Phytochemistry, Vol 24, No 5, pp 909–913, 1985
Printed in Great Britain

0031–9422/85 $3 00 + 0 00
Pergamon Press Ltd

TROPOLONE AS A SUBSTRATE FOR HORSERADISH PEROXIDASE*

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(*Revised received* 14 *September* 1984)

Key Word Index—Horseradish peroxidase, tropolone, yellow product

Abstract—Tropolone (2,4,6-cycloheptatrien-1-one), in the presence of hydrogen peroxide but not in its absence, can serve as a donor for the horseradish peroxidase catalysed reaction The product formed is yellow and is characterized by a new peak at 418 nm The relationship between the rate of oxidation of tropolone (ΔA at 418 nm/min) and various concentrations of horseradish peroxidase, tropolone and hydrogen peroxide is described The yellow product obtained by the oxidation of tropolone by horseradish peroxidase in the presence of hydrogen peroxide was purified by chromatography on Sephadex G-10 and its spectral properties at different pHs are presented The M_r of the yellow product was estimated to be *ca* 500, suggesting that tropolone, in the presence of horseradish peroxidase and hydrogen peroxide is converted to a tetratropolone

INTRODUCTION

Peroxidase is a haeme-containing enzyme that catalyses the oxidation of a variety of hydrogen donors (AH_2) in the presence of hydrogen peroxide [1, 2] In addition to this peroxidatic reaction, peroxidase in the absence of hydrogen peroxide but in the presence of oxygen can carry out oxidatic, catalytic and hydroxylation reactions [1] The data presented in this paper provide evidence that tropolone, in the presence of hydrogen peroxide can serve as a hydrogen donor (AH_2) for horseradish peroxidase (HRP)

RESULTS

Oxidation of tropolone to a yellow product by the HRP–hydrogen peroxide system

Tropolone, in the presence of 47 mM sodium phosphate buffer (pH 6 5), is characterized by an extremely high peak at 240 nm (data not shown), a high peak at 328 nm and two low peaks at 364 and 390 nm (Fig 1) The visible spectrum of tropolone was not changed by the addition of hydrogen peroxide alone (B) or HRP alone (C) (Fig 1) However, upon addition of both HRP and hydrogen peroxide, a yellow colour was detected optically immediately (hereafter referred to as the 'yellow product') and this was accompanied by a complete change in the original spectrum of tropolone (Fig 1 D) The control showed that the *A* of HRP alone, or of HRP plus hydrogen peroxide was negligible in the visible range (Fig 1 E and F) The data in Fig 2 were obtained with a constant amount of HRP and hydrogen peroxide (HRP–hydrogen peroxide system) and varying amounts of tropolone It can be seen that the yellow product is characterized by a peak at 418 nm The level of the yellow product (with a peak at 418 nm) produced by the oxidation of tropolone with the HRP–hydrogen peroxide system was dependent on the amount of tropolone present in the reaction mixture The yellow colour formed was stable for *ca* 20 min followed thereafter by a very slow decay

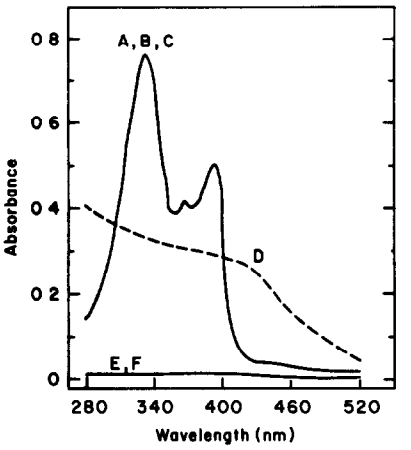


Fig 1 The oxidation of tropolone by HRP and hydrogen peroxide Reaction mixtures A–D included, in a total volume of 3 ml, 0 066 mM tropolone, 47 mM sodium phosphate buffer (pH 6 5) and the following additions A, none, B, 0 3 mM hydrogen peroxide, C, 10 μg HRP (added last), D, 0 3 mM hydrogen peroxide plus 10 μg HRP (added last) Reactions E and F included, in a total volume of 3 ml, 47 mM sodium phosphate buffer (pH 6 5) and 10 μg HRP (added last) Reaction F included, in addition, 0 3 mM hydrogen peroxide

Effect of concentration of tropolone, HRP and hydrogen peroxide on the rate of tropolone oxidation

The relationship between the rate of oxidation of tropolone (ΔA at 418 nm/min), on the one hand, and tropolone concentration (at constant HRP and hydrogen

*Contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel, No 1019-E, 1984 series

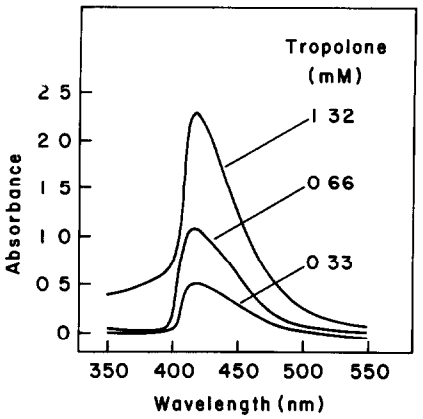


Fig 2 Visible absorption spectra of different amounts of tropolone oxidized by the HRP–hydrogen peroxide system The reaction mixture included, in a total volume of 3 ml, 3 3 mM hydrogen peroxide, 47 mM sodium phosphate buffer (pH 6 5), 1 μg HRP (added last) and tropolone as indicated The absorption spectrum was recorded 1 min after the addition of HRP against blanks that contained the above components but no hydrogen peroxide

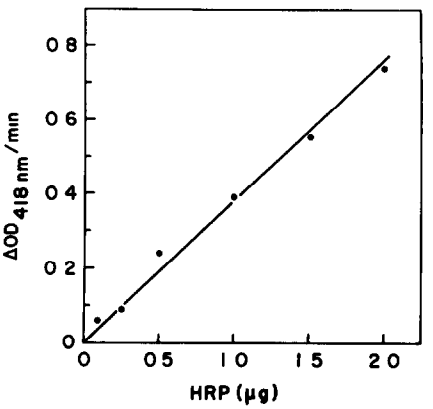


Fig 4 The rate of formation of the yellow product from tropolone at various concentrations of HRP The reaction mixture included, in a total volume of 3 ml, 6 7 mM tropolone, 3 3 mM hydrogen peroxide, 47 mM sodium phosphate buffer (pH 6 5), and HRP (added last) as indicated Change in A at 418 nm as a function of time was recorded and was linear for at least 120 sec (data not shown) Peroxidase activities (ΔA at 418 nm/min) were computed from the graphs obtained

peroxide concentrations), HRP concentration (at constant tropolone and hydrogen peroxide concentrations) and hydrogen peroxide concentration (at constant tropolone and HRP concentrations), on the other hand, was examined, the results are summarized in Figs 3–5, respectively

The data demonstrate linearity between tropolone oxidation and tropolone concentration at least up to 10 mM tropolone (Fig 3) Identical results were obtained using 3 3 or 6 6 mM hydrogen peroxide with 1 μg HRP Linearity was also observed at least up to 2 μg HRP (Fig 4) and *ca* 0 1 mM hydrogen peroxide (Fig 5) Maximum tropolone oxidation was observed at *ca* 2 mM hydrogen peroxide At much higher concentrations of hydrogen peroxide, the rate of tropolone oxidation was inhibited (Fig 5 inset), probably due to inactivation of HRP by such relatively high hydrogen peroxide concentrations [3]

Comparison of the sensitivity of assaying peroxidase activity using tropolone vs guaiacol as the substrate

The data presented so far establish that tropolone serves as a substrate for HRP and that, in the presence of hydrogen peroxide, tropolone is oxidized by HRP to a yellow product with a peak at 418 nm

Peroxidase activity can be assayed using a variety of compounds that can serve as substrates [4–7] The need to

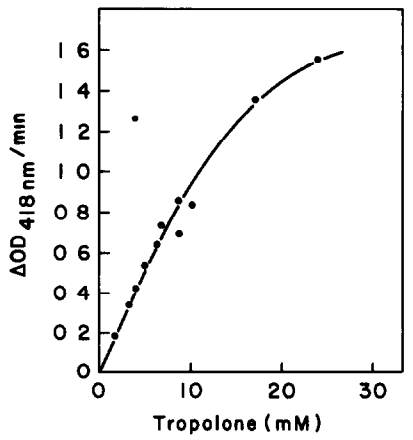


Fig 3 The rate of oxidation of different concentrations of tropolone at fixed HRP and hydrogen peroxide concentrations The reaction mixture included, in a total volume of 3 ml, 47 mM sodium phosphate buffer (pH 6 5), tropolone as indicated, 3 3 or 6 6 mM hydrogen peroxide and 1 μg HRP (added last) Identical results were obtained with 3 3 or 6 6 mM hydrogen peroxide

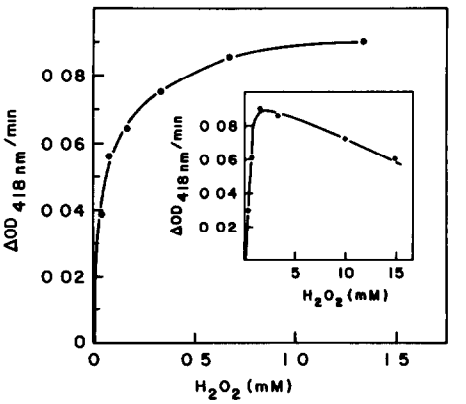


Fig 5 Effect of hydrogen peroxide on the rate of tropolone oxidation by the HRP–hydrogen peroxide system The reaction mixture included, in a total volume of 3 ml, 3 3 mM tropolone, 47 mM sodium phosphate buffer (pH 6 5), 100 μg HRP (added last) and hydrogen peroxide as indicated

find very sensitive assays for peroxidase led scientists to look for substrates that have a high E_m when oxidized Thus, relatively recently, 3,3'-diaminobenzidine [5], dicarboxidine [6] and 3,5,3',5'-tetramethylbenzidine [7] were shown to be sensitive substrates Herzog and Fahimi [5] showed that 3,3-diaminobenzidine was a substrate of high sensitivity, *o*-dianisidine of medium sensitivity and guaiacol of low sensitivity for assaying peroxidase

It was, therefore, of interest to determine whether tropolone will allow detection of lower concentrations of HRP compared with when other compounds are used as substrates However, the data presented in Fig 6 illustrate that tropolone is *ca* eight-fold less sensitive a substrate for assaying peroxidase than guaiacol

Purification of the yellow product by chromatography on a Sephadex G-10 column

In order to better characterize the yellow product obtained by the action of HRP and hydrogen peroxide on tropolone, it was necessary to separate the product from the rest of the components present in the reaction mixture This was achieved by chromatography on a column of Sephadex G-10 as described in the legend to Fig 7 The A at 280 nm was recorded as an arbitrary wavelength to aid in locating the position of elution of the various components rather than as an aid in their identification

Three regions were resolved A–C, with A eluted at the void volume of the column (V_0) (120 ml), and B and C at elution volumes of 150 and 280 ml, respectively (Fig 7)

HRP was eluted at V_0 Fractions eluted in region A sometimes had a slight yellowish tint and were characterized by a spectrum with non-resolved peaks (data not shown) Fractions eluted in region B were deep yellow and were characterized by a high A at 240 nm, a shoulder at 280 nm, a trough at 340 nm, and peaks at 390 and 418 nm (Fig 7) The peak fraction in region B will be referred to as the 'purified yellow product' The spectrum of the purified yellow product (Fig 7) was unchanged for at least 4 hr Fractions eluted in region C were colourless and were characterized by a spectrum identical to that of untreated tropolone shown in Fig 1 These fractions inhibited mushroom tyrosinase activity and also, upon addition of copper sulphate, formed a copper–tropolone complex characterized with a high peak at 320 nm and a small peak at 378 nm Moreover, when samples of fractions eluted in region C were incubated (in a total volume of 3 ml) in the presence of 47 mM sodium phosphate buffer (pH 6 5), 0 67 mM hydrogen peroxide and 20 μg HRP, a yellow product was formed similar to that shown in Fig 2 It can, therefore, be concluded that untreated tropolone was eluted in region C

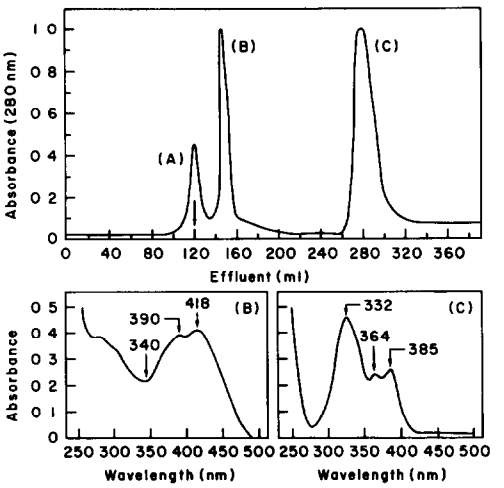


Fig 7 Purification of the yellow product by chromatography on a Sephadex G-10 column (Upper) A reaction mixture containing, in a total volume of 3 ml, 6 7 mM tropolone, 47 mM sodium phosphate buffer (pH 6 5), 0 67 mM hydrogen peroxide and 1 μg horseradish peroxidase was incubated for 5 min at room temperature The mixture was then applied to a Sephadex G-10 column (2 6 × 60 cm) and the column was eluted with water Fractions of 1 5 ml were collected in an ISCO No 328 fraction collector attached to a UA-5 monitor and the A at 280 nm was recorded (Lower) Absorption spectra of peak fractions from regions B (yellow) and C eluted off the Sephadex G-10 column

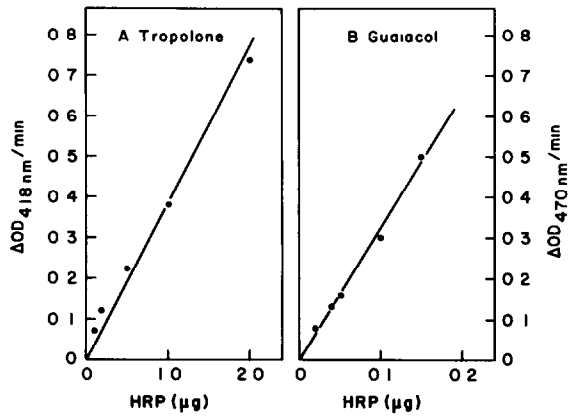


Fig 6 Comparison between the sensitivity of assaying peroxidase activity with tropolone (A) vs guaiacol (B) as the substrate (A) The reaction mixture included, in a total volume of 3 ml, 6 7 mM tropolone, 47 mM sodium phosphate buffer (pH 6 5), 3 3 mM hydrogen peroxide and HRP (added last) as indicated (B) The reaction mixture included, in a total volume of 3 ml, 6 7 mM guaiacol, 47 mM sodium phosphate buffer (pH 6 5) and HRP (added last) as indicated The change in A was followed at 418 and 470 nm in (A) and (B), respectively In all cases, the rate of increase in A was linear for at least 120 sec (data not shown) Activity was computed from the initial linear portion of the curves obtained

It was of interest to know if additional oxidation products are formed by the action of the HRP–hydrogen peroxide system on tropolone as a result of non-enzymatic polymerization In order to test this, a reaction mixture identical to that used in Fig 7 (containing tropolone, sodium phosphate buffer, pH 6 5, HRP and hydrogen peroxide) was left for 20 hr at room temperature and then chromatographed on a Sephadex G-10 column It was found that the same products were formed when tropolone was incubated with the HRP–hydrogen peroxide system for 5–10 min or for 24 hr

Characterization of the purified yellow product obtained by the action of the HRP–hydrogen peroxide system on tropolone

The effect of pH on the visible spectrum of the purified

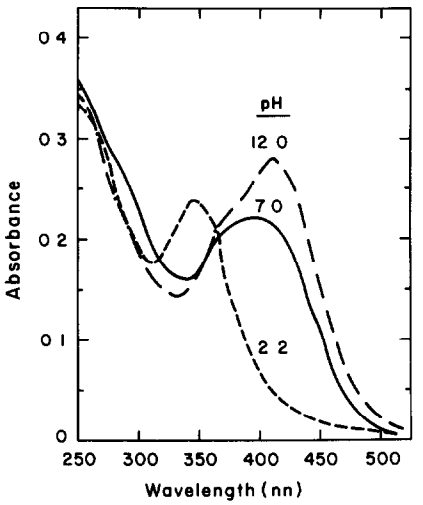


Fig 8 Effect of pH on the absorption spectra of the purified yellow product formed by the action of HRP and hydrogen peroxide on tropolone The reaction mixture included, in a total volume of 1 ml, 0 5 ml of the yellow product purified by Sephadex G-10 chromatography with 5 mM sodium citrate at pH 2 (– – –), 5 mM sodium carbonate at pH 12 (– – –), or water (pH 7) (——)

yellow product is illustrated in Fig 8 The visible spectra at pH 7 and 12 are similar, with a broader peak around 395 nm at pH 7 and a sharper peak at 416 nm at pH 12 However, at pH 2 2, the peak at 390–416 nm completely disappeared and, instead, a new one appeared at 340 nm On the basis of the determination of the elution volume of compounds of known M_r (such as ATP, tryptophan, FMN, tropolone) on a Sephadex G-10 column identical to that used in Fig 7 (not shown), the M_r of the yellow oxidation product of tropolone was estimated to be a little less than 500

Effect of pH on the spectrum of tropolone

In the course of our work we noted that when a colourless solution of tropolone is made alkaline, it becomes yellow Such an observation was reported by Cook *et al* [8], who showed that in 0 5 M sodium hydroxide, tropolone has peaks at 329 and 392 nm We examined the effect of pH on tropolone in aqueous solutions and found the following at pH 5 5 and 7 0, tropolone was colourless and characterized, in both cases, by a very high peak at 234 nm and a small peak at 324 nm (data not shown) At pH 11 5, the peak at 234 nm remained unchanged, the peak at 324 nm increased, and a new peak was observed at 390 nm

It can, therefore, be concluded that the spectrum of the yellow solution of tropolone, at pH 11 5 as described above, and that of the purified yellow product, obtained by the action of HRP on tropolone in the presence of hydrogen peroxide (Fig 8), are different The yellow colour seen when tropolone is made alkaline may, perhaps, be due to the auto-oxidation of tropolone to a yellow tropaquinone This possibility was not explored

DISCUSSION

As pointed out in the Introduction, peroxidase can carry out the following types of reactions (1) peroxidatic oxidation, (2) oxidatic, (3) catalytic, and (4) hydroxylation [1] The peroxidatic oxidation reaction occurs in the presence of hydrogen peroxide when a variety of compounds, such as guaiacol, tyrosine, *o*-dianisidine, or 3,5,3′,5′-tetramethylbenzidine, serve as the substrate (AH_2) [1, 5–7] These substrates are polymerized in the course of this reaction The catalatic reaction occurs between two molecules of hydrogen peroxide in the absence of an AH_2, while the oxidatic and hydroxylation reactions occur in the absence of hydrogen peroxide, but require oxygen instead

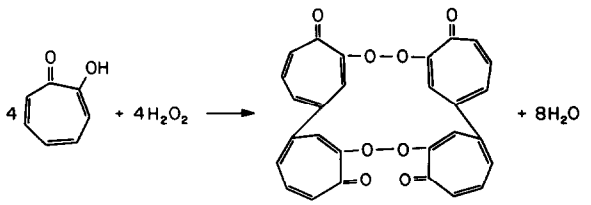
The data presented in this paper show that tropolone is oxidized to a yellow product by HRP only in the presence of hydrogen peroxide, namely that HRP acts on tropolone plus hydrogen peroxide in a peroxidatic manner The rate of tropolone oxidation to a yellow product was linearly related to tropolone concentration, HRP concentration and to low hydrogen peroxide concentrations At concentrations of hydrogen peroxide above 0 1 mM, the reaction was inhibited, probably due to inactivation of HRP as a result of such concentrations of hydrogen peroxide [3]

Substrates, such as guaiacol, *o*-dianisidine and 3,3-diaminobenzidine, are, in descending order, more sensitive substrates for assaying peroxidase [5] In view of our finding (Fig 6) that tropolone is less effective than guaiacol, we do not recommend tropolone as a substrate for detecting very low concentrations of peroxidase However, in view of our finding showing that tropolone is a very effective inhibitor of mushroom tyrosinase [9] and our present data showing that it serves as a substrate for HRP, we recommend tropolone as an aid in differentiating between these two enzymatic activities [10]

Dimeric and tetrameric oxidation products are known to be formed by the action of the HRP–hydrogen peroxide system on different substrates [1, 4, 11] Guaiacol is one of the most commonly used substrates for peroxidase Peroxidase, in the presence of hydrogen peroxide, oxidizes guaiacol to a red-brown product It is generally agreed that a tetraguaiacol is the main oxidation product of this reaction [1] However, several investigators showed that, besides tetraguaiacol, dimeric oxidation products of guaiacol, such as 2,2′-dihydroxy- and 3,3′-dimethoxydiphenyl are also formed [4, 12] Lindgren [11] attributed the red colour to diphenoquinone residues formed from some guaiacol units by oxidation, rather than to the tetramer molecule

Gross and Sizer [13] showed that monohydroxyphenols, such as tyrosine and tyramine, can be acted upon by the HRP–hydrogen peroxide system A dityrosine and a dityramine were shown to be formed in the course of the reaction [13] Similarly, homovanillic acid, in the presence of hydrogen peroxide, is dimerized by peroxidase into a dihomovanillic acid [14, 15]

The data presented show that tropolone can serve as a hydrogen donor for HRP in the presence of hydrogen peroxide as a hydrogen acceptor forming a yellow product characterized by an *A* peak at 418 nm Using Sephadex G-10 chromatography, the M_r of the yellow product was estimated to be *ca* 500 Although our calibration of the Sephadex G-10 column was not fully reproducible in different runs, we feel it is safe to suggest that a tetra-



Scheme 1

tropolone is the main product formed by the action of the HRP–hydrogen peroxide system on tropolone The tetratropolone is likely to be formed as shown in Scheme 1

Tetraguaiacol has been assigned two different structures, a symmetrical and a non-symmetrical one [4] By analogy, the structure of tetratropolone can be assigned similar alternative structures We do not have data to support one in preference to the other

EXPERIMENTAL

Materials Horseradish peroxidase (type VI) and tropolone were obtained from Sigma, H_2O_2 from Merck, and Sephadex G-10 from Pharmacia All other chemicals were reagent grade

Peroxidase activity (a) *Standard assay using guaiacol as the substrate* Peroxidase activity was assayed in a reaction mixture which included, in a total vol of 3 ml, an aliquot of mix M [freshly prepared and mixed 100 ml NaPi buffer (pH 6 5), 10 ml 1% guaiacol in 50% EtOH and 10 ml 0 3% H_2O_2] and an aliquot of the enzyme The A at 470 nm was recorded and the activity (ΔA at 470 nm/min) was calculated from the linear portion of the curve during the first 60 sec of the reaction

(b) *Assay using tropolone as the substrate* The reaction mixture included, in a total vol of 3 ml, tropolone, NaPi, H_2O_2 and HRP (added last) as indicated in the figure legends The A at 418 nm was recorded and the activity (ΔA at 418 nm/min) estimated from the initial linear portion of the curves obtained

Spectrophotometric measurements were carried out on a Varian 635 spectrophotometer equipped with a recorder

Chromatography Carried out on a column of Sephadex G-10 as described in the legend to Fig 7

H_2O_2 *concentration* Estimated at 240 nm using an E_m (240 nm) of 43 6/M cm

Acknowledgement—This research was supported by a grant from the United States–Israel Binational Agricultural Research and Development Fund (BARD)

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