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Enzymic *meso*-chlorination of chlorophylls using chloroperoxidase

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Chlorophyll *a* could be chlorinated enzymically by a chloroperoxidase (EC 1.11.1.10; chloride:hydrogen-peroxide oxidoreductase) from the fungus imperfectus *Caldariomyces fumago*. Analyses with ^{1}H-NMR, mass spectrometry, fluorescence emission and absorption spectroscopy, and chemical demetallation showed the product to be 20-chlorochlorophyll *a*. This product was formed regioselectively in about 80% yield. 13^2-Hydroxychlorophyll *a* was also converted in lower yield to 13^2-hydroxy-20-chlorochlorophyll *a*, but no evidence for an enzymic hydroxylation was found. Chlorophyll *b* was probably chlorinated to some extent while Mg-free derivatives, protochlorophyllide (17,18-didehydrochlorophyllide) and protein-bound chlorophyll could not be chlorinated. No evidence was found for a bromination or iodination of chlorophyll *a*.

Introduction

Chlorinated chlorophylls became a focus of attention with reports of the isolation, by thin-layer chromatographic methods, of a chlorophyll with spectroscopic properties similar to those of the PS I reaction center chlorophyll. This chlorophyll was designated Chl RC I and was later identified as 13^2-hydroxy-20-chlorochlorophyll *a* (13^{2}HO-20-Cl-Chl *a*) (**1**) (see Fig. 1) [1,2]. However, studies revealed that this compound was an artefact arising from Chl *a* (**3**) during the silica-gel thin-layer chromatography (TLC) procedure used for purification [3,4] and during pigment extraction [5]. The reason for the formation of chlorinated chlorophylls during the TLC procedure could be traced to heterogeneous catalysis of oxidative reactions on the chromatogram [4]. Formation during extraction procedures suggested that an enzymic reaction may be involved [5]. This raises the possibility that the enzymic chlorination of chlorophylls, if it occurs, may be another of the many examples of enzymic halogenations known in nature [6]. Chlorinated chlorophylls also deserve further attention, since their unique physical and chemical properties make them promising model compounds for comparative chlorophyll studies.

Although the great susceptibility of the C-20 methine bridge carbon of Chl *a* (**3**) to electrophilic attack is known, attempts so far to synthesize chlorinated chlorophylls chemically have been successful only on the level of Mg-free porphyrin systems [7,8] since H_2O_2/HCl was employed as halogenating agent, with the inevitable formation of a pheophytin derivative; thus, if the corresponding chlorophyll was required, remetallation became a necessary second synthetic step [9].

In this contribution, we describe the enzymic conversion Chl *a* to 20-Cl-Chl *a* (**6**) and some of its derivatives which circumvents the remetallation step and uses a commercially available chloroperoxidase (Fig. 1). This enzyme from *Caldariomyces fumago* (EC 1.11.1.10; chloride:hydrogen-peroxide oxidoreductase) is a widely studied enzyme which in the presence of H_2O_2 and KCl is capable of halogenating a variety of organic substrates including β-dicarbonyl species such as monochlorodimedone [10,11], alkenes [12–14], cyclopropanes [15], thiazole [16], antipyrine [17], NADH [18] and barbituric acid [19].

Materials and Methods

General. Chloroperoxidase (crude type) from *C. fumago* was obtained from Sigma (C-0278; Munich, F.R.G.). All chemicals used in this study were of the highest obtainable purity and were purchased from Merck (Darmstadt, F.R.G.).

Abbreviations: Chl, chlorophyll; HPLC, high-performance liquid chromatography; Pheo, pheophytin; PS, Photosystem; RC, reaction center.

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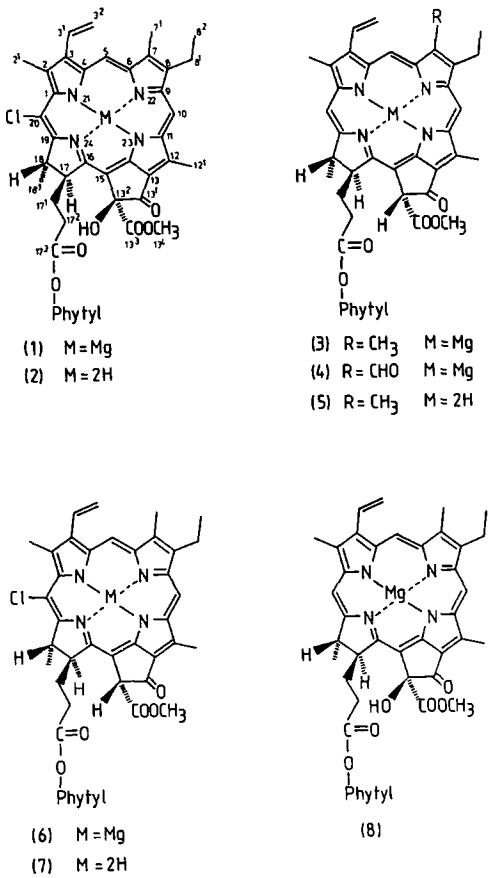

Fig. 1. Structural formulas of the compounds used in this study. (**1**) 13^2-HO-20-Cl-Chl *a*, (**2**) 13^2-HO-20-Cl-Pheo *a*, (**3**) Chl *a*, (**4**) Chl *b*, (**5**) Pheo *a*, (**6**) 20-Cl-Chl *a*, (**7**) 20-Cl-Pheo *a*, (**8**) 13^2-HO-Chl *a*. All compounds exist as epimers at C-13^2. The $13^2(S)$-configuration is distinguished by adding a prime (′) to the formula number.

HPLC measurements. The reaction of chloroperoxidase with Chl *a* (**3**) and its derivatives could be monitored by HPLC using reversed-phase silica-gel chromatography. Technical details and the quantitation of pigments by HPLC have been described elsewhere [4]. The solid phase was a Spherisorb RP-18 column (Kontron, Munich, F.R.G.) packed with 5 μm material. Solvent A (CH_3CN/CH_3OH; 3/1, v/v) was superimposed by a multilinear gradient of solvent B (H_2O). The water content was diminished within 40 min from 10% to zero, then held for 10 min at zero and finally increased again, within 10 min, to 10% H_2O. The flow rate was 1 ml/min. For semipreparative separation a reversed-phase C_{18} column (7 μm material; 250 × 10 mm; Machery & Nagel, Düren, F.R.G.) was employed. Isocratic elution with CH_3Cn/CH_3OH (3 : 1, v/v) using a flow rate of 5 ml/min yielded satisfactory separation of the chlorophylls and their epimers on a 20 min time-scale.

Thin layer chromatography. TLC separations were carried out as described by Dörnemann and Senger [1].

Spectroscopy. Absorption spectroscopy and kinetic measurements were performed with an Shimadzu-260 UV/vis or Uvikon 820 double-beam spectrophotometer (Kontron) with a 1 cm light-path. For fluorescence spectroscopy a Shimadzu RF-540 spectrofluorophotometer was used. Fast atom bombardment mass spectra and collisionally activated tandem mass spectra were recorded on a Kronos MS-50 instrument using the arrangement described by Deterding and Gross [20]. For determination of exact molecular weights and peak isotope ratios a high-resolution analysis of all $M^{+\cdot}$ peaks was performed [20]. ^{1}H-NMR spectra were measured with a Nicolet NT-360 instrument using the pulse-Fourier transform mode.

Determination of radioactivity. The localization of radioactive bands on TLC plates was tested with a Rita-3200 radio thin-layer analyser (Raytest, Straubenhardt, F.R.G.).

Membrane particles. Membrane particles were prepared from *Scenedesmus obliquus* mutant C-2A′ employing an improved version of the method from Senger and Mell [21,22].

Enzyme incubation. The following reaction mixture was used for most of the experiments: 1 ml KH_2PO_4 buffer (0.3 M), 500 μl H_2O_2 (12 mM), 200 μl potassium halide (0.3 M), 900 μl H_2O, 0.11 μmol substrate in 100 μl acetone and a suitable amount of chloroperoxidase in 300 μl NaH_2PO_4 buffer (0.1 M; pH 4.0).

Isolation of enzymic products. The reaction mixture was extracted with light petroleum benzene (20–40 °C fraction) and dried over $MgSO_4$, and the solvent was evaporated under reduced pressure at 30 °C. The residue was taken up in a small volume of acetone and final purification (and analysis) was achieved by HPLC fractionation. Traces of water were removed by codistillation with CH_2Cl_2 and evaporation under a stream of nitrogen prior to use of the samples.

Pigments. Chls *a* (**3**) and *b* (**4**) were isolated from *S. obliquus* WT-D_3 and pigment mutant C-6E after an established procedure [4] and purified by reversed-phase HPLC (see below). Pheophytins were prepared using the method of Lötjönen and Hynninen [23]. The corresponding $13^2(S)$-epimers were prepared after the procedure of Hynninen and Lötjönen [24]. Hydroxylated chlorophylls were prepared according to Senge et al. [25].

Chlorinated chlorophylls and pheophytins. For preparation of 20-Cl-Chl *a* (**6**) 10 mg Chl *a* were dissolved in 10 ml acetone and added to a solution containing 100 ml KH_2PO_4 buffer (0.3 M, pH 5.0), 50 ml H_2O_2 (12 mM), 20 ml KCl (0.3 M), 90 ml H_2O and 42 μg chloroperoxidase in 30 ml NaH_2PO_4 buffer (0.1 M, pH 4.0). The mixture was incubated at 27 °C under stirring for 10 min. The green pigments were immediately extracted with petroleum ether (20–40 °C fraction) and dried over $MgSO_4$. The solvent was evaporated at 30 °C under reduced pressure and the residue was taken up in a small volume of acetone. The reaction product was

purified by semipreparative HPLC (see above) (75% yield).

The corresponding 13^2-HO-20-Cl-Chl *a* (**1**) was prepared from 13^2-HO-Chl *a* (**8**) using the same procedure as described for 20-Cl-Chl *a*. Yield: 25%.

20-Cl-Pheophytin *a* (20-Cl-Pheo *a*) (**7**) was prepared by dissolving 2 mg 20-Cl-Chl *a* in 0.2 ml tetrahydrofuran and immediately mixing with 10 ml cold petroleum ether (20–40 °C fraction) [23]. The solution was shaken for 20 min with an equal amount of aqueous trichloroacetic acid (20%, w/w). The organic phase was separated, washed five times with 10 ml H_2O and dried over Na_2SO_4. The solvent was evaporated and the residue dried by codistillation with CH_2Cl_2. This gave (**7**) in 100% yield.

The corresponding 13^2-HO-20-Cl-Pheo *a* (**2**) was prepared in 100% yield from 13^2-HO-20-Cl-Chl *a* (**1**) as described for 20-Cl-Pheo *a*.

Results and Discussion

Chlorination of chlorophyll a

The method for the chlorination of monochlorodimedone to dichlorodimedone employed by Hager et al. [10] was adapted and optimized for chlorination of Chl *a* (**3**). The optimum pH for enzymic chlorination was a compromise between the pH of pheophytinization of Chl *a* occurring below pH 4.5 and the pH optimum of the chlorinating enzyme, occurring at pH 2.75. A kinetic analysis of Chl *a* chlorination at different pH was performed by measuring the decrease in absorbance of Chl *a* at 668 nm (Fig. 2). At this wavelength Chl *a* has a higher absorbance than both chlorinated Chl *a* (see below) and pheophytin *a*, which both have absorption maxima at longer wavelengths than Chl *a*. At pH 3.5 a fast initiation of the reaction is observed, which is then overruled by beginning of pheophytinization at pH < 5.0. A pH of 5.0 seemed to be the best compromise considering all processes. Product analysis with reversed-phase HPLC at different times of incubation confirmed the completion of the reaction within 10 min. Increasing the incubation time or the incubation temperature above the optimum of 27 °C led to a decrease in the recovery of product, probably due to competition from oxidative side-reactions. Under the optimal conditions, the highest yield (75–80%) of product (**6**) was obtained at 27 °C after 10 min.

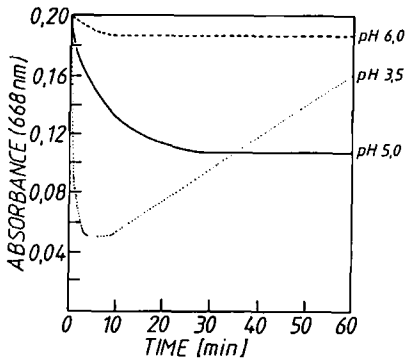

Fig. 2. Effect of pH on kinetics of chlorination of Chl *a* with chloroperoxidase. The reaction was performed in buffer of pH 3.5, 5.0 and 6.0, 0.11 μmol Chl *a* in 100 μl acetone were added to a solution of 1 ml KH_2PO_4 buffer (0.3 M), 500 μl H_2O_2 (12 mM), 200 μl KCl (0.3 M), 900 μl H_2O. 4.2 μg chloroperoxidase in 300 μl NaH_2PO_4 buffer (pH 4.0, 0.1 M) were added and the decrease in absorbance of Chl *a* was recorded at 668 nm against a reference lacking enzyme.

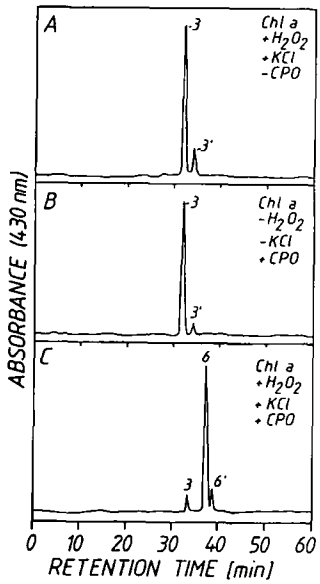

Fig. 3. HPLC elution profile of the reaction products of Chl *a* after treatment with chloroperoxidase. 200 μg Chl *a* (0.22 μmol pigment) were incubated with 8.4 μg chloroperoxidase in a solution of 2 ml KH_2PO_4 buffer (0.3 M, pH 5.0), 1.8 ml H_2O, 1 ml H_2O_2 (12 mM), 400 μl KCl (0.3 M). The pigments were extracted after 10 min with petroleum ether (20–40 °C fraction), dried by codistillation with Ch_2Cl_2 and the residue was taken up in 200 μl acetone. Aliquots of 20 μl were injected into the HPLC apparatus and separated on a Spherisorb RP-18 column with a flow rate of 1 ml/min. For details of the HPLC separation see Materials and Methods. (A) Reaction products of an incubation of Chl *a* (**3**) with chloroperoxidase and all cosubstrates; (B) incubation of Chl *a* with chloroperoxidase but without KCl and H_2O_2; (C) incubation of Chl *a* in the presence of KCl and H_2O_2 but without chloroperoxidase.

By variation of the enzyme/substrate ratio a reaction system containing KH_2PO_4 buffer of pH 5.0 and 4.2 μg chloroperoxidase per 100 μg Chl *a* (**3**) proved to be optimal (see Materials and Methods for other concentrations). An incubation with H_2O_2/KCl only or with chloroperoxidase in the absence of co-substrates gave no reaction thus showing that this reaction proceeds via an enzymic mechanism (Fig. 3B, C).

To further demonstrate that enzymic chlorination occurred, 500 μg Chl *a* (**3**) were treated with chloroperoxidase in the presence of 4.52 μCi $K^{36}Cl$: other components were the same as described in Materials and Methods section. The control incubation was identical but lacked chloroperoxidase. After extraction, the pig-

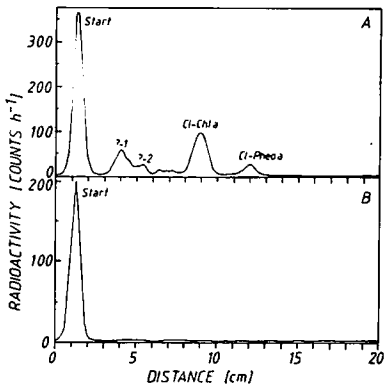

Fig. 4. Thin-layer radioscannogram of the reactions products of the enzymatic chlorination of Chl *a* in the presence of $K^{36}Cl$. 500 μg Chl *a* (**3**) (0.55 μmol) were incubated in a reaction system as described in Fig. 2 containing 4.52 μCi $K^{36}Cl$. After 10 min, pigments were extracted, dried and taken up in a small volume of acetone. This solution was applied to a silica-gel thin-layer plate and developed three times with a solvent system consisting of petroleum ether (40–60 °C fraction)/2-propanol/water (100:5:0.1, v/v). The thin-layer plate was scanned 60 min for radioactivity. (A) Test sample of the incubation of Chl *a* with $K^{36}Cl$ and enzyme; (B) reference sample, which contained the same reagents as the test sample but lacked chloroperoxidase.

ments were separated by TLC and scanned for radioactivity. In the presence of the enzyme, four chlorinated products were formed: the major one was identified as 20-Cl-Chl *a* (**6**) (Fig. 4A). Without enzyme, no labelled chlorinated products were detected (Fig. 4B). This again clearly confirms enzymic incorporation of chloride into the Chl molecule.

Analysis of the reaction products

HPLC analysis showed that Chl *a* was converted by chloroperoxidase in the presence of H_2O_2 and KCl into two compounds eluting at higher retention times (peaks 6, 6′, Fig. 3) compared to Chl *a* (peak 3, Fig. 3) (**3**). Peaks 3 and 3′ were identified as Chl *a* and Chl *a′* (i.e., $13^2(S)$-Chl *a*). The two additional compounds had identical absorption spectra and fluorescence spectra. Since the reaction conditions would not completely exclude Chl *a′* formation, these two compounds were assumed to be the chlorination products of Chl *a* and Chl *a′*; the latter compound was assumed not to be a product of the enzymic reaction but to be formed during the incubation and extraction process. This was proven by their identical absorption spectra and different circular dichroism spectra (data not shown). Initial evidence suggesting that the new substances were chlorinated Chls *a* and *a′* was obtained by comparing the fluorescence emission and absorbance elution profile. In comparison to the chlorophylls corresponding to peaks 3 and 3′ (Fig. 3), the fluorescence emission peaks for the pigments relating to peaks 6 and 6′ were smaller by a magnitude of three when compared to their corresponding absorbance peaks. Such lower fluorescence yield is typical for chlorinated chlorophylls [1].

The reaction product (**6**) of Chl *a* showed absorption maxima at 668, 628, 588 and 432 nm in acetone (Table I). Compared to Chl *a* the bathochromic shift in the absorption spectrum is consistent with theoretical expectations. Substitution of a chlorine into the con-

TABLE I

Spectroscopic properties of Chl a, 13^2-HO-Chl a, Chl b, their pheophytins and chlorinated derivatives

All spectra were recorded in acetone.

Compound	Absorption maxima (nm) (rel. absorbance)					Fluorescence maxima (nm)	
						excitation	emission
Chl *a* (**3**)	662	614	581	430		430	670
	0.79	0.17	0.09	1.0			
20-Cl-Chl *a* (**6**)	668	628	588	432		433	675
	0.71	0.18	0.06	1.0			
Pheo *a* (**5**)	666	615	534	503	408	410	674
	0.52	0.18	0.19	0.18	1.0		
20-Cl-Phe *a* (**7**)	675	615	546	515	412	412	682
	0.46	0.11	0.17	0.13	1.0		
13^2-HO-Chl *a* (**8**)	661	624	585	428		430	670
	0.74	0.12	0.06	1.0			
13^2-HO-20-Cl-Chl *a* (**1**)	668	624	586	432		433	675
	0.69	0.11	0.07	1.0			
13^2-HO-Pheo *a*	665	610	531	503	408	410	674
	0.45	0.08	0.08	0.10	1.0		
13^2-HO-20-Cl-Pheo *a* (**2**)	675	617	542	512	412	412	682
	0.49	0.05	0.11	0.08	1.0		
Chl *b* (**4**)	645	614	455			455	654
	0.60	0.09	1.0				
Cl-Chl *b*(?)	652	614	457			456	660
	0.33	0.08	1.0				

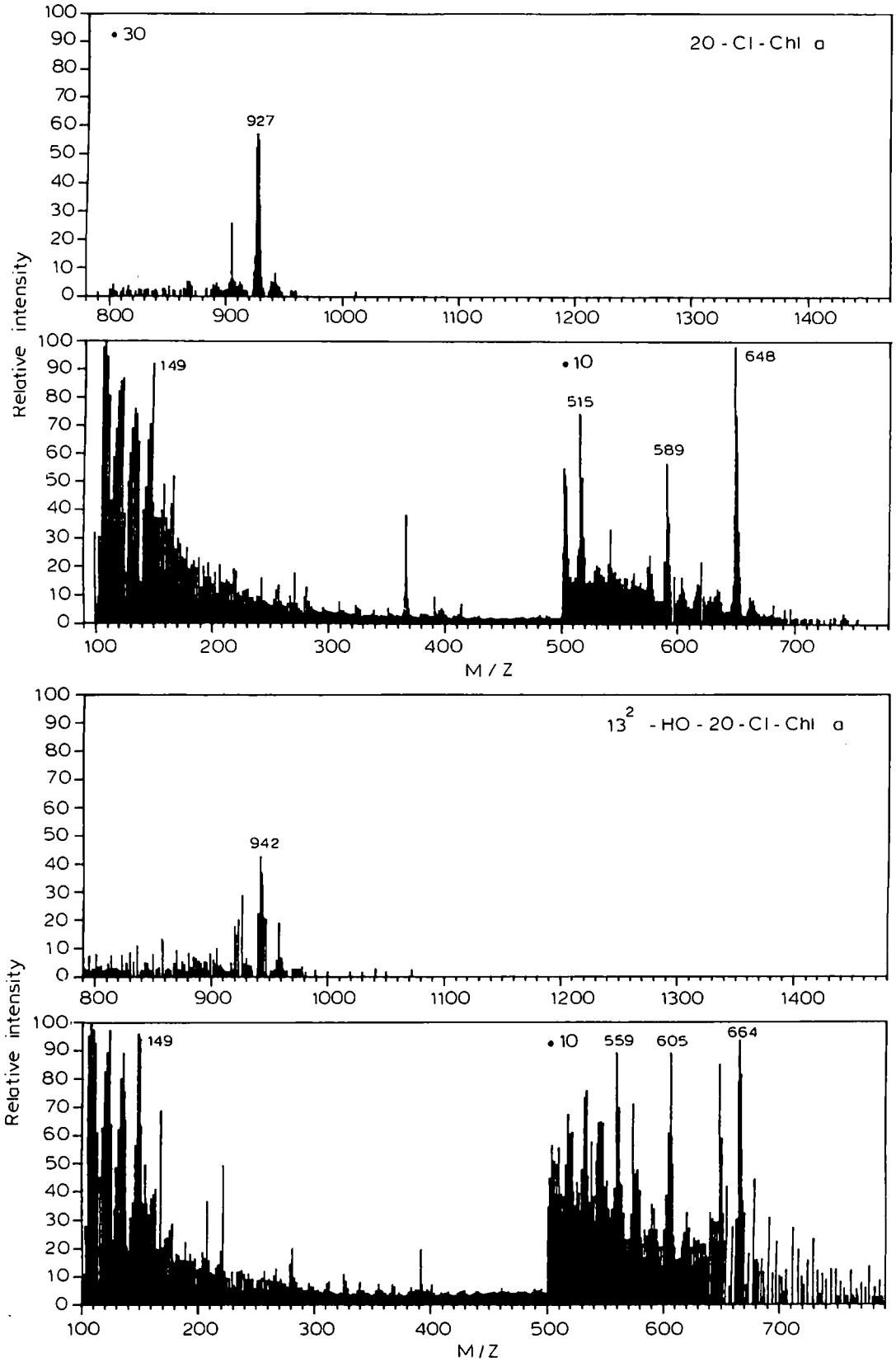

Fig. 5. Fast atom bombardment mass spectra of 20-Cl-Chl *a* (**6**) and 13^2-HO-20-Cl-Chl *a* (**1**) desorbed from a 3-nitrobenzylalcohol matrix. 1 μg chlorophyll was mixed on a probe tip with 3-nitrobenzyl alcohol and ions were produced by bombarding the samples with 7–8 keV of Ar atoms and then accelerated through a potential of 8 kV.

jugated ring system of the macrocycle lowers the excitation energy and leads to a bathochromic shift. The ratio of absorbances of the blue to red absorption maxima increased from 1.26 for Chl *a* to 1.41 in the chlorinated form.

The chlorinated pheophytin *a* (**7**), formed by mild acidification of the chlorinated Chl *a* (**6**), had absorption maxima at 675, 615, 546, 515 and 412 nm in acetone, which are at longer wavelengths than the maxima of pheophytin *a* (**5**). A useful indicator for chlorinated pheophytins can be found in the absorption spectra. For pheophytin *a* (**5**) the ratio of the absorptivities at 503 nm and 534 nm is 1.05 [23], whereas for chlorinated pheophytin *a* (**7**), the ratio of the absorptivities at 512 nm and 546 nm is 0.76. The fluorescence emission maxima of chlorinated Chl *a* (**6**) (675 nm) and the corresponding pheophytin (**7**) (682 nm) were also shifted to longer wavelengths when compared to Chl *a* (**3**) and pheophytin *a* (**5**). Only one symmetric emission band was observed in the fluorescence spectra, which is indication of sample purity.

The molecular mass of the reaction product was determined by using fast atom bombardment mass spectrometry (FAB-MS). The mass spectrum of 20-Cl-Chl *a* (**6**) is given in Fig. 5. The mass number of the $M^{+\cdot}$ peak was *m/z* 926.5, which is in accordance with the theoretical value calculated for the most abundant peak of $C_{55}H_{71}O_5N_4MgCl$ (most abundant peak, 926.5; average mass, 927.97) and with addition of a chlorine to Chl *a* (most abundant peak, 892.5; average mass, 893.5).

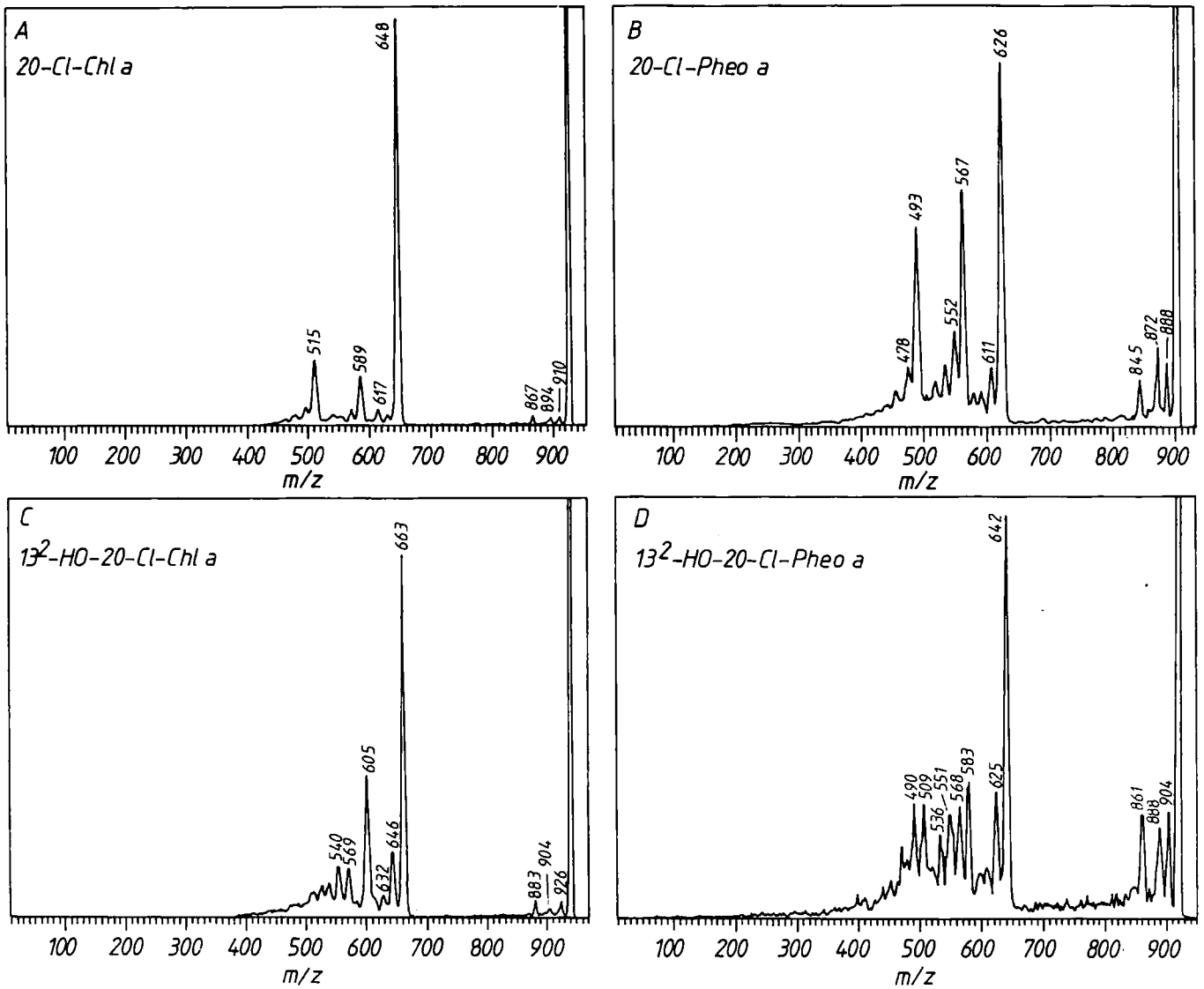

Fig. 6. Collisional activation of the $M^{+\cdot}$ ions of (A) 20-Cl-Chl *a*, (B) 13^2-HO-20-Cl-Chl *a*, (C) 20-Cl-Pheo *a*, and (D) 13^2-HO-20-Cl-Pheo *a*. $M^{+\cdot}$ ions were produced as described in Fig. 5. Ions of interest were selected at the first mass spectrometer stage at a mass resolution of approx. 1000 (width at 10% height). Mass-selected ion kinetic energy-analyzed daughter ion spectra were obtained by scanning the second mass spectrometer stage and by averaging 20 scans. Collisional activation was done by activating the mass-selected ions with helium in the third field-free region using a helium pressure that gave a 30% main beam suppression. For technical details see Deterding and Gross [20].

The corresponding pheophytin had an $M^{+\cdot}$ peak at m/z 904.5, which is also in good agreement with theoretical calculations (most abundant peak, 904.5; average mass, 905.6 for $C_{55}H_{73}O_5N_4Cl$) and the addition of Cl to pheophytin *a*. Since conventional FAB-MS leads to very complicated spectra (see Fig. 5), due to fragmentation of the matrix substance, we have employed collisional activation in tandem mass spectrometry for identification of the fragmentation pattern (Fig. 6). The fragmentation is very similar to that of Chl *a* and related compounds (Grese, R.P. et al., unpublished data), main fragmentation reactions are the loss of phytyl, peripheral substituents and cleavage of ring E. No loss of chlorine is observed in the spectrum of 20-Cl-Chl *a* (Fig. 6A). The corresponding pheophytin gives rise to the same fragments (Fig. 6B).

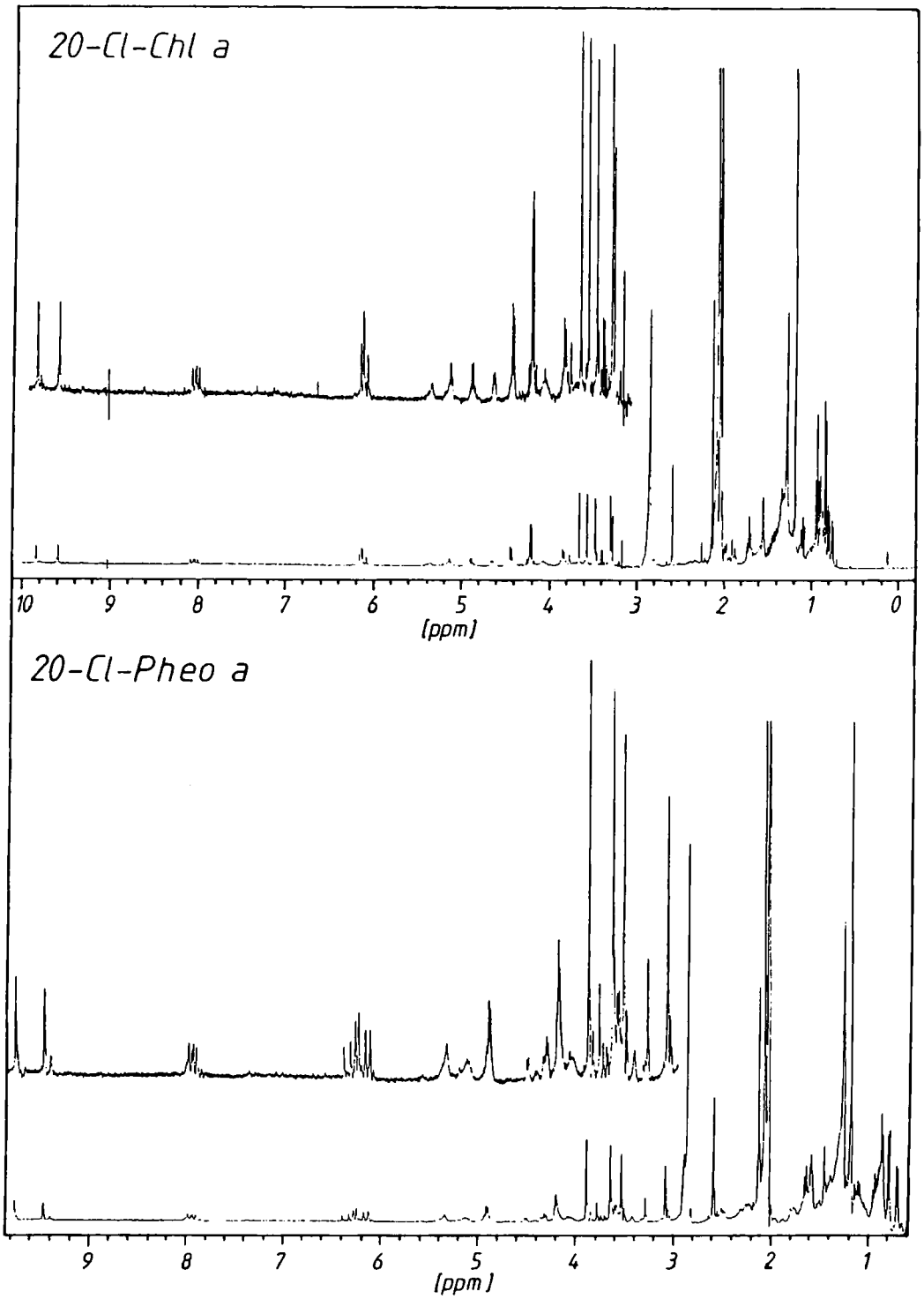

Fig. 7. 360 MHz ^{1}H-NMR spectra of 20-Cl-Chl *a* (**6**) and 20-Cl-Pheo *a* (**7**) in d_6-acetone. 36 scans were accumulated for each spectrum.

The site of chlorination was determined by ^{1}H-NMR spectroscopy. The ^{1}H-NMR spectra of 20-Cl-Chl *a* (**6**) and the corresponding pheophytin (7) are given in Fig. 7. Comparison of the chemical shifts of these compounds with those of Chl *a* (**3**) [26] shows that the main difference is the missing of the signal at 8.91 ppm. This signal is characteristic for the C-20 proton and its absence indicates that substitution has taken place at this position. Since the reactivity of this methine-bridge position is well known [7,8], an attack of chloronium ion at this position of the macrocycle is feasible. The NMR-spectra gave no indication of a substitution at C-5, C-10 or of multiple chlorination.

All the spectroscopic data presented are thus consistent with the reaction product of the enzymatic chlorination of Chl *a* being 20-Cl-Chl *a* (**6**). Further proof for this assignment was obtained by comparing the HPLC retention times from the reaction products with those of authentic samples [25].

The chromatogram of the radioactively labelled pigments showed two additional labelled peaks, more polar than chlorinated Chl *a* (Fig. 4). The radioactivity corresponded to two green, weakly fluorescent bands. Both exhibited an absorption spectrum with maxima at 696 and 432 nm in acetone and showed an $M^{+\cdot}$ peak at 912 mass units in the mass spectrum. Since these compounds could not be detected with HPLC they are assumed to be artefactual degradation or oxidation products arising from the TLC procedure [4,25].

The tetrapyrrole substrate specificity of the chloroperoxidase enzyme

To investigate the substrate specificity of the chlorination reaction, we tried to chlorinate 13^2-HO-Chl *a* (**8**) and 13^2-HO-Chl *a'* (**8'**) with chloroperoxidase. Both compounds yielded a new compound eluting at a retention time of 33 min in HPLC (Fig. 7). Comparison with authentic samples [20], the spectroscopic characteristics (Table I) and the mass spectrum with an $M^{+\cdot}$ peak at *m/z* 942.5 showed that the reaction product was 13^2(*R,S*)-HO-20-Cl-Chl *a*. This characterization is identical with that described by Dörnemann and Senger [1] for Chl RC I. 13^2-HO-Chl *a* (**8**) gave 25% chlorinated product (**1**), while 13^2-HO-Chl *a'* (**9**) gave approx. 35% of chlorinated Chl (**1'**). These data are in accordance with the results obtained by chemical chlorination [8], which indicate that the 13^2(*S*)-epimer (i.e., 13^2-HO-Chl *a'*) is more reactive than 13^2-HO-Chl *a* for chlorination. The collisional activation spectra of 13^2-HO-20-Cl-Chl *a* (**1**) and its corresponding pheophytin (**2**) are shown in Fig. 6C and D.

With Chl *b* (**4**) as substrate, a new compound could be isolated in 20% yield after incubation with chloroperoxidase (Fig. 8D, E). This chloro compound exhibited absorption maxima at 652 and 459 nm in acetone, which again shows a bathochromic shift compared to

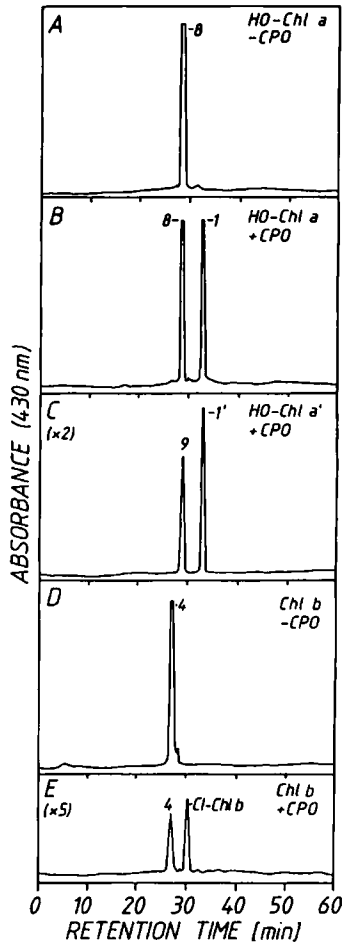

Fig. 8. HPLC chromatograms of the conversion of different Chl derivatives with chloroperoxidase. (A) 13^2-HO-Chl *a* incubated for 10 min with a reaction system lacking chloroperoxidase. (B) the same sample incubated in the presence of chloroperoxidase. (C) 13^2-HO-Chl *a'* incubated for 10 min with chloroperoxidase and co-substrates. (D) Incubation of Chl *b* without enzyme. (E) Chl *b* incubated in the presence of chloroperoxidase. For details of the experiment see Fig. 2.

the unchlorinated chlorophyll. Similar results were obtained with fluorescence spectroscopy (Table I). These results strongly suggest that this new compound is chlorinated Chl *b*.

The possibility of enzymic chlorination of pheophytin was checked using pheophytin *a* (**5**) as substrate. No chlorinated products could be detected and only pheophytin *a* was recovered from the reaction mixture. Protoporphyrin IX, an Mg-free biosynthetic precursor of Chl, could not be chlorinated. An incubation of 17,18-didehydrochlorophyllide with chloroperoxidase under the conditions used for Chl *a* gave no chlorinated product, either. Likewise, the incubation of naturally occurring Chl *a/b* protein complexes (chloroplast subparticles) with the enzyme reaction system produced no chlorinated chlorophylls. These results, taken together, indicate that chloroperoxidase requires a Mg-chlorin type structure with a reduced ring D for chlorination to occur at the C-20 methine bridge.

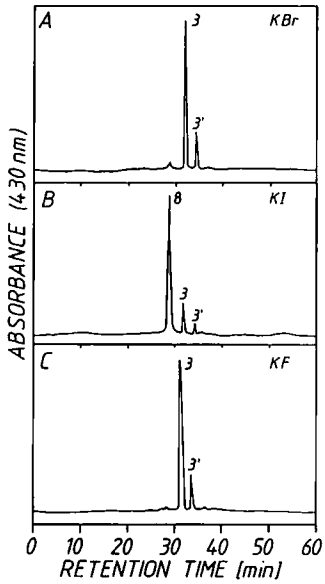

Fig. 9. HPLC chromatograms of the products from enzymic halogenation experiments. (A) Chl *a* incubated with chloroperoxidase and KBr. (B) Chl *a* incubated with chloroperoxidase and KI. (C) Chl *a* incubated with chloroperoxidase and KF. For experimental details see Fig. 2.

Other halogenations of chlorophyll a

Chl *a* was incubated with a reaction mixture containing 0.3 M KBr or KI instead of KCl, but yielded only Chl *a* (Fig. 9A). In the presence of KI, Chl *a* was largely oxidized to 13^2-HO-Chl *a*, probably by I_2 formed from I^- and H_2O_2 (Fig. 9B). This assumption is supported by the work of Smith and Simpson [9], who were able to synthesize 13^2-HO-methylpheophorbide *a* by treating methylpheophorbide *a* with iodine and sodium acetate in tetrahydrofuran [9]. Attempts to retard the formation of iodine by changing the concentrations of the reactants failed.

Furthermore, we were unable to detect enzymic fluorination of Chl *a* (Fig. 9C), which is in accordance with the results of Hager et al. [10], who described fluoride as a potent inhibitor rather than a substrate of the chloroperoxidase from *C. fumago*.

Conclusions

The chloroperoxidase from *Caldariomyces fumago*, as shown in this contribution, is capable of smoothly converting Chl *a* and some of its derivatives into the corresponding 20-chloro compounds. The very high yields of 20-Cl-Chl *a* (**6**) produced make the enzymic reaction an attractive alternative to the chemical synthesis for the production of large amounts of chlorinated chlorophylls. The procedure presented here is the first synthesis of chlorinated chlorophylls, circumventing the remetallation step necessary in all chemical syntheses.

20-Cl-Chl *a* is, to our knowledge, the first example of a Chl *a* derivative with substitution at the central ring system, in addition to the numerous known derivatives with alterations, e.g.., at ring E or at the propionic side-chain. Therefore, chlorinated chlorophylls may be useful as model compounds for photosynthetic studies.

Also this is, to our knowledge, the first proof of an enzymatic in vitro halogenation reaction taking place in the chlorophyll macrocycle. Kobayashi et al. described the isolation of varying amounts of chlorinated chlorophyll in green tissue samples, depending on the kind and age of tissue used [5]. They were not able to correlate the formation of chlorinated chlorophylls to a biological reaction or distinguish between enzymic or non-enzymic formation. Assuming that some haloperoxidase type enzyme is active during pigment extraction, our findings offer an explanation for these results. Currently under way are studies aimed at revealing the mechanism of Chl *a* chlorination and studying the use of chlorinated chlorophylls as model compounds in photosynthesis.

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