAITSQVRNGK 58
C-Miae   ------D-GASIFSANCASCHMGGKNVVNAAKTLKKEDLVKY--GKDSVEAIVTQVTKGM 51
C-Apfl   --ADTVS-GAALFKANCAQCHVGGGNLVNRAKTLKKEALEKY--NMYSAKAIIAQVTHGK 55
C-Lebo   --ADAAA-GGKVFNANCAACHASGGGQINGAKTLKKNALTAN--GKDTVEAIVAQVTNGK 55
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Fig. 7. CLUSTAL W (1.83) multiple sequence alignment: alignment of the amino acids derived from N-terminal-sequencing of the purified protein from *F. ambigua* (C-Fiam) with amino acid sequences of cytochrome c_6 from *Microcystis aeruginosa* (C-Miae), *Aphanizomenon flos-aquae* (C-Apfl), *Leptolyngbya boryana* (C-Lebo), *Monoraphidium braunii* (G-Mobr), *Scenedesmus obliquus* (G-Scob), *Cladophora glomerata* (G-Clgl) and *Bryopsis maxima* (G-Brma). Identical amino acids shaded in gray show homology especially within the cytochrome *c* typical heme-binding motif.

which a molecular mass of 19.5 kDa was determined. However, comparison of the molecular weights showed that the size of the purified protein was considerably smaller than that of other HPOs. The heme-CPO from *C. fumago* has been characterized to be 42 kDa in size (Morris and Hager, 1966), whereas Va-HPOs have been described to be composed of subunits of about 65 kDa (Coupe et al., 2007; Hemrika et al., 1999; Weyand et al., 1999). Only for the CPO of *Notomastus lobatus* it has been reported that the heme-containing subunits have a relative molecular mass of around 16 kDa. However, this HPO is unique in also requiring a flavoprotein component for peroxidase activity (Littlechild, 1999; Roach et al., 1997).

N-terminal-sequencing of the first 26 amino acids revealed the amino acid composition to be NH_2-Ala-Asp-Leu-Ala-Leu-X-Lys-Gln-Val-Phe-Asp-Gly-Asn-X-Ala-Ala-X-His-Ser-Gly-Gly-Gly-Asn-Ser-Val-Ile. In a multi-sequence alignment (Fig. 7) this fragment clearly demonstrated homology to several cytochrome c_6 proteins of other cyanobacteria (Aitken, 1977; Cohn et al., 1989; Ulrich et al., 1982) as well as green algae (Campos et al., 1993; Okamoto et al., 1987; Schnackenberg et al., 1999), including the typical heme binding motif as well as the Gly22 residue which is known to be absolutely conserved within the sequence of all cytochrome c_6 proteins (Reuter and Wiegand, 2001).

To answer the question whether the reaction of the purified *F. ambigua* protein in conjunction with MCD was truly a halogenation process HPLC-MS measurements of MCD reaction products were performed. Therefore, large scale MCD-assay reaction mixtures were analyzed, focusing on the characterization of the substrate conversion and product formation. With these techniques no di-halogenated dimedone could be observed. HPLC-MS data rather evidenced the formation of a reaction product with a UV maximum at 208 nm (MCD UV maximum at 290 nm) and a prominent signal for *m/z* at 159 in a negative mode ESI–MS experiment (Fig. 8, Supplementary data). ESI–MS measurements did not show isotope signals characteristic for halogenated compounds. Comparative studies with other cytochrome *c* proteins, i.e. from *Halorhodospira halophila* and from equine heart, showed a similar conversion of MCD during the assay, again without forming halogenated products.

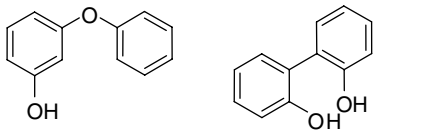

Fig. 9. Potential ambigol precursors used as substrates within the HPO-assay.

A potential involvement of the purified protein within the biosynthesis of the chlorinated compounds found in *F. ambigua* was investigated using 3-phenoxyphenol and 2,2′-dihydroxybiphenyl (Fig. 9) as substrates instead of MCD, since these substrates are more structurally related to the potential precursors of the natural products ambigol A, B, C. HPLC-MS analysis however, showed that no halogenated products were formed.

From a final comparison of absorption spectra of the MCD reaction mixture in the presence and absence of halide ions it became evident that the purified *F. ambigua* protein was not involved in halogenation processes. A decrease in the MCD absorbance was thereby visible in both spectra, as reported for lignin peroxidases of the fungus *Phanerochaete chrysosporium* (Farhangrazi et al., 1992).

Thus, in the current study the MCD-assay led to the purification of a protein not involved in halogenation, but catalyzing an unspecific reaction of MCD.

3. Discussion

Biohalogenation processes are most important due to the wide occurrence of halogenated natural products having varied biological activities. Precise understanding of biological halogenation will give rise to mild methods for modifying compounds of potential therapeutic use.

In an attempt to investigate the biohalogenation of the polychlorinated metabolites known from *F. ambigua* the routinely used MCD-assay was utilized to monitor for HPO activity. Under standard reaction conditions an active protein could be purified, using the spectrophotometrical detection method as outlined in literature (Hager

et al., 1966; Sundaramoorthy, 2001; Wever and Hemrika, 2001). Detailed analysis, however, made clear that the protein purified during this study was not linked to halogenation processes, neither in conjunction with MCD nor with potential ambigol-precursors. The MCD-assay has to be judged as extremely useful when monitoring potential HPO activity, however false positive results have to be expected. To recognize a decline in UV absorption not due to halogenation direct comparison with a control experiment conducted without halogen addition (potassium bromide, potassium chloride) is recommended.

Likewise, other literature reports indicated further trouble spots of this widely utilized method. Van Pee and co-workers (van Pee et al., 2000) identified enzymes from pyrrolnitrin-producing *Pseudomonas* strains, which showed activity within the MCD-assay but lacked a heme-chromophore or any metal prosthetic group. In these cases activity could only be detected in acetate buffer but no reactivity was determinable in phosphate buffer (Burd et al., 1995). Biochemical investigations showed that enzymatically formed peracid was responsible for the bromination of MCD. Thus, it was concluded that this was not a true peroxidase activity but rather a perhydrolysis of an ester formed between the active site of the enzyme and the acetic acid contained in the buffer. The hereby formed peracid then oxidized non-enzymatically halide ions into hypohalous acid which acted as a free diffusible halogenating agent. Purification of a protein with perhydrolysis activity by means of the MCD-assay can thus be avoided by utilizing buffer systems not capable of peracid formation. Therefore, in our study all reactions were performed in potassium phosphate buffer.

Powers and co-workers (Farhangrazi et al., 1992) demonstrated, while investigating the HPO activities of *Phanerochaete chrysosporium* lignin peroxidases (H2, H8), that MCD itself can be substrate for general peroxidase reactions. They observed that direct oxidation of MCD resulted in a decrease of UV absorption at 288 nm. However this gave a shifted absorption maximum at shorter wavelengths (270 nm), whereas the absorption band disappears completely over time when MCD was truly halogenated. In accordance with this, it was shown that HPO activity diminished due to direct reactions between MCD and the enzyme when myeloperoxidase-activity was investigated (Kettle and Winterbourn, 1994).

In all organisms, cytochrome *c* proteins are involved in many physiological processes such as electron transport chains. In cyanobacteria and algae cytochrome c_6 acts as an interthylakoidal one electron carrier between cytochrome b_6f and photosystem I (Reuter and Wiegand, 2001). Thus, the involvement of cytochrome c_6 proteins in diverse redox-systems can explain the observed direct reactivity of the purified protein with MCD in the HPO-assay.

From other data of Itoh et al. (1988) it was implied that in HPO reactions the educt was only initially converted into monobromomonochlorodimedone, followed by spontaneous elimination of halogen atoms and decomposition, to give unknown products. However, closer analytical investigations of the educt conversion and product formation performed within this study did not reveal similar degradation processes.

4. Conclusions

From our findings it has to be concluded that the spectrophotometrical MCD-assay routinely used to detect HPO activity may yield false positive results, mainly since the assay focuses on the decline of the educt and not on the formation of the product. Our data indicate that the reaction of MCD with proteins of the cytochrome *c* – family leads to unspecific products. Non-specific conversions also need to be considered when performing similar assays e.g. the recently described method utilizing the colored substrate thionin (Manoj and Hager, 2006). Buffer components convertible to peracids by perhydrolysis reaction (Burd et al., 1995) as well as proteins with redox activity, as described herein, can yield false positive effects. In any case, results of the MCD-assay have to be judged critically and should be confirmed by analytical product characterization.

Our results suggest that the enzymes responsible for the halogenation of ambigols and tjipanazole D are not CPOs.

5. Experimental

5.1. General experimental procedures

UV/Vis spectral characterizations were performed with a Lambda 40 UV/Vis spectrophotometer (Perkin–Elmer Instruments). HPLC separation of the large scale MCD-assay mixture was performed using a Macherey–Nagel Nucleodur 100-5 C_{18} column (5 μm, 250 × 4.6 mm) on a Merck-Hitachi system equipped with an L-4500A photodiode array (PDA) detector. Elution was conducted with a gradient from 10/90 to 35/65 methanol/water containing ammonium acetate (2 mM), over 15 min at a flow rate of 1 ml/min. ^{1}H NMR spectra were recorded using a Bruker Avance 300 DPX spectrometer operating at 300 MHz. Samples were dissolved in deuterated methanol. For HPLC-(ESI)-MS experiments samples to be analyzed were diluted in methanol to a concentration of 1 mg/ml. A Macherey–Nagel Nucleodur 100-5 C_{18} column (5 μm, 125 × 2 mm) was used on an Agilent 1100 HPLC system and chromatograms were monitored at 250 nm with a PDA detector. As mobile phase a methanol/water system buffered with 2 mM ammonium acetate was utilized and a gradient was applied from 10/90 to 100/0 in 20 min at a flow rate of 0.25 ml/min, ending isocratic for another 10 min. Ionization of characteristic peaks was achieved by an electro spray ionization (ESI) source and MS measurements were performed on an Applied Biosystems MDS Sciex API 2000 MS spectrometer. In MALDI-TOF-MS experiment 3,5-dimethoxy-4-hydroxycinnamic

acid was used as matrix and the spectrum was recorded on an Applied Biosystems Voyager System 4337. Polyacrylamide gel electrophoresis was performed with 12.5% or 20% polyacrylamide gels, respectively, containing sodium dodecyl sulfate on a Mini-Protean system (Bio-Rad Laboratories, Inc.). Gels were stained either with Coomassie dye or silver nitrate. Multisequence alignments were obtained with the ClustalW sequence analysis program of the European Bioinformatics Institute.

5.2. Materials

BG11-medium concentrate, MCD and cytochrome *c* from equine heart were purchased from Sigma–Aldrich Inc., semi purified cytochrome c_{551} from *H. halophila* was made available by the Institute for Microbiology and Biotechnology, University of Bonn. Butyl Sepharose 4FF resin, DEAE Sepharose FF resin, Mono Q HR 5/5 anion exchange column and Superdex 200 gel filtration column, as well as PD-10 desalting columns were purchased from GE Healthcare. Amicon Ultra centrifugal filters were acquired from Millipore Corporation. All other chemicals used were obtained in analytical grade from commercial sources.

5.3. Culture

F. ambigua (Näg.) Gomont, designated strain number 108b, was obtained from the Culture Collection of Algae of the Swiss Federal Institute for Water Resources and Pollution Control (EAWAG), Dübendorf, Switzerland. The strain was continuously cultivated in BG11-medium, in a 25 l photobioreactor (Planctotec, Type: Pluto) at 25 °C, under constant illumination with white fluorescent lamps (Osram L 58W/25) and was steamed by a sterile-filtered air flow. Biomass was harvested monthly by flow-through centrifugation (Heraeus Contifuge Stratos, Kendro Laboratory Products GmbH) and stored at −20 °C.

5.4. Protein purification

Approximately 210 g wet weight biomass was suspended in potassium phosphate buffer (0.1 M, pH 7) and progressively homogenized. Cell disruption was achieved by sonication (Brandson Sonifier 250) and the crude protein extract was kept on ice. After separation of the cell debris by centrifugation, the supernatant was concentrated with Amicon Ultra centrifugal filters to a volume of about 60 ml. For ammonium sulfate precipitation the solution was progressively brought to 40% saturation with solid ammonium sulfate while stirred on ice.

After centrifugation the supernatant was passed onto a Butyl Sepharose 4FF hydrophobic interaction column which had been equilibrated with potassium phosphate buffer (20 mM, pH 7, saturated to 40% ammonium sulfate) and a gradient of 40–0% ammonium sulfate was applied over 600 ml. Fractions assayed positive for protein content (A_{280}) were tested for bromination activity.

Cell disruption and pre-purification as described above were repeated three more times, starting with the same amount of biomass. Those fractions having activity in the MCD-assay were pooled from all four Butyl Sepharose 4FF separations and dialyzed against Tris buffer (20 mM, pH 7.2) overnight. After concentration the protein solution was passed onto a DEAE-Sepharose FF anion exchange column equilibrated with Tris buffer (20 mM, pH 7.2). A gradient from 0 to 0.5 M NaCl was applied over 300 ml. Fractions active in the MCD-assay were pooled and desalted with PD-10 columns before their application onto a Mono Q HR 5/5 anion exchange column, which was equilibrated with Tris buffer (20 mM, pH 7.2). A gradient was run from 0 to 0.5 M NaCl over 75 ml. Protein fractions (A_{280}) were assayed as above and those active were applied to a Superdex 200 gel filtration column. Equilibration and elution was performed with Tris buffer (20 mM, pH 7.2, 150 mM NaCl). The purified protein was stored at −20 °C.

5.5. Monochlorodimedone assay

Bromination and chlorination activity was measured by the method described previously (Hager et al., 1966) with MCD (42 μM) as substrate in presence of hydrogen peroxide (8.8 mM) and potassium bromide (100 mM) or chloride (100 mM), respectively, in a total volume of 1 ml potassium phosphate buffer (0.1 M, pH 5.5) at room temperature. The reaction was started by adding the protein fractions and the decrease of MCD absorbance was recorded at 290 nm ($\varepsilon = 1.99 \times 10^4\ M^{-1}\ cm^{-1}$). Specific activity was expressed as unit per milligram protein, in which a unit was defined as μmol MCD decrease per minute.

To analytically characterize the substrate conversion and product formation, the reaction was carried out in a 150 ml scale overnight. After incubation the reaction mixture was extracted three times with ethyl acetate and the combined extracts were evaporated to dryness.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2007.07.020.

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