STUDIA UNIVERSITATIS BABEŞ-BOLYAI, CHEMIA, XLVIII, 2, 2003

HORSERADISH PEROXIDASE - CATALYZED OXIDATION OF WATER - INSOLUBLE PHENOTHIAZINES

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ABSTRACT. Horseradish peroxidase catalyzes the oxidation of N-alkyl phenothiazine derivatives to the corresponding sulfoxides and sulfones in the presence of a co-solvent. The reaction conditions may also be controlled so as to yield the sulfoxide in quantitative yields.

INTRODUCTION

Horseradish peroxidase (HRP) is a hemoprotein known to catalyze the oxidation of a wide range of compounds. Its normal catalytic cycle involves the oxidation by hydrogen peroxide of the native state of the enzyme (ferric), forming an intermediate, "Compound I", which is two oxidation equivalents above the resting state and contains a porphyrin radical cation and a ferryl group. Compound I then interacts with the substrate, yielding Compound II (one oxidation equivalent above the resting state) and substrate radical cation. Compound II similarly reacts with another substrate molecule to yield the ferric state of the enzyme and a substrate radical cation [1,2].

Peroxidases have been associated with several physiological events [1,2]. From this point of view phenothiazine derivatives have already been studied as substrates for enzymatic oxidation with HRP. This is due to the fact that phenothiazines demonstrate antihistamine, cholinolytic, sedative or neuroleptic activity [3,4]. While the influence of all components of the reaction mixture has been examined in previous studies, little attention was paid to the possibility of using non water-soluble phenothiazines. Performing the reaction in organic media would greatly enhance the range of prospective substrates for HRP.

Our interest in the chemistry of heterocycles has recently led to the synthesis of some N-alkyl-phenothiazines as well as of the respective sulfoxides and sulfones [5]. Here, we report the HRP-catalyzed oxidation of these water-insoluble N-alkyl-phenothiazines in the presence of co-solvents (cf. Figure 1).

RESULTS AND DISCUSSION

As reported elsewhere [3,4], the oxidation of phenothiazines may be conveniently monitored as a decrease in absorbance at 300 nm as well as an increase in absorbance at 340 nm. The 340-nm peak is taken to indicate formation of the phenothiazine sulfoxide, which is one of the determined reaction products (cf. Figure 1). We therefore monitored our reaction mixtures at 340 nm. In addition,

a pink phenothiazine radical cation has been reported to transiently form upon oxidation of water-soluble phenothiazine derivatives [3,4]. We did indeed observe such pink intermediates in our own experiments, but no further data is reported on them here.

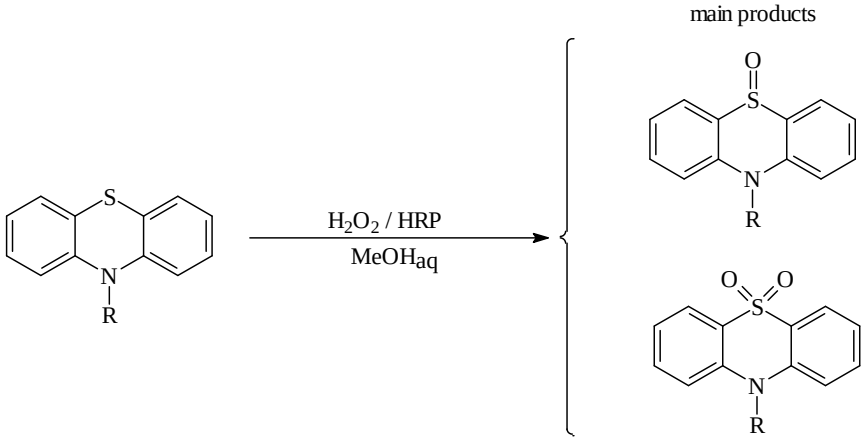

Figure 1. *HRP catalyzed oxidation of N-substituted phenothiazine derivatives (R = propyl, iso-propyl, amyl, methyl, ethyl)*

Figure 1 shows the change of absorbance during the HRP-catalyzed oxidation of N-propyl phenothiazine (PrFT) with 1.6 mM H_2O_2. Initial rates are shown in Table 1. This data allows the apparent K_m for PrFT to be estimated at 60 μM (a similar value was obtained by employing 160 mM H_2O_2).

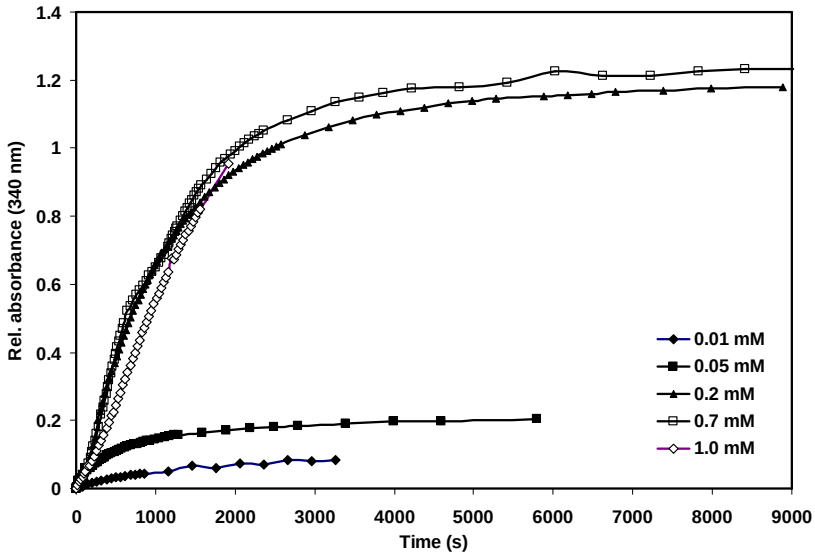

Figure 2. *The influence of phenothiazine concentration on the HRP catalyzed oxidation of PrFT (monitored as absorbance increase at 340 nm).*

Table 1.

Initial rate vs. PrFT concentrations.

c(PrFT)/mM	v/min^{-1}
0.01	0.0038
0.05	0.0102
0.2	0.041
0.7	0.0372
1.0	0.0312

The presence of phenothiazine sulfoxide as well as sulfone in the reaction mixtures used for kinetic studies was verified using thin layer chromatography against chemically synthesized authentic samples. Besides these two products, which were expected based on previous studies [3,4], we also found other three to four products, of unknown identity. This disagrees with previously published reports, that only sulfoxide and sulfone were products of the HRP-mediated oxidation of N-substituted phenothiazines. Possible explanations for this difference may include the presence of a co-solvent in our experiments, or differences in HRP preparations used. Perhaps relevant to this issue, we found that the sulfoxide and the sulfone of PrFT, under the same conditions as used for HRP-catalyzed oxidation of PrFT, did induce slight increases in the extinction at 340 nm, and unidentified products of sulfone and sulfoxide metabolism by HRP were found by thin layer chromatography.

Figure 3 and Table 2 indicate that the optimum pH for the HRP-catalyzed oxidation of PrFT is between 6 and 6.5.

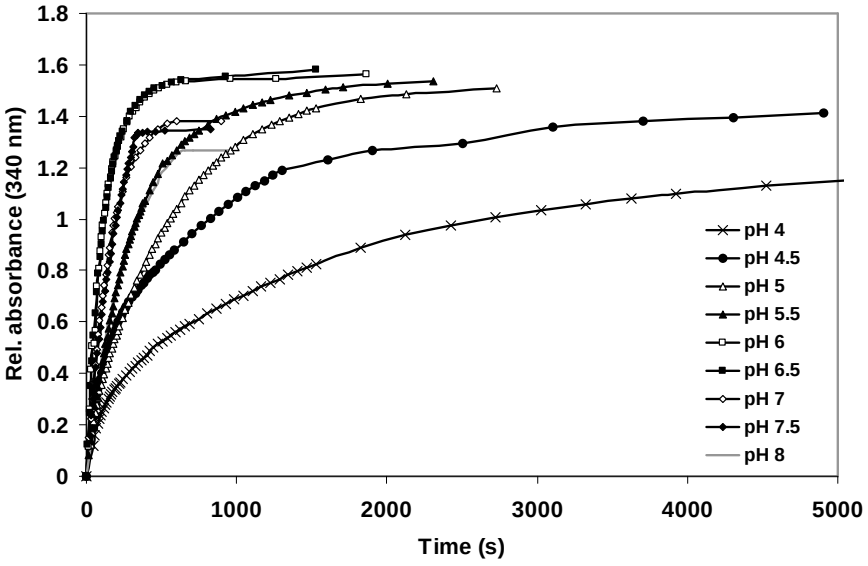

Figure 3. *pH dependency of PrFT oxidation catalyzed by HRP, monitored as absorbance increase at 340 nm.*

Table 2

Initial rate of the oxidation in dependence of the pH.

pH	v/min^{-1}
4	0.204
4.5	0.276
5	0.267
5.5	0.324
6	0.894
6.5	0.681
7	0.441
7.5	0.426
8	0.291

Figure 4 and Table 3 illustrate oxidation of various N-alkyl phenothiazines by HRP (methyl – Me, ethyl - Et, propyl- Pr, iso-propyl – iPr, amyl – Am). Notably, oxidation of PrFT was more than three times faster than that of iPrFT, although these two phenothiazine derivatives are structurally very similar.

Table 3

Initial rate of the oxidation depending for various N-alkyl-phenothiazines.

substrate	v/min^{-1}
MeFT	0.354514
EtFT	0.498
PrFT	0.531429
iPrFT	0.153943
AmFT	0.310971

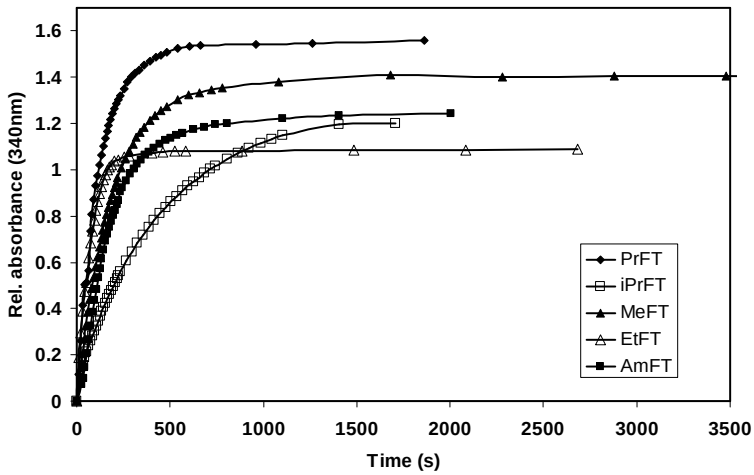

Figure 4. *HRP-catalyzed oxidation of different phenothiazines, monitored as absorbance increase at 340 nm.*

Since TLC analyses of the reaction mixtures used for kinetic studies revealed the presence of both the sulfoxide and sulfone as reaction products, we sought to optimize reaction conditions so as to obtain phenothiazine sulfoxide as the sole reaction product. Indeed, we found that simply adding the hydrogen peroxide in small increments over the course of 1 to 2 hours, allowed formation of PrFT as the sole reaction product and in quantitative yields. The product was extracted with ethyl acetate from the reaction mixture and analyzed independently to confirm identity with a chemically synthesized sample.

In conclusion, we have shown that water-insoluble N-alkyl phenothiazines are selectively oxidized by HRP in the presence of a co-solvent, and that the reaction may be applied for preparation in very good yields of N-alkyl phenothiazine sulfoxides.

EXPERIMENTAL

Horseradish peroxidase was an aqueous preparation, prepared and analyzed as described elsewhere [6].Phenothiazine derivatives were chemically prepared as previously described [5]. All other reagents were of analytical grade.

The HRP, extracted from horseradish was used as an aqueous solution to catalyze the oxidation of 2-propyl- (PrFT), 2-i-propyl- (iPrFT) 2-methyl- (MeFT), 2-ethyl- (EtFT) and 2-amylphenothiazine (AmFT) by H_2O_2. The oxidation of PrFT (0.01; 0.05; 0.2; 0.7; 1.0mM) by H_2O_2 (0.16; 1.6 mM) was done in a medium containing acetate buffer (pH 5.5), methanol (25-60% vol.), and HRP solution (0.1ml).

Alternatively, the reaction of PrFT (0.2mM) with H_2O_2 was carried out in different media containing acetate buffer (pH 4;4.5; 5; 5.5; 6) or phosphate buffer (pH 6.5; 7; 7.5; 8), methanol (52%vol.), and HRP solution (1μM).

This led to the optimization of the reaction, so that different derivatives of phenothiazine could be oxidized under the same conditions: Phenothiazine (0.2 mM), H_2O_2 (0.45mM), HRP (1 μM), methanol (52% vol.) in acetate buffer (pH 6).

All reaction mixtures were extracted with ethyl acetate in order to analyze the reaction products by TLC. In selected cases, melting points and the spectral data of reaction products were obtained, and shown to be identical with those previously described independently synthesized samples [5], confirming the reaction products.

ACKNOWLEDGEMENT: We are grateful to Mr. R. Dumitru for his help on the enzyme preparations.

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