Purification and characterization of rat intestinal peroxidase

Its activity towards 2-t-butyl-4-methoxyphenol (BHA)

Massimo VALOTI,* Laura DELLA CORTE,† Keith F. TIPTON‡ and Giampietro SGARAGLI*

*Centro di Ricerca Interdipartimentale sul Metabolismo dei Farmaci Psicotropi, Istituto di Scienze Farmacologiche, Università di Siena, Siena, Italy, †Dipartimento di Farmacologia Preclinica e Clinica 'M. Aiazzi-Mancini', Università di Firenze, Firenze, Italy, and ‡Department of Biochemistry, Trinity College, Dublin 2, Ireland

Impure preparations of rat intestinal peroxidase were shown to aggregate at low ionic strengths and to disaggregate at higher values. This aggregation was accompanied by a decrease in specific activity, which could lead to hysteretic behaviour of reaction progress curves. Advantage was taken of this reversible aggregation to obtain a relatively pure extract, which was subsequently purified to apparent homogeneity by affinity chromatography on concanavalin A–Sephadex followed by hydrophobic chromatography. The purified enzyme did not show the ionic-strength-dependent aggregation behaviour, behaving as a monomer of M_r 50 000. The purified enzyme was shown to catalyse the peroxidatic conversion of the commonly used antioxidant 2-t-butyl-4-methoxyphenol (butylated hydroxyanisole, BHA) to form 3,3'-di-t-butyl-2,2'-dihydroxy-5,5'-dimethoxybiphenyl, with a K_m value of $176 \mu\text{M}$ and a maximum velocity of $8 \mu\text{mol/min per mg}$. The specificity constant, $k_{\text{cat.}}/K_m$, for this substrate was similar to that shown towards the substrate guaiacol.

INTRODUCTION

Although considerable peroxidase (EC 1.11.1.7) activity is associated with mammalian intestine (Neufeld *et al.*, 1958), it is still unclear whether it is intrinsic to the intestinal cells or whether it arises from eosinophil infiltration (Rytömaa & Teir, 1961; Kimura *et al.*, 1983; Keeping & Lyttle, 1984). However, Venkatachalan *et al.* (1970) have shown peroxidase activity in the mucus-secreting cells of the cryptae in rat large intestine.

Whatever the origin of this enzyme, however, it might be expected to affect the behaviour of some ingested xenobiotics. Previous work has shown, for example, that the commonly used antioxidant 2-t-butyl-4-methoxyphenol (butylated hydroxyanisole, BHA) is oxidized by peroxidase from horseradish or mammalian systems to the dimer 3,3'-di-t-butyl-2,2'-dihydroxy-5,5'-dimethoxybiphenyl (di-BHA) via the generation of a free-radical intermediate, which was detected by e.p.r. spectroscopy in conditions *in vitro* (Sgaragli *et al.*, 1980). This pathway has been subsequently shown to occur in everted intestinal preparations (Della Corte *et al.*, 1984) and *in vivo* in the rat (Guarna *et al.*, 1983). It has been suggested that oxidation of this compound by intestinal peroxidase might partially account for its lower toxicity on oral administration, which is some two orders of magnitude lower than after intraperitoneal administration (Della Corte & Sgaragli, 1984).

Peroxidases, however, can catalyse the oxidation of phenol or amine compounds to reactive species which can bind to both protein and DNA (Smart & Zannoni, 1984; Epe & Metzler, 1985; Subrahmanyam & O'Brien, 1985; Auclair *et al.*, 1986). Thus, in certain tissues where peroxidase activities are high, peroxidatic oxidation of xenobiotics may play a significant role in induction of toxicity.

Partly purified (Sgaragli *et al.*, 1984b) and apparently

homogeneous preparations (Stelmaszyńska & Zgliczyński, 1971; Kimura & Jellinck, 1982) of intestinal peroxidase have been obtained, but few studies have been carried out on their kinetic behaviour.

The present paper reports the purification and characterization of the enzyme from rat intestine and some studies on its interactions with BHA. During the course of this study an ionic-strength-dependent aggregation–disaggregation of the enzyme that had previously been noted in our laboratory (Sgaragli *et al.*, 1984a) was further characterized and exploited in the enzyme purification.

MATERIALS AND METHODS

Materials

Sephacryl S-300, concanavalin A–Sephadex, phenyl–Sephadex, Sephadex and Blue Dextran 2000 were purchased from Pharmacia A.B. (Uppsala, Sweden). Aldolase, chymotrypsinogen A, cytochrome *c*, glyceraldehyde-3-phosphate dehydrogenase, catalase, horseradish peroxidase, lactoperoxidase, Tris base and α -methyl mannoside were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ovalbumin and bovine serum albumin were from Boehringer A.G. (Mannheim, Germany). Acrylamide, *NN'*-methylenebisacrylamide, *NNN'N'*-tetramethylethylenediamine and Coomassie Brilliant Blue were from Bio-Rad (Richmond, CA, U.S.A.). All other chemicals, the purest available, were purchased from Merck A.G. (Darmstadt, Germany). BHA was recrystallized before use, and di-BHA was prepared as previously described (Sgaragli *et al.*, 1980).

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All procedures were carried out at 4°C unless otherwise stated.

Abbreviations used: BHA, 2-t-butyl-4-methoxyphenol (butylated hydroxyanisole); di-BHA, 3,3'-di-t-butyl-2,2'-dihydroxy-5,5'-dimethoxybiphenyl.

1: Homogenization and extraction. Rat intestines (approx. 70 g) were removed and washed in 0.9% NaCl solution, cut into small pieces and homogenized in 300 ml of 0.25 M-sucrose with a Polytron, followed by a Potter-Elvehjem homogenizer with a Teflon pestle. After filtration through muslin, the homogenate was centrifuged at 35000 *g* for 30 min. The pellet was homogenized in 180 ml of 1% Triton X-100 in 5 mM-Tris/HCl buffer, pH 7.0, stirred for 1 h and centrifuged at 35000 *g* for 30 min. The pellet was then homogenized in 150 ml of 10 mM-acetate buffer, pH 5, containing 0.05 M-BaCl₂, and centrifuged at 35000 *g* for 30 min. To solubilize the enzyme, the pellet was homogenized in 110 ml of 0.5 M-BaCl₂ in 10 mM-acetate buffer, pH 5.0, and centrifuged at 35000 *g* for 30 min. The supernatant was retained.

2: Gel filtration. The BaCl₂ extract was concentrated by ultrafiltration with a PM10 filter and loaded on a Sephacryl S-300 column (4 cm × 58 cm) previously equilibrated with 0.5 M-BaCl₂ in 10 mM-acetate buffer, pH 5.0. The enzyme was eluted with the same buffer. The fractions (10 ml) with peroxidase activity were collected and concentrated as above. Then 10 mM-acetate buffer, pH 5.0, was added to dilute the BaCl₂ to 0.05 M, and the suspension was centrifuged at 100000 *g* for 45 min. The pellet was suspended in 25 ml of 10 mM-acetate, pH 5.0, containing 0.5 M-BaCl₂ and 0.02% *N*-cetyl-*NNN*-trimethylammonium bromide (buffer A) and stirred overnight.

3: Affinity chromatography. The peroxidase was passed through a small column (3 ml) of concanavalin A-Sepharose equilibrated with buffer A. The column was washed with buffer A until the *A*₂₆₀ and *A*₂₈₀ of the effluent decreased to zero. The peroxidase activity was then eluted with buffer A containing 0.2 M- α -methyl mannose.

4: Hydrophobic chromatography. The active fractions were pooled and loaded on a column (3 ml) of phenyl-Sepharose. The column was washed with buffer A until the *A*₂₈₀ of the effluent was zero. The peroxidase activity was eluted with buffer A containing 25% (v/v) glycerol. The fractions containing peroxidase were collected and diluted 3-fold with buffer A, and dialysed overnight against buffer A containing 25% glycerol.

Assay of peroxidase activity

The method was based on that of Chance & Mahely (1955). The reaction mixture contained, in a total volume of 1.5 ml, 33.3 mM-Tris/HCl buffer, pH 8.0, 0.33 mM-H₂O₂, 6.6 mM-guaiacol and the enzyme sample. The peroxidatic oxidation of guaiacol was measured at 37 °C by monitoring the increase in *A*₄₇₀. One unit of enzyme activity is defined as the amount catalysing the formation of 1 μ mol of tetraguaiacol in 1 min, based on a molar absorption coefficient of the latter of 26600 l · mol⁻¹ · cm⁻¹.

Activity towards BHA was determined in a reaction mixture containing, in a total volume of 1.0 ml, 50 mM-Tris/HCl buffer, pH 8.0, 0.1 mM-H₂O₂, BHA and enzyme. After incubation at 37 °C for various times, the reaction was stopped by the addition of 0.8 ml of aq. 25% (v/v) NH₃ plus 0.5 ml of dimethyl sulphoxide, and the *A*₃₄₀ of the mixture was determined against a blank containing the initial concentration of BHA. The BHA

was stored as a stock 5.0 mM solution in dimethyl sulphoxide. The concentrations of dimethyl sulphoxide present in the assay mixture were shown to have no significant effects on the activities of peroxidase with guaiacol as substrate. A standard assay time of 2 min was used, and it was shown that the time course of the reaction was linear for this period under all conditions used in this work.

Protein concentration was determined by the method of Bensadoun & Weinstein (1976), with bovine serum albumin as the standard.

Gel filtration

The *M_r* of the enzyme was determined by gel filtration through a 60 cm × 20 cm column of Sephadex G-150. The conditions used were the same as those described for the enzyme purification. The standard proteins used to calibrate the column were: cytochrome *c* (*M_r* 13300), chymotrypsinogen A (25000), horseradish peroxidase (40000), ovalbumin (43500), bovine serum albumin (67000), lactoperoxidase (77500) and aldolase (158000). The *M_r* values in parentheses are taken from Dixon & Webb (1979).

Electrophoresis

Electrophoresis in the presence of SDS was carried out in slab gels containing 10% acrylamide as described by Laemmli (1970). Enzyme samples were precipitated with trichloroacetic acid and boiled in the presence of 5% (w/w) 2-mercaptoethanol before application to the gels. After electrophoresis, protein bands were stained with Coomassie Brilliant Blue. The subunit *M_r* of peroxidase was determined by comparison with the mobilities of the same standard proteins as used in the gel-filtration study, except that chymotrypsinogen A and aldolase were replaced by glyceraldehyde-3-phosphate dehydrogenase and catalase, which were taken to have subunit *M_r* values of 36000 and 57500 respectively (Dixon & Webb, 1979).

T.l.c.

This was carried out on pre-coated 0.25 mm-thick 5 cm × 20 cm silica-gel GF₂₅₄ plates (Merck). Chromatograms were usually developed to a distance of 15 cm in the dark and at room temperature with methylene chloride as the solvent. After drying, the plates were examined under u.v. light to allow the separated reactants to be seen. The *R_f* values of BHA and di-BHA were 0.52 and 0.90 respectively, and those of guaiacol and tetraguaiacol were 0.61 and 0.75 respectively.

RESULTS AND DISCUSSION

Enzyme purification and characterization

Table 1 shows the results of a typical purification procedure. The repeated suspension and centrifugation of the intestinal homogenate (step 1) gave a considerable decrease in protein concentration, which facilitated later stages of the purification procedure. The strong binding to concanavalin A-Sepharose and its elution by α -methyl mannoside are consistent with the enzyme being a glycoprotein, as has been shown for peroxidases from several other sources (Lyttle & De Sombre, 1977; De Sombre & Lyttle, 1978; Keeping *et al.*, 1981; Kimura & Jellinck, 1982; Olsen & Little, 1983). Fresh batches of

Table 1. Purification of peroxidase from rat intestine

Purification step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification (fold)	A_{413}/A_{280}
Homogenate	300	525	4723	0.11	100	1	—
1. BaCl_2 extract	12	312	180	1.73	59.4	15.7	—
2. Sephacryl S-300	20	175	12.18	14.37	33.3	130.6	0.18
3. Concanavalin A-Sepharose	12.5	65	1.08	60.18	12.3	547	0.56
4. Phenyl-Sepharose	5	51.6	0.392	131.53	9.8	1195	0.73

concanavalin A-Sepharose were found to bind the enzyme so tightly that elution could not be achieved even at concentrations of α -methyl mannoside as high as 1.0 M. However, on subsequent uses the material behaved reproducibly, as described in the purification procedure.

The purified enzyme was homogeneous by the criterion of SDS/polyacrylamide-gel electrophoresis. The M_r determined by this procedure was 48000, whereas gel filtration gave a value of 50000 for the native enzyme. These values are similar to those reported for other preparations of the enzyme from intestine (Stelmaszyńska & Zgliczyński, 1971; Kimura & Jellinck, 1982; De *et al.*, 1986) and other sources (De Sombre & Lyttle, 1978; Olsen & Lyttle, 1981; Hosoya & Saito, 1981; De & Banerjee, 1986).

The crude (after step 2 of the purification procedure) and purified enzyme preparations showed similar initial rates of guaiacol oxidation at pH 8.0, but purification resulted in a shift of the pH/activity curve to lower pH values (Fig. 1). In view of the complicated effects of salt concentration on the activity of the crude preparation of the enzyme, discussed below, no attempts were made to

Fig. 1. Dependence of the initial velocity of guaiacol oxidation on pH

The activities of the pure (●) and the crude (○; after step 2 of the purification procedure) preparations of peroxidase were assayed as described in the text, but in the presence of 33.3 mM-sodium acetate buffer for the pH range 4–6.5 and 33.3 mM-Tris/HCl for the pH range 6.5–9.5.

correct for any effects of ionic-strength changes in these experiments. The effects of pH were reversible by adjusting back to pH 8.0, in the range pH 4–9. K_m values for guaiacol and H_2O_2 determined for the crude preparation of the enzyme were 23 mM and 100 μM respectively, consistent with the values obtained for the purified preparation (see Table 2).

Fig. 2 shows the absorbance spectra of the pure enzyme and a sample obtained after step 2 of the purification procedure. The former preparation showed the typical haem absorbance at 413 nm. Native peroxidases purified from intestine (Kimura & Jellinck, 1982), stomach (De & Banerjee, 1986), uterus (Olsen *et al.*, 1982) and milk (Morell, 1954; Carlström, 1969) show a characteristic Soret absorbance maximum at 412 nm. Changes of the Soret peak from 412 nm to a longer wavelength (414 or 415 nm) have been described

Table 2. Kinetic constants for guaiacol and BHA as substrates for purified rat intestinal peroxidase

Substrate	$V_{\max}$ (units/mg of protein)	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1} \cdot \text{mM}^{-1}$)
Guaiacol	244	21	9.8×10^3	4.67×10^2
BHA	8.0	0.18	1.12×10^2	6.36×10^2

Fig. 2. Absorption spectra of rat intestinal peroxidase

The spectra of purified (—) and crude (after step 2 of the purification procedure; ---) preparations are shown. The left-hand absorbance scale refers to the absorbance of the purified preparation above 500 nm.

to occur with pig intestinal peroxidase (Kimura & Yamasaki, 1978), rat intestinal peroxidase (Kimura & Jellinck, 1982; De *et al.*, 1986) and lactoperoxidase (Kimura & Yamasaki, 1978) when the enzyme was suspended in the presence of high Cl^- concentrations. It is possible that the small change of the Soret peak of the pure enzyme from 412 to 413 nm was dependent on the relatively high Cl^- concentration used for the enzyme purification and storage. For the pure enzyme, the A_{413}/A_{280} ratio was 0.73, a value within the range reported for apparently pure preparations of this enzyme from a number of mammalian sources (Stelmaszyńska & Zgliczyński, 1971; Olsen & Little, 1981; Jörg *et al.*, 1982; Kimura & Jellinck, 1982; Olsen & Little, 1983; De & Banerjee, 1986). At higher wavelengths, small absorbance peaks at 510 nm and 640 nm were evident (Fig. 2), similar to those reported for the enzyme from rat uterus (Olsen *et al.*, 1982). The absorbance spectrum of the less pure preparation (after step 2) was dominated by a strong band at 260 nm. This contaminating material was removed by chromatography on concanavalin A-Sepharose (step 3).

Aggregation behaviour

Aggregation of impure preparations of the enzyme at low ionic strengths has been previously reported (Sgaragli *et al.*, 1984a). Fig. 3 compares the behaviour of a partly purified preparation of the enzyme (after step 2 of the procedure) on gel filtration in the presence of 0.5 M- and 0.05 M- BaCl_2 . At this stage of the purification procedure, this behaviour was fully reversible and the enzyme could be taken through several aggregation-disaggregation cycles by adjusting the BaCl_2 concentration. No such behaviour was, however, observed after step 3 of the purification procedure, when the elution volume corresponded to that of the lower- M_r species at both salt concentrations. This behaviour of the crude enzyme preparations may be related to the material absorbing at 260 nm which is removed in step 3 of the purification procedure, but further work would be necessary to show whether that was the case.

The purified enzyme was shown to pass through an Amicon PM-100 membrane (approx. exclusion $M_r = 100\,000$) on ultrafiltration in solution in either 0.05 M- or 0.5 M- BaCl_2 . In contrast, the enzyme preparation after step 2 of the purification procedure passed through this membrane in 0.5 M- BaCl_2 , but was retained by it in 0.05 M- BaCl_2 . Similar behaviour was observed with NaCl, in place of BaCl_2 at similar high and low ionic strengths ($I = 1.5$ and 0.15 respectively). However, it was less effective in extracting the enzyme. Although CaCl_2 and SrCl_2 were as effective as BaCl_2 in extracting the enzyme, the last of these salts was preferred during the initial extraction and purification stages, since the enzyme was found to lose activity on storage in the other salts. After step 1 of the purification procedure, over 50% of the activity was lost after storage of the enzyme in buffers containing NaCl, CaCl_2 or SrCl_2 ($I = 1.5$) for 10 days at -20°C . In contrast, preparations stored in the same concentration of BaCl_2 showed an increase in activity, to about 120% of that originally present, over 2 days of storage at -20°C , and the activity then remained constant for at least 30 days. As discussed below, this initial gain in activity probably resulted from disaggregation of the enzyme to the higher-specific-activity form. Instability of crude preparations of

Fig. 3. Gel filtration of rat intestinal peroxidase on Sephacryl S-300

Samples of partly purified preparations, after step 2 of the purification procedure, were subjected to gel filtration in the presence of 10 mM-acetate buffer, pH 5.0, containing (a) 0.5 M- BaCl_2 or (b) 0.05 M- BaCl_2 . Enzyme activity ($\blacktriangle$) and A_{280} ($\triangle$) were monitored in the effluent.

peroxidase in CaCl_2 at -20°C has also been reported by Kimura & Jellinck (1982).

The aggregation of crude preparations of the enzyme that occurs at the lower concentration (0.05 M) of BaCl_2 is associated with a decrease in enzyme activity. This can result in reaction progress curves which show apparently hysteretic behaviour, as shown in Fig. 4. Such behaviour, which was freely reversible by changing the BaCl_2 concentration (see Fig. 4), was not observed with the purified enzyme. Fig. 5 shows the time courses of the changes in specific activity after dilution of enzyme samples to change the BaCl_2 concentration. Crude enzyme preparations (after step 2 of the purification procedure) which had been allowed to equilibrate in either 0.5 M- or 0.05 M- BaCl_2 , however, gave linear dependencies of initial velocity on enzyme concentrations in the range 0–3 μg of protein in the assay. When preparations of the crude and purified enzyme that had been equilibrated in 0.5 M- BaCl_2 were assayed in the presence of BaCl_2 concentrations of 2 mM or less, the initial velocities were found to be proportional to enzyme concentration, but the specific activities in 0.05 M- BaCl_2 were $25 \pm 3\%$ less than those of preparations assayed in the presence of 0.5 M- BaCl_2 . The non-linearity of the reaction progress curves at longer incubation times, shown in Fig. 4, was not due to the effects of BaCl_2 in the

Fig. 4. Time courses of the oxidation of guaiacol by rat intestinal peroxidase

Activities were determined with a partly purified preparation (after step 2 of the purification procedure; *a*) or a pure preparation (*b*). Assays with the pure enzyme contained $0.15 \mu\text{g}$ of protein, whereas those with the cruder preparation contained $0.8 \mu\text{g}$. Curves 'a' show progress curves of preparations suspended in 0.5 M-BaCl_2 and assayed in buffer containing the same BaCl_2 concentration. For curves 'b' the enzyme samples in 0.05 M-BaCl_2 were assayed in buffer containing 0.5 M-BaCl_2 . Curves 'c' show activities of samples suspended in 0.5 M-BaCl_2 assayed in buffer containing 0.05 M-BaCl_2 , and curves 'd' were obtained with samples suspended in 0.05 M-BaCl_2 assayed in the presence of the same concentration of that salt.

assay mixture. This is shown in Fig. 6, where data are presented in the form of Selwyn (1965) plots. The concentration-dependent differences in the curves obtained are indicative of instability of the enzyme during the time period of the assay. Similar results were obtained whether the purified enzyme preparation was assayed in 0.5 M-BaCl_2 or in the absence of added BaCl_2 from the assay. Preparations of the enzyme after step 2 of the purification procedure in 0.5 M-BaCl_2 also gave similar families of curves when assayed in the presence or absence of that concentration of BaCl_2 . At longer times a decrease in A_{470} was observed. This has been shown to result from instability of the guaiacol oxidation product (Pütter, 1975).

Activity towards BHA

The time-dependent change in absorbance, determined after the addition of 8.7% (w/w) NH_3 during the oxidation of BHA by the pure preparation of peroxidase, is shown in Fig. 7. Insolubility of the product of the reaction at pH 8.0 led to the development of turbidity, which prevented the determination of reaction progress at that pH. However, the product appeared to be fully soluble at the concentrations obtained here at the higher pH afforded by the addition of NH_3 to terminate

Fig. 5. Effects of dilution on the specific activity of rat intestinal peroxidase

The partly purified preparation, after step 2 of the purification procedure (Δ), suspended in 10 mM-acetate buffer, pH 5.0, containing 0.5 M-BaCl_2 was diluted 10-fold with 10 mM-acetate buffer, pH 5.0, and activities were determined at intervals. After 80 min the same sample was diluted 10-fold with 0.55 M-BaCl_2 in 10 mM-acetate buffer, pH 5.0, and samples were assayed at intervals for a further 2 h. A similar crude extract ($\blacktriangle$) was diluted 10-fold with 0.5 M-BaCl_2 in 10 mM-acetate buffer, pH 5.0, and after 80 min was diluted again 10-fold with the same buffer/salt mixture. Each value represents the mean of three separate determinations.

the reaction. The molar absorption coefficient for di-BHA was determined under these conditions to be $14100 \pm 345 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at 340 nm . The decrease in absorbance after 30 min may be due to instability of di-BHA or to its further peroxidase-catalysed oxidation, as has been reported for the oxidation of BHA by horseradish peroxidase (Valoti *et al.*, 1985).

The initial rate of BHA oxidation was proportional to enzyme concentration, and the time course of the reaction was linear for at least 2 min. The effects of BHA and guaiacol concentrations on the observed reaction rates are shown in Fig. 8. The dependence on H_2O_2 concentration gave inhibition at higher concentrations of this substrate (results not shown), consistent with the behaviour reported for peroxidase preparations from some other sources (Stelmaszyńska & Zgliczyński, 1971; Shindler *et al.*, 1976; Olsen & Little, 1979). The kinetic parameters determined for these two substrates are shown in Tables 2 and 3. The similar K_m values for H_2O_2 with guaiacol and BHA would be consistent with the same active site being involved in the peroxidation of both substrates.

In order to identify the nature of the reaction products, 1 ml samples of reaction mixture were quenched after 1 min by the addition of $500 \mu\text{l}$ of 3 M-HCl , and the products were extracted into methylene chloride and analysed by t.l.c. Spots corresponding to tetraguaiacol and di-BHA were easily seen under u.v. light when guaiacol and BHA, respectively, was used as substrate. When the reaction was carried out in the presence of K_m concentrations of both BHA and guaiacol, an additional spot with an R_F value intermediate between that of guaiacol and tetraguaiacol was observed. This may correspond to a mixed adduct between BHA and

Fig. 6. Inactivation of rat intestinal peroxidase during assay

The partly purified (after step 2 of the purification procedure; *a*) was assayed at protein concentrations of 0.8 μg (○), 1.6 μg (△) and 2.4 μg (□) per assay, and the pure preparation (*b*) was assayed at concentrations of 0.109 μg (○), 0.212 μg (△) and 0.327 μg (□). Assay mixtures contained 0.5 M-BaCl₂. The data are plotted in accordance with the procedure of Selwyn (1965).

Fig. 7. Absorption spectra during the oxidation of BHA by rat intestinal peroxidase

The reaction mixtures contained 50 mM-Tris/HCl buffer, pH 8.0, 50 mM-H₂O₂, 0.141 mM-BHA and 1 μg of enzyme. After incubation for 0, 1, 3, 15 and 30 min at 37 °C, the reactions were stopped by the addition of aq. NH₃ and DMSO (final concns. of 8.7% and 21%, v/v, respectively) and the absorbance spectra were determined.

guaiacol similar to those that have been reported to be formed between the former compound and *p*-phenetidine and benzidine (Ross *et al.*, 1985; Josephy & Van Damm, 1984).

Fig. 8. Double-reciprocal plots for the oxidation of BHA and guaiacol (inset) by purified rat intestinal peroxidase

Each value is the mean of three separate determinations. An enzyme concentration of 0.8 μg per assay was used for the determinations with BHA, and 0.11 μg per assay was used when guaiacol was the substrate.

Table 3. Kinetic constants for the interactions of H₂O₂ with purified rat intestinal peroxidase determined in the presence of guaiacol or BHA

Equal relative concentrations, corresponding to $K_m/3.18$, of the two substrates were used.

Substrate	Concn. (mM)	K_m (μM)	K_i (mM)
Guaiacol	6.6	85	536
BHA	0.055	60	602

The demonstration that BHA is a substrate for purified rat intestinal peroxidase with a specificity constant, $k_{\text{cat.}}/K_m$, similar to that shown towards guaiacol (Table 2) suggests that it may compete effectively with other substrates for oxidation, and that the activity of that enzyme may be involved in the potentially toxic effects of this antioxidant.

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