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Chloroperoxidase Mediated Halogenation of Phenols

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Halophenols are among the many varied types of halogenated organic compounds occurring in the ecosystem. Primarily because of pollution research, the chlorophenols found in most environmental samples are believed to be the result of man-related activities. However, several of the halophenols also have a natural origin. Indeed, hundreds of halogenated compounds occur as secondary metabolites of living organisms ranging from bacteria to higher plants and animals (Neidleman and Geigart 1986; Siuda and DeBernardis 1973). Both quantitative and qualitative evaluations of organohalogens may in some cases be misleading due to background levels of naturally occurring substances or to significant contributions of closely related structures generated by living organisms. Since environmental investigation of halogenated pollutants usually leads to the ultimate source(s) of these substances, careful assessment must be made regarding the presence, causes and effects of halophenols found in the biosphere.

Nature then, represents a supplemental route to the presence of a number of organohalogens some of which have been classified as pollutants. The emerging picture strongly indicates that many organisms contain haloperoxidase enzymes which catalyze the formation of a carbon-halogen covalent bond leading to the formation of halogenated natural products (Hager 1982). In this study, we employed a fungal enzyme, chloroperoxidase (CPO) obtained from Caldariomyces fumago. Chloroperoxidase has been well utilized as a model in the study of biological halogenation (Neidleman and Geigart 1986). Our intent was to use the enzyme as a means to predict potential halogenation of phenols in the ecosystem.

MATERIALS AND METHODS

Phenols were obtained from various suppliers. In all cases, minimum purity was greater than 95% and usually >98% as determined by GC. CPO was commercially available (Sigma).

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Halogenation activity was determined as previously described by Morris and Hager (1966) whereby the decrease in absorption of monochlorodimedone (MCD) was followed at 278 nm.

Reaction conditions -- To a teflon-capped 10 mL vial was added the following: 2.1 mL of 0.05M phosphate buffer pH 2.75, 40 μL of 0.1 M of the phenolic substrate (4 μmoles) in MeOH, 200 μL of 2 M aqueous KCl (400 μmoles), 300 μL of 0.02M H_2O_2 (6 μmoles) followed by a volume of CPO solution containing 2.5 units of haloperoxidase activity (ca. 200 μL of CPO solution in buffer). The final total volume of the reaction medium was adjusted with buffer to 4.0 mL. The amount of enzyme used represented a saturated system, i.e., the substrates were in excess. After addition of the enzyme, the solution was shaken a few times and left for 0.5 hr. At the end of this time, 0.2 mL of 1.0 N HCl was added to adjust the pH to 2.0. Fifteen μL of a 0.1 M methanolic solution of the internal standard 3,5-dichlorophenol (1.5 μmoles) was finally added. The aqueous solution was then extracted with CH_2Cl_2 (2 x 2 mL) for 30 sec with a vortex mixer for each extraction. The combined organic extracts were then dried with 0.1g $MgSO_4$, decanted and evaporated to dryness at ice bath temperature using a rotary evaporator. The residue was taken up in 200 μL of MeOH and placed in a teflon capped vial. Two μL of the methanolic solution were injected onto a 4' 1% SP-1240 DA glass column (3mm i.d.) contained in a Hewlett-Packard 5720 A FID gas chromatograph. Conditions: N_2 flow, 30 mL/min; injector 250°C; detector 275°C; column temperature 85° for 1 min then programmed at 8°/min up to 180°C; range 10; attenuation 4/8.

Qualitative analysis was first performed by comparison of retention times of reaction products versus times of known substances. Coinjection of the peaks in question was then conducted with the known compounds. Further confirmation of structure was obtained from mass spectral analysis using a Finnigan 3200 GC-MS system. For quantitative analysis, the peak heights of both the internal standard and the product under study were measured. Concentrations of products were determined from a previously established standard curve. Reactions were run in duplicate and reproducibility was 6-7%.

RESULTS AND DISCUSSION

Chloroperoxidase catalyzed chlorination of phenolic substrates led to the results illustrated in Table 1. Overall, the results are those expected for chlorination by HOCl which is representative of the mechanism of action of CPO (Brown and Hager 1967). Enzyme-mediated chlorinations were consistent with chloronium ion attack at sites ortho or para to the directing phenolic moiety. In this study, the enzyme was saturated with the substrate and dichlorination was minimal. Thus, phenol yielded both the 2- (ortho) and 4- (para) isomers. When 2-chlorophenol was used as a substrate, very small amounts of the 2,4- and 2,6- isomers as well as the 2,4,6-trichloro product were formed. During chlorination, both the 2,4- and 2,6-isomers can function as substrates

Table 1. Reaction products obtained from chloroperoxidase induced halogenation of phenolic substrates

Substrate	Phenolic Products	% Measured
phenol	2-chloro	---
	4-chloro	25
2-chlorophenol	2,4-dichloro	trace
	2,6-dichloro	trace
	2,4,6-trichloro	trace
4-chlorophenol	2,4-dichloro	5
2,4-dichlorophenol	2,4,6-trichloro	9-12
2,6-dichlorophenol	2,4,6-trichloro	12-15
2,3,5-trichlorophenol	2,3,4,5-tetrachloro	15
	2,3,5,6-tetrachloro	5
2,4,5-trichlorophenol	2,3,4,6-tetrachloro	---
2,4,6-trichlorophenol	none	0
2,3,4,5-tetrachlorophenol	pentachloro	5
2,3,5,6-tetrachlorophenol	pentachloro	2
2,3,4,6-tetrachlorophenol	none	0
o-cresol	2-methyl-4-chloro	38
	2-methyl-6-chloro	---
m-cresol	3-methyl-4-chloro	33
	5-methyl-2-chloro	15
	3-methyl-2-chloro	---
p-cresol	4-methyl-2-chloro	---

since each would produce the same trichlorinated product. With other chlorophenols, monochlorination was typically observed. Yields of products generally increased with increasing activation of the aromatic nucleus. Cresols offer the greatest conversion whereas very low yields of pentachlorophenol (PCP) were achieved by chlorination of the lesser activated tetrachloro compounds. The results adequately demonstrate that EPA priority pollutants such as 2-chlorophenol, 2,4-dichloro-phenol, 2,4,6-trichloro-phenol and 4-chloro-m-cresol can be formed enzymatically and quite possibly by living systems. Furthermore, extrapolation of the data suggests that similar compounds could arise by chemical halogenation of candidate precursors in the environment.

The pattern of substitution was that expected for substrates undergoing attack by an electrophilic chloronium ion at the most activated ring sites. On the other hand, the pollutant 2,4,5-trichlorophenol (2,4,5 TCP) was not observed in these enzyme reactions. Again this is consistent with electrophilic attack by Cl^+ on the phenolic nucleus. If meta substitution occurred on any of the employed substrates, estimations of this kind of attack are much less than 1-2% based on the GC results. The point to be noted here is that accumulation of 2,4,5-TCP in the environment by direct pollution is more probable than environmental halogenation by chloronium ions of any 2,4-dichlorophenol or its immediate precursors present in the biosphere. If CPO is a useful model for enzyme-mediated chlorination, these results suggest that the 2,4,5- isomer is not a natural product. When using 2,4,5-TCP as a substrate, the structure of the only product obtained was found to be 2,3,4,6-tetrachlorophenol. Based on our experiments, a similar argument can be made for the occurrence of the ultimate polyhalophenol, pentachlorophenol (PCP). Theoretically, PCP could possibly arise from several chlorophenolic substrates made available to a haloperoxidase or to HOCl. However, if the biomimetic reactions do suffice as a reasonable model for "natural" halogenation, 2,4,5-TCP would not be a likely candidate precursor to PCP if one assumes chlorination only at the activated positions of the aromatic ring. One of the two halogenation reactions leading from 2,4,5-TCP to PCP would necessitate substitution at a deactivated meta position, viz. the C-3 atom. Extending this rationale, the bulk of the PCP found in the environment would, as commonly believed, be a consequence of direct pollution, since little if any PCP would be formed from environmental halogenation of 2,4,5-TCP or the 2,3,4,6- substituted phenol. Although it has been shown that small amounts of PCP may be obtained from 2,3,4,5- and 2,3,5,6-tetrachlorophenols, these substances do not appear to be used commercially to any great extent, nor are they recognized as natural products. In contrast, 2,3,4,6-tetrachlorophenol is a fungicide but does not yield PCP during enzymatic chlorination. Additionally, the lesser substituted chlorinated phenols are not precursors to PCP. The evidence accumulated here strongly suggests that PCP found in the environment is unrelated to normal biosynthetic processes.

The enzymatic chlorination of cresols was conducted primarily due to the fact that these substrates are available as by-products from coal combustion (coking). Again, the results are consistent with attack at the most activated positions of the ring. Under the conditions of the experiment, dichlorination was not observed. Furthermore, no reaction was observed to take place at sites solely directed by the methyl group, viz., p-cresol produced only the 2-chloro and not the 3-chloro isomer. In the reaction of m-cresol, three products were observed all of which gave characteristic 142, 144 m/e ions in the mass spectra. The structures of both 4-chloro- and 6-chloro-m-cresol were determined by comparison with known substances by the peak enhancement method.

As previously mentioned, dichlorination did not occur. Based on the small amounts of dichlorinated products obtained from 2-chlorophenol, one would expect dichlorination of o-cresol to provide at least small amounts of 4,6-dichloro-o-cresol since both monochloro products would furnish the same dichloro analog. Again, the explanation above for the results of the 2-chlorophenol does not satisfactorily suffice for observations noted with the cresols. Chlorination of cresols with excess CPO did result in multi-halogenation. Using phenol as a substrate, 2-chlorophenol and 4-chlorophenol were obtained in 38% and 36% respectively. However, CPO chlorination of chlorophenols with excess enzyme still did not produce significant dihalogenation (data not shown).

Although complete halogenation would not be expected when the enzyme was not in excess, another possible explanation for the limited halogenation may rest with the characteristics of the enzyme. CPO is a suicidal enzyme and in generating HOCl serves as its own substrate by halogenating tyrosine residues of the protein (Hager et al. 1966). Thus, chlorination of the phenols may proceed in competition with halogenation of CPO until the enzyme is no longer active and/or all HOCl is used. We have assumed that the enzyme no longer exhibits catalytic action at the end of 0.5 hr of incubation and that little HOCl remains. This assumption is supported somewhat by the fact that when p-ethylphenol was added to the enzyme catalyzed mixture after 0.5 hr, halogenated ethylphenols were not observed.

During the chlorination of phenols and cresols we occasionally observed traces of brominated substances by GC-MS techniques. These brominated substances most likely arise from the small amounts (0.01%) of bromide ion present in the reagent grade potassium chloride. Since bromination takes place much faster than chlorination, it is not surprising to observe some of these brominated phenols. These results further point to potential products that exist or could arise from biological or chemical chlorination. Brominated phenols have been found in sediments (Watanabe et al. 1985) and in treated water (Sithole and Williams 1986).

The investigation of the presence of certain halogenated pollutants, but especially the halophenols, appears to cross several scientific disciplines. The halogenated phenols are examples of substances found in the biosphere and which have multiple geneses. Sources of halophenols include: (a) direct discharge, (b) metabolism and/or environmental transformation of other pesticides, (c) chlorination of water, and (d) natural production by plants, microorganisms and other living species. Toxic chlorophenols are also known to be generated by the bleaching process in pulp and paper treatment (Landner et al. 1977).

Biodegradation by living organisms represents one method of control and detoxication. Enzyme mediated reactions may not only be partly responsible for degradation of halogenated

products, but also may play an important role in the biosynthesis of such compounds. The availability of haloperoxidases which exhibit a wide range of pH optima for both halogenating and peroxidase activity affords an unique opportunity to study the generation and potential oxidation of halogenated compounds under diverse environmental conditions. Enzymatic halogenation via haloperoxidases or chlorination of water inherently involves generation of HOCl and either path could qualitatively lead to similar products. Our data indicates that some of the lesser substituted chlorinated phenols may be formed from suitable substrates by a halonium ion mechanism. Neither enzymatic halogenation nor chlorination of water would seem to offer a plausible explanation for significant generation of 2,4,5-TCP or for phenols with four-five halogen atoms.

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