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First detection of a chloroperoxidase in bryophytes

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Abstract

Chlorinated cyclic bisbibenzyls of the isoplagiochin type are the first verified halometabolites from bryophytes. They could be obtained by in vitro chlorination of isoplagiochin C with chloroperoxidase from *Caldariomyces fumago*. Furthermore, an enzyme of this type was detected for the first time in bryophytes namely in the liverwort *Bazzania trilobata* using the monochlorodimedon assay.

Keywords: *Bazzania trilobata*; Liverworts; Enzymes; Peroxidase; Chloroperoxidase

1. Introduction

Chlorinated metabolites were isolated over the last 5 years from bryophytes (liverworts), namely *Plagiochila deflexa* (Anton et al., 1997), *Bazzania trilobata* (Martini et al., 1998), *Lepidozia incurvata* (Schmidt, 1996), *Herbertus sakuraii* and *Mastigophora diclados* (Hashimoto et al., 2000).

All these compounds like the highly chlorinated bazzanin Q (**1**) are obviously derived and are biogenetically related to isoplagiochin C (**2**) or isoplagiochin D (**3**), two known bryophyte constituents (Hashimoto et al., 1996) (Fig. 1).

In a preceding paper we reported on MALDI-TOF mass spectrometry investigations on crude plant extracts and we could demonstrate that these chlorinated compounds are not artefacts of an incidental occurrence or of the sample preparation, but should be genuine and produced by the liverwort or an endosymbiotic metabolism (Speicher et al., 2001). So, these compounds are the first verified type of chlorometabolites from bryophytes. We are interested in the biogenesis of these chlorinated isoplagiochins. Haloperoxidases are enzymes with a low substrate specificity which catalyze the electrophilic incorporation of halogen atoms into organic molecules in the presence of a halide and hydrogen peroxide and which are thought to be involved in the biosynthesis of most halometabolites (Neidleman and Geigert, 1986). They were detected in many organisms including higher plants (Monde et al., 1998) and lichens (Cohen and Towers, 1996) but not yet in bryophytes (Fig. 2).

More recently a nucleophilic pathway involving so called halogenases (Keller et al., 2000) as well as a radical mechanism (Sitachitta et al., 1998; Hartung, 1999) were discussed for some specific substrates.

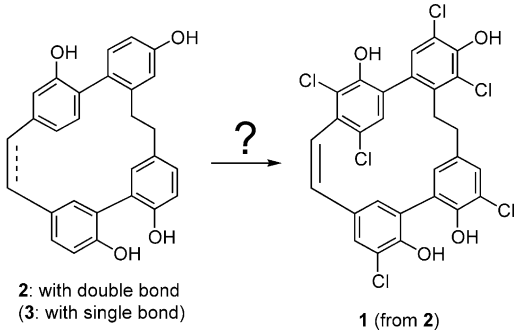

Fig. 1. Chlorinated derivatives like **1** derived from isoplagiochin C **2** or D **3**.

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$$R{-}H + H_2O_2 + Cl^- + H^+ \xrightarrow{\text{Haloperoxidase}} R{-}Cl + 2\,H_2O$$

Fig. 2. Enzymatic halogenation with haloperoxidase.

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2. Results and discussion

2.1. In vitro chlorination of isoplagiochin C 2

Numerous in vitro reactions are described for the commercially available chloroperoxidase (CPO, EC 1.11.1.10) from *Caldariomyces fumago* (Franssen, 1994). We subjected a synthetic sample of isoplagiochin C **2** (Eicher et al., 1998) to a chemo-enzymatical transformation (Speicher, 2000) with KCl/H_2O_2 in the presence of CPO in a buffered aqueous medium (see Fig. 3) and the crude reaction mixture was analyzed using MALDI-TOF-MS (Speicher et al., 2001). The in vitro chlorination of **2** resulted in the incorporation of 1–6 chlorine atoms. In control experiments the addition of an enzyme solution proved to be essential to achieve this halogenation.

2.2. Peroxidase and haloperoxidase assays

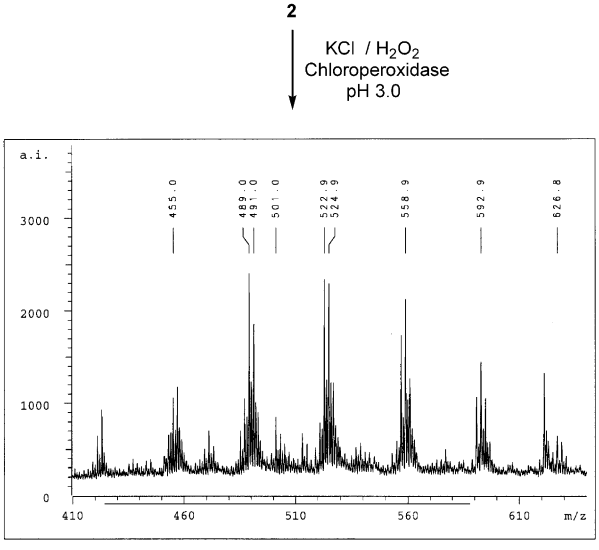
Crude enzyme fractions of pH 4.5 and 6.0 were prepared from fresh plant material of *B. trilobata* from Lautzkirchen, Saarland, Germany and first subjected to a simple guaiacol peroxidase assay (Kariya et al., 1987). Peroxidase activity (EC 1.11.1.7) was already detected in bryophytes (Hilgenberg et al., 1978; Szweykowski et al., 1981). We measured a peroxidase activity of 1–2 units/ml (compared to a commercially available horseradish peroxidase) in the enzyme fraction of pH 6.0 (Fig. 4 and Table 1). Enzyme extracts of pH 4.5 showed lower activity (0.25-0.6 units/ml).

Table 1
Peroxidase activity in *Bazzania trilobata*

Enzyme soln pH 6.0 (µl)	Abs/min	mAbs/min/µl	u	u/ml
10	0.019	1.92	0.019	1.92
50	0.099	1.97	0.099	1.97
100	0.177	1.77	0.177	1.77
200	0.245	1.22	0.245	1.22

The standard test for the detection and activity measurement of a haloperoxidase (bromo-, chloro-) is the so called monochlorodimedon (MCD) assay (Hager et al., 1966). Chlorination or bromination of this 1,3-diketone (see Fig. 5) results in the decrease of UV-absorption at 278 nm ($\varepsilon = 19\,000$). We optimized and calibrated this assay to a sensitivity of 0.1 units/ml (total 0.1 milli-units) by dilution of the chloroperoxidase from *C. fumago*.

Crude enzyme fractions of pH 4.5 (25–500 µl) gave positive results for bromide and chloride as halide source (Fig. 6 and Table 2). Control experiments clearly clarified that the activity depends on H_2O_2, halide and enzyme preparation (no blind activities). So, a chloroperoxidase activity (0.14–2.23 milli-units/ml) was detected for the first time in bryophytes. The activity was significantly higher in extracts of pH 4.5 than in pH 6.0 (0.04–0.17 milli-units/ml) which might be due to the known enzyme stability (Neidleman and Geigert, 1986).



426-422 (422)	$C_{28}H_{22}O_4$
456-460 (456)	$C_{28}H_{21}ClO_4$
490-495 (490)	$C_{28}H_{20}Cl_2O_4$
524-529 (524,526)	$C_{28}H_{19}Cl_3O_4$
558-564 (560)	$C_{28}H_{18}Cl_4O_4$
592-598 (594)	$C_{28}H_{17}Cl_5O_4$
626-636 (628)	$C_{28}H_{16}Cl_6O_4$

Fig. 3. Incorporation of 1–6 chlorine atoms through in vitro chlorination of **2** using chloroperoxidase (detection: MALDI-TOF-MS).

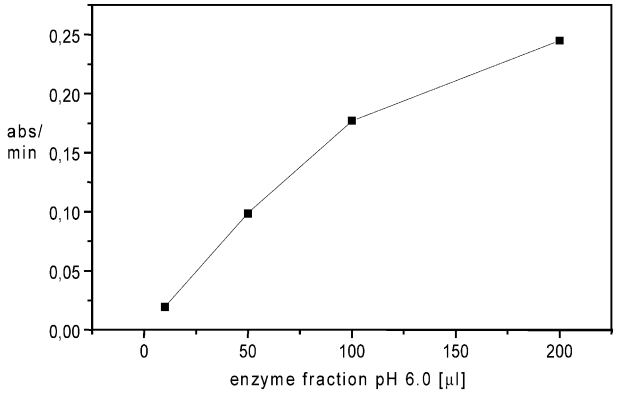


Fig. 4. Peroxidase activity in *Bazzania trilobata* (see Table 1).

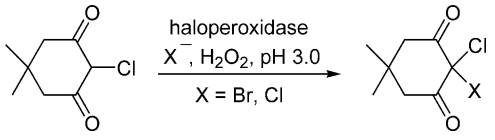


Fig. 5. The monochlorodimedon assay.

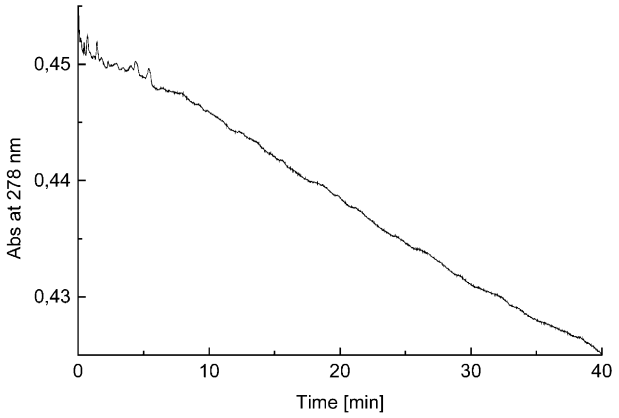


Fig. 6. Chloroperoxidase activity in *Bazzania trilobata* (see Table 2). (2.0 ml 0.2 M K-phosphate buffer pH 3.0, 50 μl (50 nmol) 1 mM MCD 50 μl (5 μmol) 0.1 M KCl, 50 μl (0.5 μmol) 10 mM H_2O_2, 500 μl enzyme preparation pH 4.5).

Table 2
Haloperoxidase activity in *Bazzania trilobata*: (a) collected 12/2000, enzyme fraction pH 4.5; (b) collected 11/2001, enzyme fraction pH 4.5

	μl	ΔE/min $\times 10^{-3}$		nmol/min (mu)		Activity (mu/ml)	
		KCl	KBr	KCl	KBr	KCl	KBr
(a)	25	0.53	0.53	0.056	0.056	2.23	2.23
	125	1.2	1.35	0.118	0.142	0.94	1.13
	500	4.12	3.71	0.433	0.390	0.87	0.78
(b)	25	0.18		0.019		0.75	
	125	0.37		0.039		0.31	
	500	0.68		0.072		0.14	

3. Experimental

3.1. In vitro halogenation of isoplagiochin C 2

126.6 mg (0.30 mmol) of isoplagiochin C **2** (pre-dissolved in 1 ml ethanol) were added to a solution of 3.00 mmol potassium chloride in 120 ml 0.1 M potassium phosphate buffer (pH 3.0). The temperature of the mixture was kept constant to 30–35 °C and 6.0 mmol H_2O_2 (30% in H_2O) as well as 1000 units of the chloroperoxidase (CPO, 11.1.10 from *C. fumago* obtained from Fluka as suspension in 0.1 M sodium phosphate pH 4.0, 1717 U/mg, 8 mg protein/ml) were added in 10 portions each during 10 h. After five portions an additional amount of the halide (3.00 mmol) was added. The mixture was extracted with ethyl acetate, dried ($MgSO_4$) and concentrated.

3.2. Mass spectrometry

The MALDI-TOF measurements were performed using a Bruker Reflex III MALDI-TOF mass spectrometer equipped with a delayed extraction system facility using a N_2-Laser (337 nm) with a single pulse duration of 3 ns and 10^7–10^8 W/cm^2 irradiation. The sample preparation for MALDI-TOF analysis was performed by dissolving the oily or powdered samples in methanol at concentrations of about 1 mg/ml. 1 μl of the solutions were spotted on the metal target plate and dried at room temperature. MALDI-TOF mass spectra were recorded in reflectron mode with negative acceleration voltage without matrix.

3.3. Plant material and preparation of crude enzyme solutions

The plant materials of the liverwort *B. trilobata* (L.) S. F. Gray (family: Lepidoziaceae) were collected in Lautzkirchen, Saarland, Germany during December 2000 and November 2001 and identified by Professor Dr. R. Mues (Institut für Botanik der Universität des Saarlandes, Saarbrücken, Germany). Voucher specimens are deposited in the herbarium SAAR, Saarbrücken.

The fresh plant material (100 g) was carefully cleaned and washed several times with deionized water, surface-dried, flooded with liquid nitrogen and crushed vigorously. The material was homogenized (Vortex vibration 1 min, rotatory shaker 60 min) with 100 ml 0.1 M phosphate buffer (0–5 °C, pH 6.0 or 4.5) and centrifuged (4 °C, 15 min at 15 000 rpm/20 000 G). For UV detection the supernatants were cleared by adding active charcoal for 30 s.

3.4. Guaicol assay for peroxidases

To 200 μl (20 μmol) 100 mM guaiacol (2-methoxyphenol) in 1.8 ml 0.1 M acetate buffer (pH 4.5) was added the enzyme solution. The reaction was initiated by adding 20 μl (0.2 μmol) 10 mM H_2O_2 and UV-monitored at 470 nm (formation of phenol oxidation products). The activity was calibrated with solutions of horseradish peroxidase (EC 1.11.1.7, Fluka 727 U/mg) in acetate buffer (pH 4.5; 200–0.1 u/ml/2 u–1 mu).

3.5. MCD assay for haloperoxidases

To 50 μl (50 nmol) 1 mM monochlorodimedon (MCD), 50 μl (5 μmol) 0.1 M KCl or KBr and 50 μl (0.5 μmol) 10 mM H_2O_2 in 2.0 ml 0.2 M K-phosphate buffer (pH 3.0) was added the enzyme preparation (25–500 μl) for initiation. The reaction was monitored at 278 nm. 1 unit of CPO activity means the enzyme amount which catalyzes the transformation of 1 μmol monochlorodimedon to dichlorodimedon per min at 25 °C. The activity was calibrated with solutions of CPO from *C. fumago* in phosphate buffer (pH 4.5).

Acknowledgements

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