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CHEMICAL BROMINATION OF PHENOL RED BY HYDROGEN PEROXIDE IS POSSIBLE IN THE ABSENCE OF HALOPEROXIDASES

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ABSTRACT

The dye phenol red (phenolsulphonphthalein) was chemically brominated to bromophenol blue (3',3",5',5"-tetrabromophenolsulphonphthalein) directly by hydrogen peroxide (20 mM or higher) under acidic pH (0.5-4.5) and moderate temperature (30°C). Since this bromination reaction takes place in the absence of any chemical or biological catalyst, the results represent an important warning concerning a commonly used assay for detecting or screening haloperoxidases. Bromophenol red (5',5"-dibromophenolsulphonphthalein) and another unidentified compound were observed as temporary intermediates. Incubation of the reaction mixture with KCl instead of NaBr did not yield any chlorinated products under the same conditions..

1. INTRODUCTION

Organohalogens have generally been regarded as pollutants of anthropogenic origin, however these compounds can also be produced naturally by many different kinds of organisms [1-3]. Haloperoxidases are enzymes which are capable of halogenating a variety of organic compounds using hydrogen peroxide and halide ions as substrates [4-5]. A considerable number of bromoperoxidases and chloroperoxidases have been

isolated and described from many different terrestrial and marine organisms. Usually these enzymes contain heme or vanadium as prosthetic group, although in few of them the cofactor is unknown or absent. The reaction mechanism by which haloperoxidases are able to halogenate organic compounds is not completely clarified; for several enzymes, however, it has been proved that hypohalous acid (HOX) or X_2 (in most cases these species are in equilibrium with each other), are the active halogenating agents in solution that in turn halogenate the organic substrates [4-5].

Phenol red has been used as a standard substrate of haloperoxidases [4]. This compound can be brominated [6] and chlorinated [7] by the vanadium-containing haloperoxidase from the marine brown alga *Ascophyllum nodosum*.

In the course of our investigations on halogenating enzymes, we have observed that the chemical bromination of phenol red by hydrogen peroxide (20 mM or higher) is also possible in the absence of haloperoxidases. The reaction takes place at a moderate temperature (30°C), at acid pH, in the presence of NaBr, that is, in conditions that are not uncommonly used in routine haloperoxidase assays.

2. MATERIALS AND METHODS

The standard compounds bromophenol red (5',5"-dibromophenolsulphonphthalein), and bromophenol blue (3',3",5',5"-tetrabromophenolsulphonphthalein) were obtained from Fluka Chemie AG (Buchs, Switzerland) and phenol red (phenolsulphonphthalein) from Merck (Darmstadt, Germany) (Figure 1). Catalase (EC 1.11.1.6), used to remove the excess of H_2O_2 from the samples, was from *Aspergillus niger* (Sigma Aldrich, Steinheim, Germany).

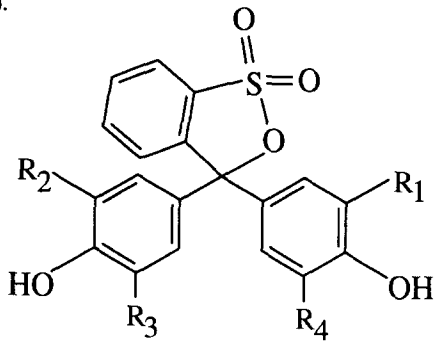

Figure 1. Chemical structure of phenol red ($R_1 = R_2 = R_3 = R_4 = H$); bromophenol red ($R_1 = R_2 = Br$ and $R_3 = R_4 = H$) and bromophenol blue ($R_1 = R_2 = R_3 = R_4 = Br$).

Reaction conditions

The final concentration of substrates used in all assays was 0.1 mM for phenol red and 0.1 M for NaBr or KCl. The concentration of H_2O_2 and the final pH of the reaction mixture were variable and are indicated in the results section. The buffer KH_2PO_4 (0.1 M) was used to adjust the different final pHs in the reaction

mixture. All of the reagents were prepared in demineralized water.

In all cases, samples without H_2O_2, and samples without any halide (NaBr or KCl) were incubated in parallel as negative controls. The final volume of the reaction mixture (1 ml) was incubated in 50 ml closed glass bottles at 30°C, under shaken conditions, during the different incubation times indicated. The reaction was stopped at the indicated time by diluting 100 µl of the reaction mixture into 900 µl of buffer KH_2PO_4 0.1 M pH 7 and analyzed by a UV/VIS spectrophotometer or by high-pressure liquid chromatography (HPLC).

When phenol red was incubated at pH 2.8, 5.0 and 7.0 with a H_2O_2 concentration of 441 mM but in the presence of KCl (0.1M) as halide ion donor instead of NaBr, no increase in the absorbance was observed indicating that hydrogen peroxide at the concentration tested could not carry out the chlorination of phenol red. The spectra of the chlorinated reaction products of phenol red have been recently published by Soedjak and Butler (1990) [7].

Determination of phenol red, bromophenol red and bromophenol blue

UV/VIS spectra

The UV/VIS-spectra of phenol red, bromophenol red and bromophenol blue are very distinct at pH 7 (Figure 2).

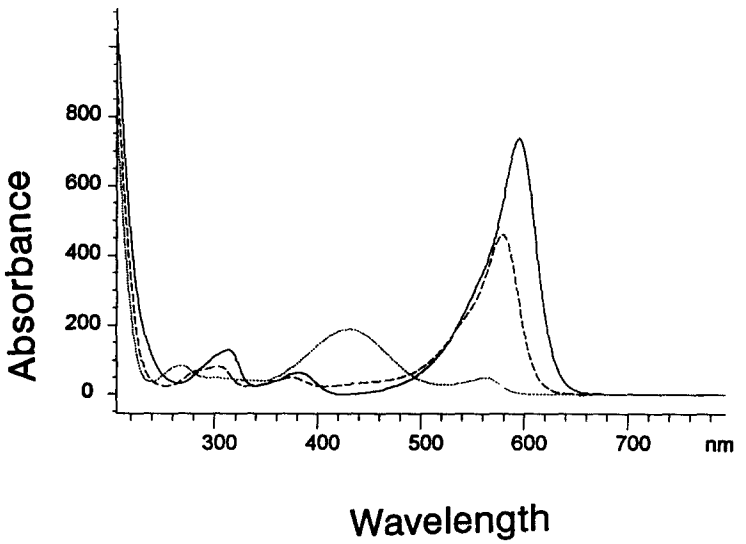

Figure 2. Optical spectra of the standards phenol red (.....), bromophenol red (----) and bromophenol blue (__) (0.01mM) in buffer KH_2PO_4 0.1 M at pH 7.

Phenol red shows a small absorption maximum at 268 nm, a high absorption maximum at 436 nm (ε_{436} = 19,700 M^{-1} cm^{-1}) and another small maximum at 557 nm. Bromination of phenol red results in a decrease of the absorption maximum at 436 nm and a strong increase of the third absorption maximum. The third absorption maximum changes from 557 nm to 592 nm due to the progressive halogenation of the two

phenolic ring systems. The third absorption maximum is located at 575 nm in the case of bromophenol red and at 592 nm in the case of bromophenol blue [6]. The increase of the third absorption maximum was used to follow the formation of bromophenol blue measuring the absorbance on a Perkin Elmer lambda 2 UV/VIS spectrophotometer (scanning mode from 200 to 700 nm).

HPLC conditions

Samples were analyzed in duplicate using a HPLC Hewlett-Packard 1100 series equipped with a diode array detector (DAD). The data were processed with a Hewlett-Packard Chemstation (Waldbronn, Germany). Samples (50 µl) were injected on a ChromSpher 5 C18 reversed phase column (Chrompack Internat., Middelburg, The Netherlands). The mobile phase used was acetonitrile:phosphate buffer (0.1 M pH7) at relative concentration of 10:90 for the first 10 min. and, thereafter, 50:50 till 17 min. of total run. Flow rate was set at 0.5 ml min^{-1} at 30°C. Phenol red, bromophenol red and bromophenol blue were detected at 570 and 436 nm. In both cases the reference wavelength was 750 nm. The compounds were identified based on matching retention times and UV spectra with standards. Samples with concentrations of H_2O_2 in excess of 441 mM were treated overnight and before HPLC analysis with 1µl catalase to avoid interferences in the HPLC detection system.

3. RESULTS

Time course of phenol red bromination

The reaction of phenol red in the presence of NaBr and 441 mM H_2O_2 at pH 2.8 was monitored in time (Figure 3). Through the analysis of the products by HPLC, we demonstrated that phenol red is brominated via the dibrominated intermediate (bromophenol red) and an unidentified compound before it is converted into the final tetrabrominated product (bromophenol blue).

After 30 min. of incubation the chromatogram in the HPLC displayed a profile of peaks corresponding to phenol red (retention time 11.03 min.), the unidentified intermediate (retention time 11.99 min.), bromophenol red (retention time 12.14 min.), and small amounts of bromophenol blue (retention time 12.77 min.). The intermediates bromophenol red and the unidentified compound reached their maximum concentrations at 60 min. and 90 min., respectively. After 150 min., the substrate phenol red has been totally consumed and converted into the intermediates and the final product. The intermediates have disappeared completely at 250 min. of incubation. The amount of final product (bromophenol blue) progressively increased from 30 min. until 180 min., when the reaction was practically complete. The yield of bromophenol blue after 360 min. of incubation was 89%.

The optical absorption UV/VIS spectrum of the final product in the reaction mixture was identical to that of a standard solution of bromophenol blue in KH_2PO_4 buffer (0.1 M pH 7) and to that described by de Boer et al. (1987) [6]. Its retention time also coincided with that of the bromophenol blue standard

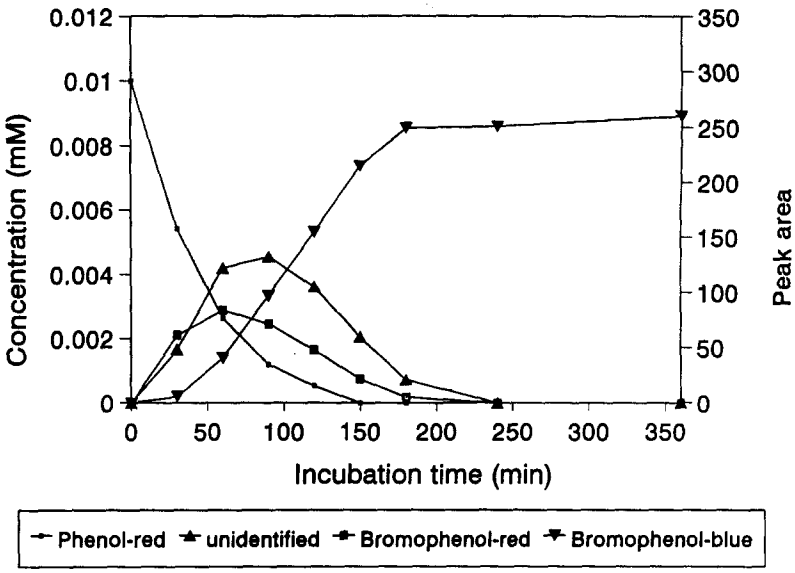

Figure 3. Concentration of phenol red, the intermediate (bromophenol red) and the final product (bromophenol blue) as a function of the incubation time during the bromination of phenol red by hydrogen peroxide (441 mM). The peak area refers to the unidentified intermediate compound.

Both H_2O_2 and NaBr were essential for the bromination reaction since the brominated products of phenol red were never observed in any of the negative controls in which one of these two compounds was absent.

Bromination of phenol red at different concentrations of H_2O_2.

The bromination of phenol red was carried out at different concentrations of H_2O_2 in the presence of NaBr and KH_2PO_4 0.1 M at pH 2.8 (Figure 4).

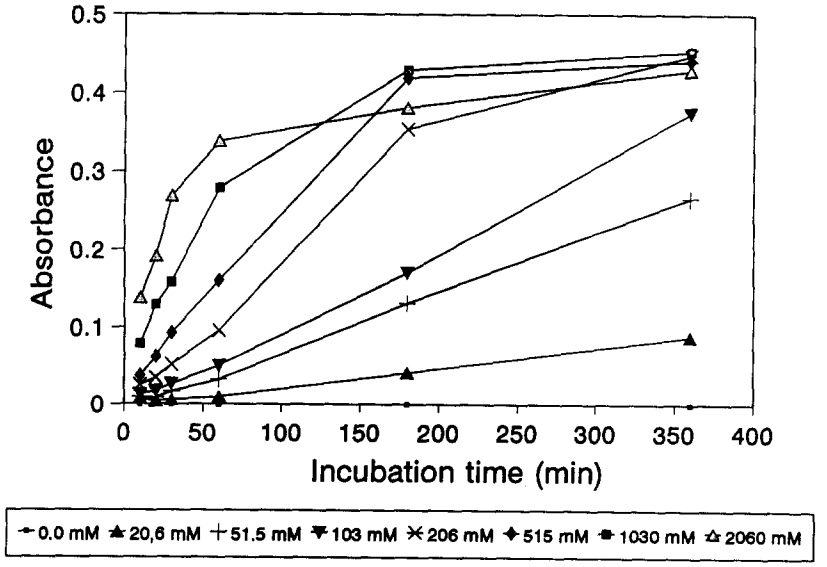

Figure 4. Effect of hydrogen peroxide concentration on the bromination of phenol red at pH 2.8.

The formation of the final product (bromophenol blue) was monitored spectrophotometrically between 575 and 592 nm. The absorbance maxima between these wavelengths increased with the concentration of H_2O_2 and with the incubation times (Figure 4). The absorbance increase was more rapid at higher hydrogen peroxide concentrations. In similar experiments at pH 3.5 with 20 mM H_2O_2, we confirmed that the increase in absorbance after 50 h of incubation was due to the formation of bromophenol blue based on HPLC analyses.

To check what is the minimum concentration of H_2O_2 required to carry out the bromination reaction, phenol red was incubated with 1 mM H_2O_2 at pH 4, but the bromophenol blue could not be detected in the HPLC chromatograms even after 117 hours of incubation.

Bromination of phenol red by hydrogen peroxide at different pHs.

In Figure 5, the effect of pH on the bromination of phenol red in the presence of NaBr and hydrogen peroxide (441 mM) is shown. The increase in absorbance was only detectable in a range of acidic pHs between pH 0.5 to 4.5 with an optimum at pH 3, indicating that the reaction is highly pH dependent.

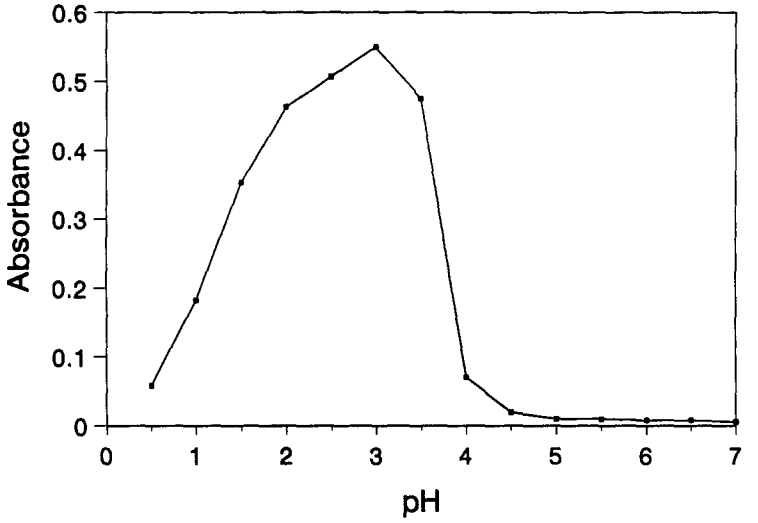

Figure 5. Effect of pH on the bromination of phenol red by hydrogen peroxide (441 mM) after 20 hours of incubation.

4. DISCUSSION

The results indicated that hydrogen peroxide at high concentrations (20 mM) are able to brominate phenol red at room temperature at acid pH in the absence of any catalyst. Halogenation of organic compounds (*e.g.*, barbituric acid) with halide ions and hydrogen peroxide in the absence of a catalytic element has been observed before [8]. However, this reaction was only practical at 100°C using very high concentrations of hydrogen peroxide (3 M). Other reports of chemical bromination reactions carried out in presence of H_2O_2 have been described for several aromatic compounds (acetanilide, 4-methylacetanilide, and salicylaldehyde)

but in these cases, the presence of vanadium pentoxide as a catalytic element was indispensable to observe the brominated products [9]. Another procedure to brominate several aromatic hydrocarbons and methoxy benzenes has been described utilizing the catalyst molybdenum(VI) instead of vanadium [10-11]. Molybdenum or vanadium catalyze the bromination reaction by means of peroxo- vanadium or molybdenum species which are much stronger oxidants than hydrogen peroxide.

Our results demonstrate that a 20 mM concentration of hydrogen peroxide can lead to chemical bromination. This H_2O_2 concentration is not uncommon in haloperoxidase-assays, so caution has to be exercised by the scientists using the phenol red assay to detect or screen haloperoxidases.

It has been described that the oxidation of Br^- by H_2O_2 is also highly pH dependent and in the case of the vanadium(V)-catalyzed reaction it was found that this is only possible when the pH of the reaction medium was highly acidic (pH ca. 1-2) [11]. The optimum pH for the chemical bromination in our reaction mixture (pH 3) coincided with the pH at which the heme chloroperoxidase from the deuteromycete *Caldariomyces fumago* has its maximal halogenating activity [5]. It is feasible that acidic pHs may be necessary to give rise to the active brominating agents HOBr and Br_2.

Although it cannot be concluded from our results, it is probable that under the conditions assayed, such a high concentration of H_2O_2 is sufficient to produce HOBr and/or Br_2 as the active brominating agents which could transfer the bromine to the substrate, in the same way that has been reported for the reaction mechanism of some algal bromoperoxidases [4]. Therefore, assays for bromoperoxidase activity using the phenol red method should preferably be carried out above pH 4.

Furthermore, our results indicate that the use of hydrogen peroxide as disinfectant or as a bleaching agent (*e.g.*, in the paper industry) may lead to the formation of undesirable organohalogen compounds.

Acknowledgments

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