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PEROXIDASE CATALYSED FORMATION OF PROSTAGLANDINS FROM ARACHIDONIC ACID

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Abstract—Horseradish peroxidase and bovine lactoperoxidase (EC 1.11.1.7), when incubated aerobically with arachidonate, gave rise to the formation of substances identified by bioassay as prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)- and prostaglandin E_2 (PGE_2)-like compounds.

Boiling of enzymes, which suppressed their capacity to peroxidise guaiacol, also destroyed their capacity to convert arachidonate into PG-like compounds. The rates of formation of PG-like compounds rapidly declined with time, approaching zero after 10 and 20 min for $PGF_{2\alpha}$- and PGE_2-like compounds, respectively. Addition of more enzyme further promoted the reaction. Horseradish and lacto-peroxidases showed optimum pH values of 9.0 and 10.0, respectively. Both enzymes exhibited apparent K_m values of about 5×10^{-5} M for arachidonate. Some reducing agents such as ascorbic acid, NADH and adrenaline dose-dependently inhibited this reaction. The haem poison, phenylhydrazine, also inhibited, with an IC_{50} of 1×10^{-7} M. Indomethacin inhibited only the formation of PGE_2-like compounds with an IC_{50} of about 3×10^{-6} M. As compared to a standard commercial preparation of horseradish peroxidase, the purified horseradish basic and acidic isoenzymes exhibited a higher activity, towards arachidonate whereas other haemoproteins, possessing peroxidase activity, were less active. TLC and GC–MS analyses performed on the reaction products led to the identification of $PGF_{2\alpha}$, PGE_2 and $PG6K_{1\alpha}$ and other unidentified arachidonate derivatives. At 25°, pH 9.5, horseradish peroxidase, acting on saturating concentration of arachidonate, catalysed the formation of 60 μmol/min/mmole enzyme of $PGE_2 + PGF_{2\alpha}$. This appears to be the first report of the synthesis of prostaglandins catalysed by peroxidases.

Prostaglandin endoperoxide synthetase (PES, EC 1.14.99.1) and peroxidase (POD) share some catalytic properties. PES catalyses both the bis-dioxygenation of arachidonic acid (AA) to prostaglandin G_2 (PGG_2) (fatty acid cyclo-oxygenase) and the subsequent reduction of PGG_2 to the corresponding alcohol PGH_2 (PG hydroperoxidase). Both reactions are catalysed by an enzyme preparation from bovine seminal vesicles which was purified to apparent homogeneity by Miyamoto *et al.* [1]. This enzyme preparation, which had both cyclo-oxygenase and hydroperoxidase activity, had a 71 kD molecular mass and contained one molecule of ferriprotoporphyrin IX [2]. The questions about the location of the active sites, the role of peroxidase activity in cyclo-oxygenase catalysis and the reason for the presence of both enzyme activities in a single protein remain unanswered.

In the last few years a number of drugs and xenobiotics that are known to be substrates for POD, have been shown to be reducing cofactors for the hydroperoxidase of PES, donating electrons and undergoing "co-oxidation" in the process. This electron transfer appears to occur via a free-radical mechanism [3–6]. The source of the oxygen incorporated into the co-oxidized substrate varies, and depends on the nature of substrate itself [3, 7, 8]. In some experiments both the hydroperoxides, hydrogen peroxide and 15-hydroperoxyarachidonic acid, have been shown to support the co-oxidation promoted by the hydroperoxidase activity of PES [9].

There are some data directly comparing the peroxidative mechanism of PES with that of other peroxidases, and similarities between horseradish peroxidase (HRPOD) and PES have been reported for the one-electron oxidation of xenobiotics [10]. Studies on the peroxidative oxidation of benzidine [11], 2-aminofluorene [9], *p*-aminophenol and *p*-phenetidine [12–13] catalyzed by PES or HRPOD have shown that the main products formed are identical with both enzymes. These studies also suggest that the mechanisms of the reactions catalysed by both enzymes are very similar.

Further support for the operation of similar mechanisms comes from a recent study by Kulmacz [14] in which the peroxidase activity of PES was investigated spectrophotometrically during incubation with hydrogen peroxide or 15-hydroperoxyeicosatetraenoic acid (15-HPETE). Under these conditions the

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† Abbreviations: PG, prostaglandin; HRPOD, horseradish peroxidase; LPOD, lactoperoxidase; AA, 5,8,11,14-eicosatetraenoic acid (arachidonic acid); PES, prostaglandin endoperoxide synthetase; TMSI, trimethylsilylimidazole; SOD, superoxide dismutase; TLC, thin layer chromatography; FAB–MS, Fast Atom Bombardment–Mass Spectrometry; CAD, Collisionally Activated Decomposition.

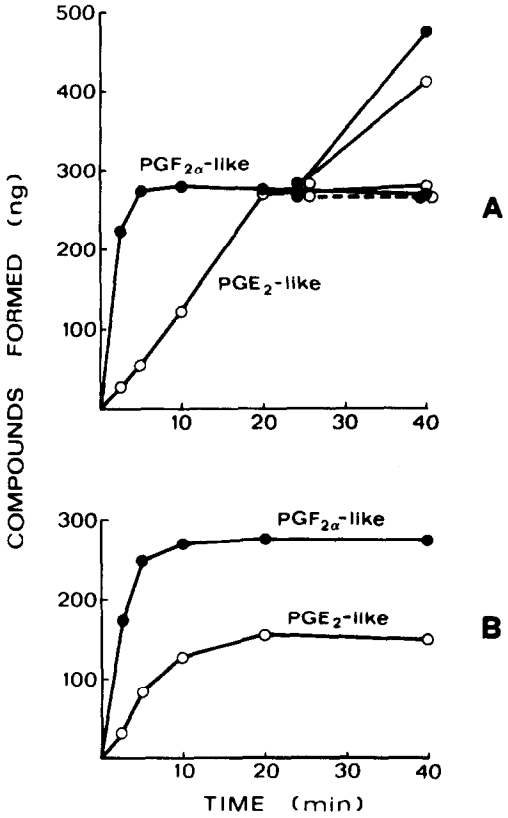

Fig. 1. Time-course of the formation of PG-like substances from arachidonate catalysed by HRPOD (A) and LPOD (B). Formation of PG-like substances performed at 25° in air in 8 ml of a reaction mixture containing (final concentrations) 50 mM sodium carbonate buffer, pH 9.5, 0.33 mM AA (starter of the reaction) and 0.12 μM enzyme. The reaction was blocked with HCl addition. PG-like substances were determined by bioassay in the ethyl acetate extracts of the reaction mixture. Further synthesis was promoted by addition of fresh enzyme at 25 min. Points represent the averages from triplicated experiments.

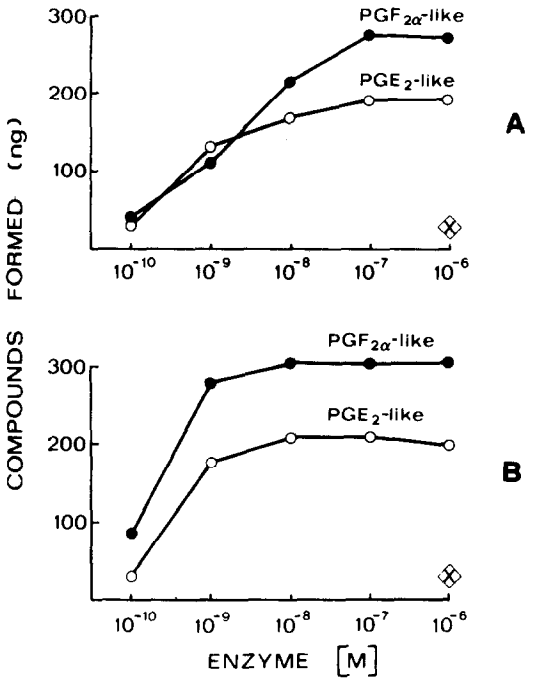

Fig. 2. Dependence of formation rate of PG-like substances from arachidonate on HRPOD (A) and LPOD (B) concentration and on oxygen. Experimental conditions are those reported in Fig. 1. Enzymes were inactivated by heat treatment (◇). Oxygen was reduced to 2.3% saturation in the incubation medium by continuous bubbling of N_2 for 5 min (×). Points represent the averages from triplicated experiments.

enzyme was transformed into Compound I, similar to that observed when HRPOD reacts with hydrogen peroxide.

Identical spectral changes were shown to occur when both PES or HRPOD catalysed the reduction of 5-phenyl-4-pentenyl hydroperoxide [15]. Furthermore, during this reaction PES exhibited a turnover number comparable to that observed with either HRPOD or lactoperoxidase (LPOD). However, PES was shown to be more sensitive than HRPOD to inactivation by peroxides and to show different efficiencies in the reduction of 5-phenyl-4-pentenyl hydroperoxide in the presence of different hydrogen donor substrates [16].

A comparison of the properties of PES with those of peroxidases, indicated that one aspect of the latter enzymes which has so far been underestimated is their capacity to catalyse oxidations by molecular oxygen (for reviews, see Mason [17] and Ray [18]). Following the observation that HRPOD can aerobically oxidise indole-3-acetic acid and studies aimed at elucidating the mechanism of this reaction [19–22], it was shown that 5,6,7,8-tetrahydropterin could generate the quinone-7,8-dihydro(6H)-pterin in the presence of HRPOD and either hydrogen peroxide [24] or oxygen. Furthermore, 5,6,7,8-tetrahydrobiopterin could be converted by HRPOD into the quinonoid-7,8-dihydro(6H)-biopterin in the presence of oxygen [25]. More recently, the mechanism by which HRPOD introduces an OH group into phenol in the presence of molecular oxygen and dihydroxyfumaric acid has been analysed [26]. It has been shown that dihydroxyfumaric acid acts by first activating O_2 to O_2^-, necessary for the transformation of HRPOD-Fe^{3+} into HRPOD-Fe^{2+}, and secondly, participates with the latter enzyme form to generate OH which is subsequently introduced into phenol.

All these findings indicate that PES shares with POD the ability to promote peroxidative oxidation of xenobiotics and that POD exhibits, a still incompletely explored, oxygenase activity which might have some similarities with the cyclo-oxygenase activity of PES.

We thus attempted to test the hypothesis that AA is a substrate for POD, in a reaction that forms biologically active products, by using commercial preparations of POD. The results presented here show that plant and mammalian POD aerobically convert AA into biologically active PGE_2- and $PGF_{2\alpha}$-like compounds. Among these, PGE_2 and $PGF_{2\alpha}$ have been identified by thin layer chromatography (TLC) and by GC–MS analysis.

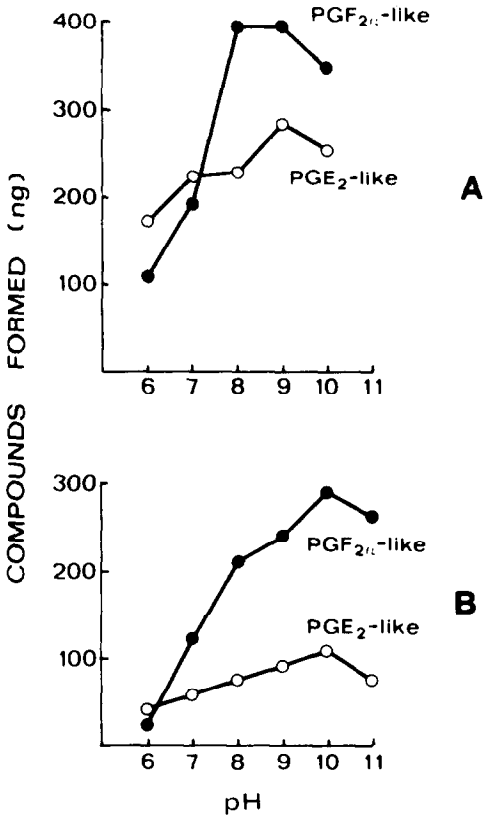

Fig. 3. Dependence on pH of formation rate of PG-like substances from arachidonate catalysed by HRPOD (A) and LPOD (B). Experimental conditions are those reported in Fig. 1. Different pHs were obtained by using 50 mM Tris–HCl buffer and 50 mM sodium carbonate buffer. Points represent the averages from triplicated experiments.

MATERIALS AND METHODS

Materials

Chemicals. HRPOD grade VI and LPOD from bovine milk, HRPOD acidic isoenzyme, HRPOD basic isoenzyme, cytochrome *c* from bovine heart, catalase and superoxide dismutase (SOD) from bovine liver, arachidonic acid, adrenaline HCl and indomethacin were purchased from Sigma Chemical Co. (St Louis, MO). Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), E_2 (PGE_2) and $6KetoF_{1\alpha}$ ($PG6KF_{1\alpha}$) standards were kindly provided by Upjohn (Kalamazoo, MI). Methysergide dimaleate was from Sandoz (Basel, Switzerland), mepyramine maleate was from Prodotti Gianni (Milano, Italy), bovine haemoglobin was from Calbiochem (Frankfurt, F.R.G.) and trimethylsilylimidazole (TMSI) was from Supelco Inc. Bellefonte, PA). Hyoscine hydrobromide and phenylhydrazine were from BDH (Milano, Italy). Bio-SilA (100–200 mesh) was from Bio-Rad Labs (Segrate, Italy). All other reagents were of the highest purity commercially available.

Apparatus. Bioassays were performed by using a Marb isometric transducer and responses were recorded on a Linseis Polygraph. Mass spectrometry studies were performed on a VG 7070-EQ (VG Analytical, Manchester, U.K.) interfaced to a Digital PDP8/A Data System.

The 7070-EQ consists of a VG 7070-E double focusing mass spectrometer coupled to a quadrupole analyser. A Beckman Acta III spectrophotometer equipped with a recorder was used for spectrophotometric determinations. O_2 content was measured polarographically by using a Model 5300 Biological Oxygen Monitor (Yellow Spring Instrument Co., Yellow Spring, OH).

Incubations. Incubations were carried out in unstoppered 25 ml glass tubes at 25° in a shaking water-bath (60 orbital oscillations/min, 3 cm stroke radius).

The standard incubation mixture contained 3.3×10^{-4} M AA (to start the reaction), 1.2×10^{-7} M enzyme and 5×10^{-2} M Na carbonate buffer, pH 9.5 in a total volume of 8 ml. Incubation time varied from 2.5 to 40 min (under standard conditions, 25 min) and the reaction was stopped by acidification to pH 3 with 1 M HCl. The pH-dependence of the reaction was assessed by using 50 mM Tris–HCl buffer, and 50 mM sodium carbonate buffer. Dependence on substrate concentration was determined by incubating a fixed amount of enzyme with various concentrations (1×10^{-5}–1.2×10^{-4} M) of AA. A standard assay time of 5 min was used because it was shown that the time-course of the reaction, giving rise to the synthesis of PGE_2-like compounds was linear for this period under standard conditions. K_m values were calculated from double-reciprocal plots, as described by Dixon and Webb [27]. In some experiments hypoxic conditions were maintained by using stoppered tubes provided with inlet and outlet connections which allowed the reaction mixture to be bubbled with N_2 for 5 min before addition of AA. In these conditions O_2 saturation dropped from 100% value to 2–2.5%.

When the effects of different compounds on the reaction were studied, these were preincubated with the enzyme for 5 min before the addition of AA.

Inactivation of peroxidases. In some experiments inactive enzyme preparations were used. In order to destroy their activity, HRPOD and LPOD preparations were boiled for 30 min. The residual, heat resistant peroxidase activity associated with low molecular weight enzyme forms present in HRPOD commercial preparations was removed by ultra-filtration with a PM 10 filter of the enzyme solution before the heat treatment.

Peroxidase assay. The method was based on that described by Chance and Mahely [28] which utilises guaiacol as the hydrogen donor, as previously described [29].

Sample processing. The reaction products were extracted into ethyl acetate according to the method of Piper and Vane [30] and the aqueous phase was discarded after centrifugation at 1000 *g*. The organic phase was evaporated to dryness, in a gentle stream of N_2, and the residue was solubilised in ethanol for TLC purification and separation of the reaction products which were subsequently analysed by MS or by bioassay; alternatively, the residue was suspended directly in Krebs–Henseleit solution for immediate bioassay.

Bioassay. The PG-like activity of the reaction

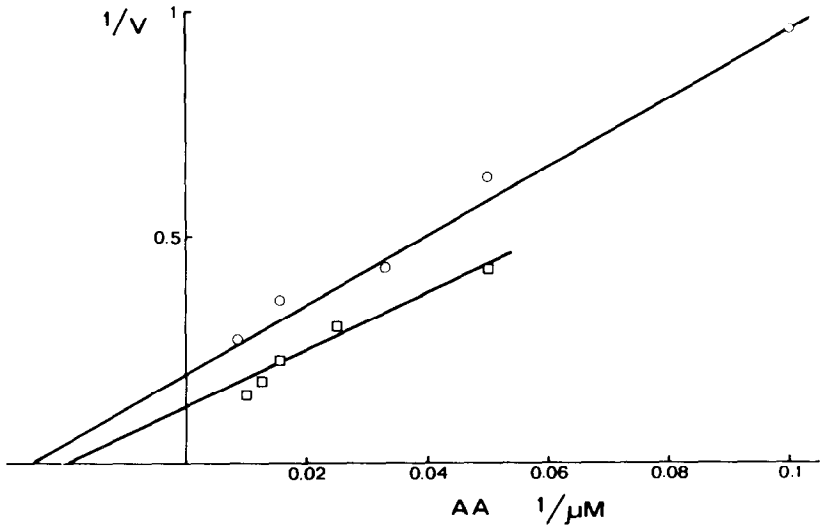

Fig. 4. Dependence on arachidonate concentration of PG_2-like compounds catalysed by HRPOD (A) and LPOD (B). Experimental conditions are those reported in Fig. 1. Arachidonate concentration varied from 10 to 123 μM. Points represent the averages from triplicated experiments.

products was determined as PGE_2 by bioassay with a strip of rat stomach fundus [31] and as $PGF_{2\alpha}$ with rat colon [32] in the presence of 0.56 mM methysergide, 0.33 mM hyoscine, 0.35 mM mepyramine and 2.8 mM indomethacin. Tests were carried out, at least in duplicate, and biological contents were determined at three dose levels. PGE_2 (1–4 ng) elicited a good contractile response from rat stomach fundus, while it did not cause the contraction of rat colon. $PGF_{2\alpha}$ (20–40 ng) evoked a good contraction of rat colon while it elicited a weak contraction of rat stomach fundus. Blank samples (buffers plus AA, no PODs in them) were always carried out and the occasional presence of PG-like material was subtracted from the amounts of PG-like substances found in the experimental samples.

TLC purification and identification. The residue obtained after evaporation under N_2 of the ethyl acetate extract, was suspended in 0.2 ml ethanol, and submitted to two subsequent purification steps on TLC (DC Alufolien Kieselgel 60 F254, Merck) using two eluting systems: *n*-hexane: diethyl ether: acetic acid (15:85:0.1) for the first chromatography and benzene: dioxane: acetic acid (60:30:0.3) for the second [33]. Prostaglandin standards were co-chromatographed. In the first chromatography authentic PGs did not migrate; the compounds present in gel areas with $R_f = 0$, after elution with ethyl alcohol, were applied to another plate for the second chromatography. Some lanes of both chromatograms were developed with anisaldehyde reagent [34], while the remainders (undeveloped lanes) were used for the bioassay and mass-spectrometric identification. The gel areas in the undeveloped portions of the plate, corresponding to authentic PG or to other unidentified compounds, were scraped off, suspended in 2 ml of Krebs solution, centrifuged and bioassayed. Alternatively, they were scraped off, suspended in ethyl alcohol, centrifuged and analysed by mass-spectrometry. The recovery of standard $PGE_2 + PGF_{2\alpha}$, taken through the entire procedure, was compared with bioassay values determined by direct addition to the organ bath. Each sample was supplemented with the same known amounts of PGs. The recovery for both PGs was found to be almost quantitative.

Mass spectrometric identification. This was performed by Fast Atom Bombardment Mass Spectrometry (FAB–MS) and by Mass spectrometry–Mass spectrometry (FAB–MS/MS), without previous derivatisation of the compounds identified as PGE_2 and $PGF_{2\alpha}$ on TLC by the criterion of co-chromatography. FAB–MS analysis was performed by using Argon atoms at 7 keV kinetic energy. Full (mass) spectra in negative-ion mode at $M/\Delta M$ 2500 resolution (10% valley definition) were recorded with a run speed of 20 sec/decade and data were processed by a PDP8/A computer system. The samples, suspended in 3 μl glycerol, were directly transferred to a FAB target and inserted into the ion source. The characteristic glycerol peaks were then removed via computer from all spectra. FAB–MS/MS analyses in negative-ion mode were performed by selecting the quasi-molecular ion $(M\text{-}H)^-$ of the expected compound by using double-focusing section at $M/\Delta M$ 1000 resolution (10% valley definition) and by focusing it onto the entrance of the quadrupole collision gas cell for Collisionally Activated Decomposition (CAD). Daughter ions of the decompositions produced in the collision cell were mass analysed by using a run speed of 10 sec per scan at unit mass resolution in the second quadrupole and detected by using a second off-axis electron multiplier. Air, as the collision gas, was used at a cell pressure of 1.5×10^{-6} torr. To confirm the results obtained in FAB–MS and FAB–MS/MS analysis, GC–MS analysis with electron impact ionisation was performed on ethyl acetate extracts after purification on a Bio-Sil A column and derivatisation with TMSI [35].

Table 1. HRPOD-catalysed formation of PG-like substances from AA in the presence of different compounds as percent of the values found in control conditions

Compounds	M	PGE_2-like	$PGF_{2\alpha}$-like
Ascorbic acid	5×10^{-5}	71	51
	1×10^{-4}	57	33*
	5×10^{-4}	31**	17**
NADH	1×10^{-7}	69	57
	1×10^{-6}	45*	54*
	1×10^{-5}	35*	43*
Adrenaline	1×10^{-5}	65	90
	1×10^{-4}	42*	64
	1×10^{-3}	47*	70
Propylgallate	1×10^{-5}	100	105
	1×10^{-4}	91	84
H_2O_2	1×10^{-11}	115	101
	1×10^{-9}	108	104
	1×10^{-7}	109	95
Catalase	1.2×10^{-8}	107	104
SOD	3×10^{-8}	87	90

Standard reaction incubation: 8 ml at 25° containing 50 mM sodium carbonate pH 9.5, 0.12 μM peroxidase and 0.35 mM AA. Incubation time 25 min. The mean values (± SE) of the actual formation of PG-like compounds for control experiments (N = 7) in the absence of additives were 43.43 ± 6.60 ng and 442.14 ± 59.43 ng for PGE_2-like and $PGF_{2\alpha}$-like activities, respectively. ** $P < 0.01$; * $P < 0.05$ against controls (no compounds added) calculated by comparing experimental values.

RESULTS

Formation of PG-like substances from AA catalysed by peroxidases under aerobic conditions

HRPOD and LPOD, when incubated aerobically with AA, gave rise to the formation of substances which were identified by bioassay as $PGF_{2\alpha}$- and PGE_2-like compounds. The formation rate of these compounds with both enzymes, as shown in Fig. 1, was well sustained in the first few min (5 and 10 min for the formation of $PGF_{2\alpha}$- and PGE_2-like substances, respectively), then decreased rapidly to zero after 20 min.

Furthermore, the two enzymes behaved differently as to the maximum amounts of compounds obtained. While HRPOD gave rise to about the same maximum amounts of $PGF_{2\alpha}$- and PGE_2-like compounds, LPOD formed about twice as much of the $PGF_{2\alpha}$-like compounds as PGE_2-like compounds.

When the synthesis of PG-like compounds was exhausted, the addition of fresh enzyme samples, but not of AA, promoted the additional formation of PG-like compounds as shown in Fig. 1. For both HRPOD and LPOD the formation of PG-like products increased with increasing enzyme concentrations at low concentrations of the latter but tended to plateau values at higher concentrations (Fig. 2). Such behaviour would not be expected for simple mechanism-based inhibition by AA and may indicate some component of the reaction mixture to become limiting. This, however, did not seem to be the case with O_2. When the reaction was carried out in a closed system, in fact the O_2 concentration did not fall below 95% saturation after 30 min incubation.

Inactivation of enzyme preparations by heat suppressed their capacity to convert AA into PG-like compounds. The same result was obtained by incubating active enzyme preparations in the absence of O_2 (Fig. 2). This catalytic activity of POD towards AA was pH-dependent. As shown in Fig. 3, apparent optimum pH values for HRPOD and LPOD were 9.0 and 10.0, respectively. The two enzymes showed similar K_m values of about 5×10^{-5} M for AA, as calculated from the rate of the synthesis of PGE_2-like products (Fig. 4).

The total yields of PGE_2 and $PGF_{2\alpha}$ activities were found to depend on the sample of AA used. When freshly prepared solutions were used the amounts formed were about 70 and 600–650 ng PGE_2- and $PGF_{2\alpha}$-like compounds, respectively. However, after storage of solution at 4° for 3 days the responsive values fell to less than 30 ng and less than 300 ng for the two products. It is possible that the decreased production of PG-like substances in these latter

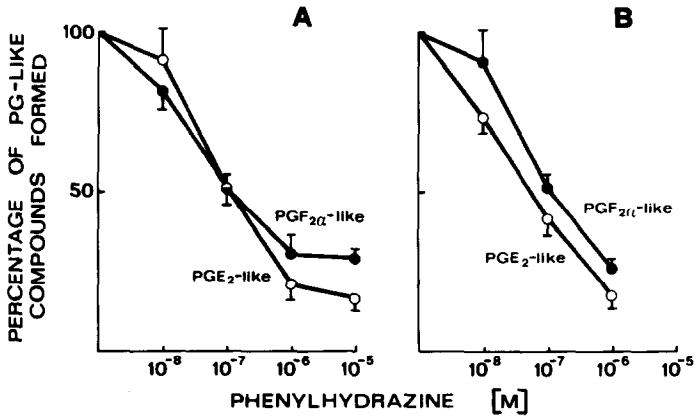

Fig. 5. Inhibition by phenylhydrazine of synthesis of PG-like substances catalysed by HRPOD (A) and LPOD (B). Experimental conditions are those reported in Fig. 1. Enzymes were preincubated with phenylhydrazine for 5 min. The percentage of PG-like substances formed was calculated by comparison with the amount produced in the absence of the inhibitor. Points are means of four experiments ± SE.

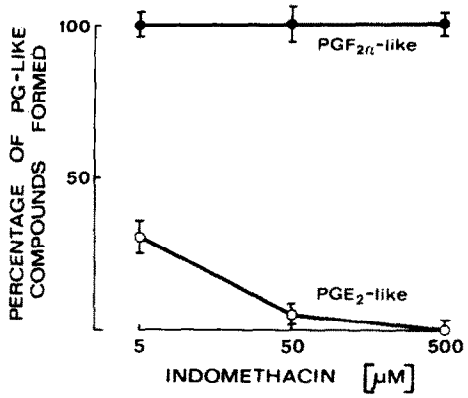

Fig. 6. Inhibition by indomethacin of HRPOD-catalysed formation of PGE_2-like substances. Experimental conditions are those reported in Fig. 1. HRPOD was pre-incubated with indomethacin for 5 min. The percentage of PG-like substances formed was calculated by taking as 100% the amount produced in the absence of the inhibitor. Points are means of four experiments ± SE.

AA preparations was dependent on their reduced AA content.

Inhibition of peroxidase-catalysed conversion of AA into PG-like substances under aerobic conditions

The results in Table 1 show that ascorbic acid, NADH and adrenlaine dose-dependently inhibited the conversion of AA to PG-like compounds when catalysed by HRPOD. Among these reducing agents, ascorbate and adrenaline are well-known substrates for HRPOD-catalysed oxidation [36, 37]; and were probably inhibiting this reaction by competing with AA for the enzyme active site.

The weak or null effect of hydrogen peroxide and catalase clearly indicates that hydroperoxides were not participating in this reaction. Propylgallate, a potent inhibitor of cytochrome P-450 [38] was shown to only moderately inhibit the conversion of AA into PG-like substances, perhaps by interacting with the enzyme haemoprotein. That the integrity of the haem-group is a prerequisite for POD to catalyse this conversion is shown in Fig. 5. The haem poison phenylhydrazine, in fact, dose-dependently inhibited this reaction, showing an IC_{50} of about 10^{-7} M towards activities of both plant and mammalian enzymes catalysing the formation of either PGE_2- or $PGF_{2\alpha}$-like substances form AA.

The effects of indomethacin, a powerful inhibitor of cyclo-oxygenase activity of PES, are shown in Fig. 6. Interestingly, this drug inhibited only the formation of PGE_2-like compounds from AA catalysed by HRPOD but left the capacity of the enzyme to synthesise $PGF_{2\alpha}$-like compounds unaltered. Similar results were obtained with LPOD. The apparent IC_{50} for the action of indomethacin on the HRPOD-catalysed formation of PGE_2-like compounds was about 3×10^{-6} M.

Formation of PG-like substances from AA catalysed by some haemoproteins in aerobic conditions

The capacity of POD to catalyse the formation of PG-like substances from AA under aerobic conditions was shared by some haemoproteins, which also to some extent, exhibit peroxidase activity. As shown in Fig. 7, haemoglobin, cytochrome *c* and catalase exhibited a much lower capacity for synthesising PG-like substances from AA when compared to a commercial preparation of HRPOD. Of

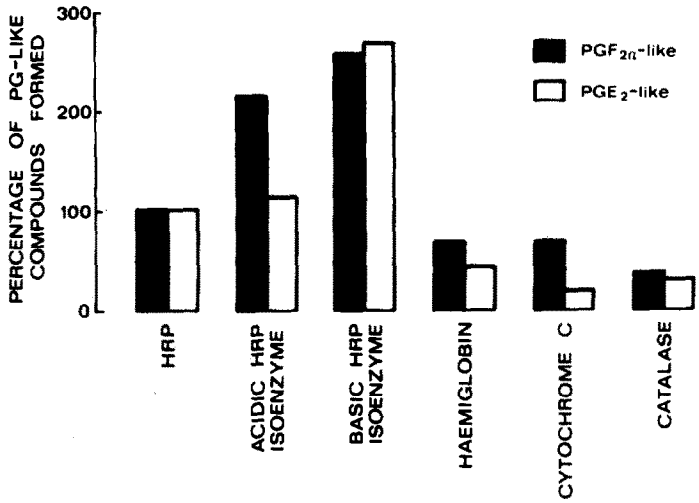

Fig. 7. Formation of PG-like substances from arachidonate, catalysed by different HRPOD preparations and some other haemoproteins. Experimental conditions are those reported in Fig. 1. The percentage of PG-like substances formed was calculated by taking as 100% the amount produced in the presence of a standard HRPOD commercial preparation (see Materials and Methods). Bars represent the averages from triplicated experiments.

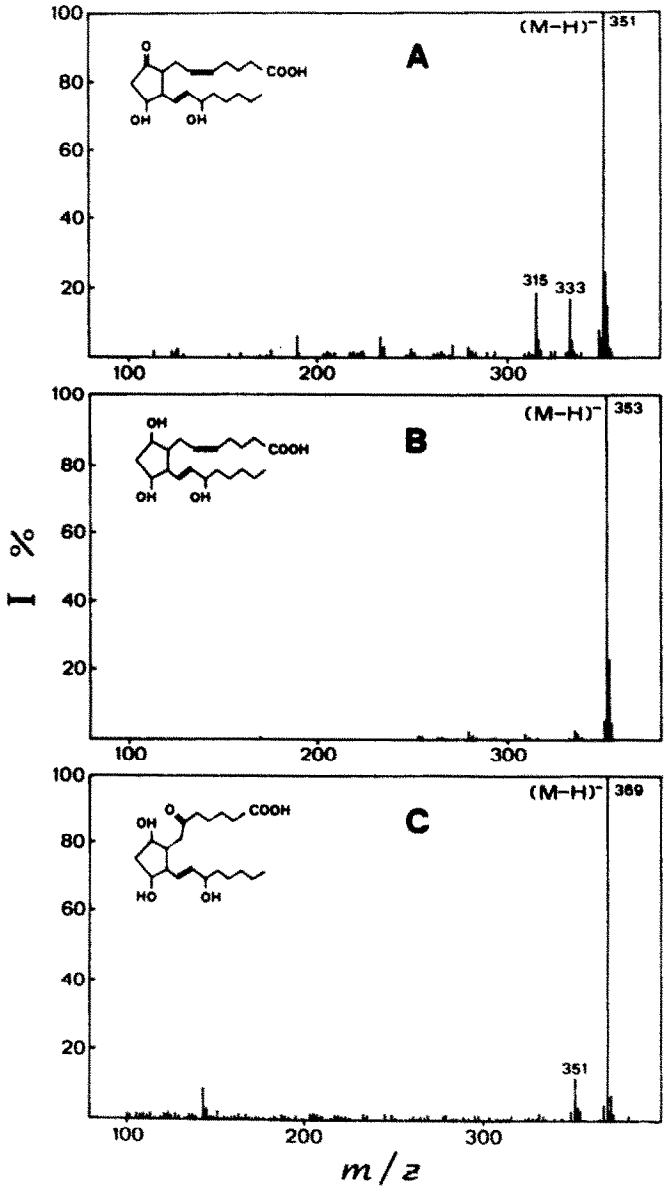

Fig. 8. FAB negative-ion mass spectra of PGE_2 (A), $PGF_{2\alpha}$ (B) and $PG6KF_{1\alpha}$ (C). For details see text.

the two isoenzymes purified from HRPOD which were tested, the basic one was the more active.

Identification of PGE_2, $PGF_{2\alpha}$ and $PG6KG_{1\alpha}$ among the products of the aerobic transformation of AA catalysed by POD

Some of the compounds which had not migrated in the first TLC performed on the products extracted into ethyl acetate from the POD–AA reaction mixture, when submitted to the second TLC (see Methods section) and to visualisation with anisaldehyde reagent, gave rise to the formation of two spots with R_f values of 0.13 and 0.23 which corresponded to those of authentic $PGF_{2\alpha}$ and PGE_2, respectively. The identity of the PGs was confirmed by MS analysis. The FAB ionisation technique in the negative-ion mode performed on standard PGs, gave rise to spectra which were characterised by the almost exclusive presence of the quasi-molecular ions $(M–H)^-$. Since with this technique the co-production of fragment ions was negligible or totally absent, the method was characterised by a high detection sensitivity (Fig. 8). PGE_2, $PGF_{2\alpha}$ and $PG6KF_{1\alpha}$, in fact, exhibited almost exclusively peaks corresponding to $(M–H)^-$ ions at m/z 351, 353 and 369, respectively. As shown in Fig. 9, when the ethanol extract of the gel area of the first TLC which contained the compounds which had not migrated was submitted to FAB–MS, the spectrum showed the same peaks

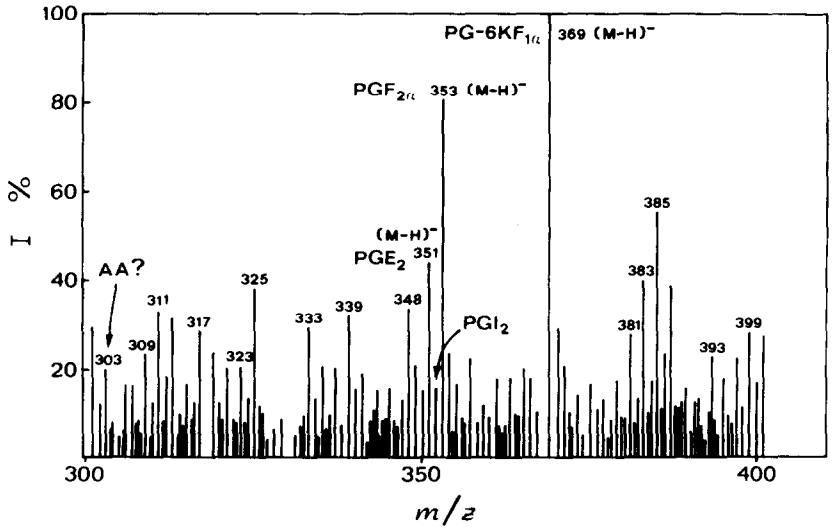

Fig. 9. FAB negative-ion mass specrum of products formed from arachidonate in the presence of LPOD. The ethyl acetate extract of the incubation mixture was submitted to a first TLC (see Methods). MS analysis was performed on the ethanol eluate from the TLC areas which were presumed to contain PGs.

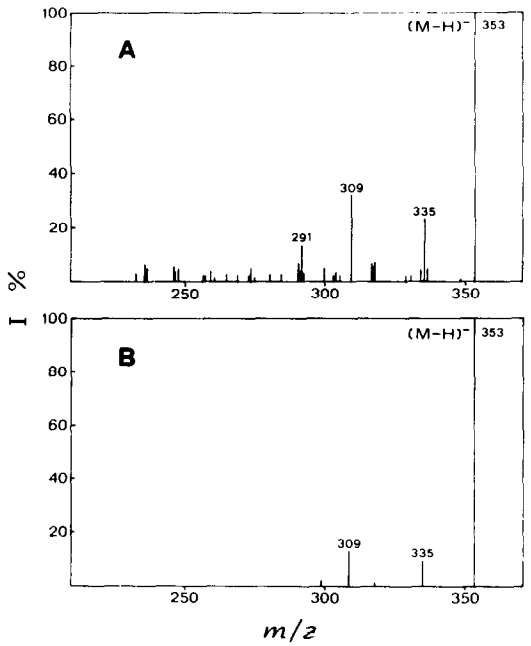

Fig. 10. CAD FAB negative-ion mass fragmentation spectra of m/z 353 $(M-H)^-$ $PGF_{2\alpha}$ standard parent ion (A) and of m/z 353 ion obtained from the MS analysis performed on the reaction products of LPOD and arachidonate (B), as mentioned in the caption to Fig. 9.

at m/z 351, 353 and 369 which corresponded to the $(M-H)^-$ ions produced by the authentic PGs.

It is notable that when the ethanol extract was submitted additionally to CAD FAB–MS, the resulting spectrum exhibited the same peak at m/z 353 as that obtained with authentic $PGF_{2\alpha}$ (Fig. 10). Therefore, the FAB–MS/MS technique confirmed the identity of the peak at m/z 353 with the $(M-H)^-$ ion of $PGF_{2\alpha}$. The identification of the PGs was confirmed by GC–MS analysis with electron impact ionisation of the compounds derived with TMSI. As shown in Fig. 11, in fact, peaks eluting with a R_f of 3:42, 4:29 and 5:22 were found to correspond to authentic $PGF_{2\alpha}$, $PG6KF_{1\alpha}$ and PGE_2, respectively. Other peaks present in the mass chromatogram were not identified.

Yield of PGs from AA in the reaction catalysed by HRPOD under aerobic conditions

The capacity of HRPOD to catalyse the synthesis of PGE_2 and $PGF_{2\alpha}$ from AA under aerobic conditions was evaluated by bioassaying the amount of PGs formed in 20 min when a fixed amount of enzyme was incubated at 25° with a saturating concentration of AA (0.33 mM). The results of this study are shown in Table 2. It is remarkable that the amount of authentic PGs, isolated by TLC, represented only a fraction of PG-like compounds formed. PGE_2 and $PGF_{2\alpha}$, in fact, accounted for 46.4 ± 10.2 and 26.6 ± 3.6% (mean values ± SE from eight separated determinations) respectively, of the total PGE_2- and $PGF_{2\alpha}$-like activity of the crude extract of the reaction mixture. Most of the PG-like biological activities were, however, due to unidentified AA derivatives which were separated from the authentic PGs by the first TLC (see Table 2).

The results from the quantitative bioassay analyses indicated that the biological activity of the single purified fractions considered together was higher than that of the crude extract. This discrepancy might be explained by the presence in the crude extract of compounds having biological activities antagonistic to those of PGs. Further work will be necessary to resolve this problem. Nevertheless, under the present experimental conditions HRPOD catalyses the formation from AA of 60 μmol/min/mmol enzyme of PGE_2 + $PGF_{2\alpha}$.

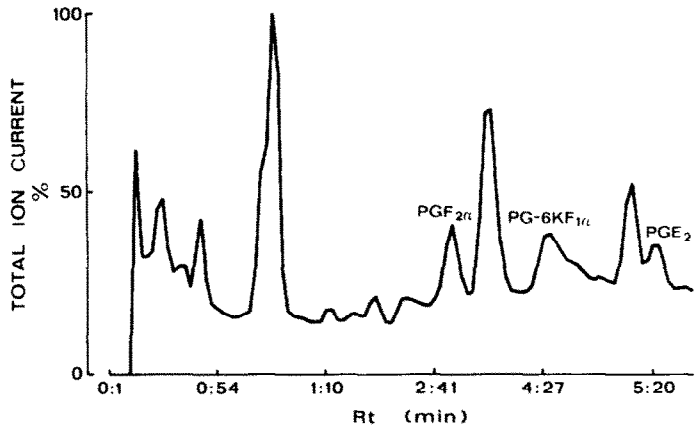

Fig. 11. GC–MS analysis with electron impact ionisation of substances formed from arachidonate incubated with LPOD. The compounds present in the ethylacetate extract of the incubation mixture were derived with TMSI after purification on Bio-SiLA column (see Methods). Chromatographic peaks were attributed to different PGs by the criterion of the R_f identity.

DISCUSSION

These findings clearly show that aerobic incubation of AA with POD gives rise to the formation of a number of compounds with PG-like biological activities. Among these compounds PGE_2 and $PGF_{2\alpha}$ were identified by TLC and GC–MS analysis. That POD was responsible for this catalysis is further supported by the findings obtained when highly purified HRPOD isoenzymes were used. Since this catalytic property was shared by different haemoproteins (cytochrome *c*, catalase and haemoglobin), it can be inferred that the haem group is of primary importance. Furthermore, phenylhydrazine, which is known to inactivate HRPOD irreversibly by complexing haem in the presence of oxygen at doses as low as 10^{-6} M [39], strongly inhibited the HRPOD-catalysed conversion of AA into PG-like compounds at a concentration of 10^{-7} M. Thus, the integrity of the haem group appears to be essential for this catalytic property of POD.

The formation of PG-like compounds appeared to be completed within 20 min of incubation under our standard experimental conditions. The aerobic conversion of AA into PG-like substances — according to the bioassay determination — accounted only for a very limited amount of the AA initially present in the assay (about 1%). Since O_2 concentration in our conditions was not limiting, it might be concluded that there was a self-deactivation of POD. Strong support for this hypothesis comes from the observation that the addition of new enzyme to the reaction mixture after the synthesis of PG-like substances had been completed, promoted the synthesis of additional amounts of PG-like compounds whereas additional AA did not. This phenomenon might be explained by the formation of intermediates or by-products of the reaction which lead to enzyme deactivation. This is known to occur with PES during oxygenation of AA. Smith and Lands [40] first reported self-deactivation of PG–cyclo-oxygenase. Egan *et al.* [41] studied the mechanism of this phenomenon and devised a free-radical mechanism for enzyme destruction; in addition, a deactivating effect caused by PGG_2 was reported. Van der Ouderaa *et al.* [42] also suggested that the accumulation of PGG is harmful and leads to enzyme deactivation. Porter [43] hypothesised that the conversion of PGG_2 to PGH_2, a peroxidase reaction catalysed by PES, generates oxygen-centred radicals that occasionally attack the enzyme and lead to deactivation. In a recent study [14] carried out on PES purified to homogeneity from sheep seminal

Table 2. HRPOD catalysed conversion of AA into PG-like compounds and authentic PGs determined by biological assay

		PGE_2-like	$PGF_{2\alpha}$-like	Authentic PGE_2	Authentic $PGF_{2\alpha}$
Crude extract		400	1700		
1st TLC	R_f 0.50	150	1050		
	0.45	150	200		
	0.15	50	400		
2nd TLC	0.23			100	
	0.13				300

Figures indicate ng of compounds formed in the course of a single experiment and carried through the entire analytical procedure.

vesicles, the mechanism by which inactivation of the peroxidase and cycloxygenase activity of PES takes place has been analysed. It is suggested that some enzyme intermediates in the peroxidase or in the cyclo-oxygenase catalytic cycle are susceptible to an internal rearrangement or a side-reaction that leads to inactivation.

Phenol, methional and other radical-scavenging reducing agents have also been shown to act by stabilising the cyclo-oxygenase activity of PES, probably owing to their capacity to be oxidised in its place and thereby preserve it, or by trapping any radicals before they can damage the enzyme [44]. In the case of AA oxidation catalysed by POD, however, some radical scavengers and/or reducing agents which are substrates for POD-catalysed oxidation, namely adrenaline [37], L-ascorbic acid [36] and NADH [45], were shown in the present work to inhibit the reaction in a dose-dependent way, perhaps by competing with AA for the enzyme.

The lack of effect of the addition of SOD, catalase, or H_2O_2 is an indicator that in our experimental conditions both O_2^- or H_2O_2, either as products of the reaction or as contaminants (H_2O_2) in the reaction mixture, do not participate in the reaction itself.

The powerful inhibitory effect of indomethacin was not surprising. Non-steroidal anti-inflammatory agents have been shown, in fact, to inhibit *in vitro* both the HRPOD-catalysed oxidation of *o*-dianisidine [46] and iodination of bovine serum albumin catalysed by a number of PODs [47]. It was surprising, however, that this inhibition was not extended to the synthesis of $PGF_{2\alpha}$-like substances. The different degrees of oxidation, which characterise PGE_2 and $PGF_{2\alpha}$ structures, might be responsible for the different capacities of indomethacin to impair the synthesis of these two PG-like compounds.

In the present study it was shown that under standard experimental conditions HRPOD had the capacity of synthesising about 60 μmol/min/mmol enzyme of $PGE_2 + PGF_{2\alpha}$ from AA, with the latter representing the largest portion. It might be worth comparing this figure with that reported for PES, highly purified from bovine seminal vesicle microsomes, which was about 700 μmol/min/mmol enzyme of PGH_1 synthesised as calculated from the initial rate of the synthesis [2]. Thus, taking into account the values found after a 20 min incubation with the enzyme (see Table 2), HRPOD exhibited about 10% of the capacity of PES in catalysing the synthesis of PGs. This figure, however, does not reflect the effective capacity of the enzyme because it is not based on the initial rate values which would be considerably higher (see Fig. 1) and because it does not include the other unidentified biologically active compounds which were synthesised along with PGs.

In conclusion, this appears to be the first report of the synthesis of PGs by peroxidases. This, however, does not seem the only way in which POD metabolises AA. The ability of POD to catalyse peroxidation of AA in the presence of hydroperoxy AA is under investigation in this laboratory.

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