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Fluorimetric Measurement of Hydrogen Peroxide Produced During Aldehyde Oxidase Catalysed Oxidation Using Scopoletin

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A scopoletin-based fluorimetric assay for the measurement of hydrogen peroxide formed by aldehyde oxidase was developed using sodium azide as an inhibitor of scavenging enzymes. The assay involves the coupled oxidation of scopoletin by hydrogen peroxide, catalysed by horseradish peroxidase and subsequent measurement of the change in scopoletin fluorescence. Hydrogen peroxide concentrations were measured against scopoletin fluorescence using two different scopoletin standards ranging from 0.1-2 and 4-40 μM. The overall linear correlation coefficient (r) for hydrogen peroxide concentrations ranging from 0.1-40 μM against fluorescence response is 0.998 (n = 3). The initial rates of hydrogen peroxide production by aldehyde oxidase were similar for both nitrogen heterocycles and aldehyde substrates. Initial rates of hydrogen peroxide formation during the oxidation of 100 μM substrates, indole-3-aldehyde or 2-pyrimidinone by aldehyde oxidase in the presence of 1 mM sodium azide were found to be 86-89% of substrate oxidation. All specific aldehyde oxidase inhibitors such as chlorpromazine ($p<0.001$), menadione ($p<0.05$) and β-estradiol ($p<0.001$), caused highly significant inhibition of hydrogen peroxide production during the oxidation of phthalazine, indole-3-aldehyde, 2-pyrimidinone and phenanthridine by guinea pig liver aldehyde oxidase. The kinetic constants, K_m and V_{max}, for hydrogen peroxide production during phthalazine and indole-3-aldehyde oxidation by guinea pig liver aldehyde oxidase have been determined for the first time using this method.

Key words: Scopoletin, hydrogen peroxide, Aldehyde oxidase, Horseradish peroxidase, fluorimetry

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INTRODUCTION

Reactive Oxygen Species (ROS) play an important role in a variety of physiological and pathological processes[1,2]. As oxidative stress can often be linked to hydrogen peroxide production, quantitative work in this medically and biochemically important field requires reliable methods for measuring rates of hydrogen peroxide formation under varying conditions[3]. Several analytical techniques have been employed for the detection and measurement of hydrogen peroxide concentrations. Thus a manometric method which involved monitoring the gas/solid reaction at an oxygen electrode interface in the presence of hydrogen peroxide and catalase[4]. Several spectrophotometric methods including measurement of hydrogen peroxide absorbance at 230 nm, oxidation of luminol by sodium hypochlorite in the presence of hydrogen peroxide to an excited aminophthalate, which has a maximum wavelength at 431 nm[5] and a method involving oxidation of *N,N*-diethyl-*p*-phenylenediamine (DPD) to the purple coloured species DPD^+ by hydrogen peroxide in presence of peroxidase has been developed[6]. An electrochemical method which determines the voltage difference between working and reference electrodes that is proportional to hydrogen peroxide concentrations was reported[7]. Fluorimetric methods such as oxidation of 10-acetyl-3, 7-dihydroxy-phenoxazine (Ample x Red), 2,2'-azino-bis (3-ethylbenzthiazoline)-6-sulfonic acid, phenol red, homovanillic acid, acetaminophen or 7-hydroxy-6-methoxy-coumarin (scopoletin). Fluorimetric methods are generally based on the oxidation of a non-fluorescent reagent to a fluorescent compound[8-10]. These reactions are catalysed by a peroxidase, usually horseradish peroxidase, in an acidic medium in the presence of hydrogen peroxide as hydrogen donor. In contrast, scopoletin is oxidised by hydrogen peroxide changing from a fluorophore to a non-fluorescent compound[8,11]. However, none of the above methods have been applied for the measurement of hydrogen peroxide production by aldehyde oxidase. Several methods have been developed and employed successfully for monitoring and measurement of enzyme kinetics from the oxidation of substrate in reactions catalysed by aldehyde oxidase; these include High Performance Liquid Chromatography (HPLC) and spectrophotometric monitoring of absorbance changes using either artificial electron acceptors or direct measurement of substrate oxidation or product formation[12]. However, fewer methods have been reported for the measurement of hydrogen peroxide produced during reactions catalysed by aldehyde oxidase[13] or xanthine oxidase[14]. In the present study, a fluorimetric method, utilising the oxidation of scopoletin in the presence of horseradish peroxidase, has been developed to measure hydrogen peroxide production during aldehyde oxidase catalysed oxidation.

MATERIALS AND METHODS

Reagents and materials: Allopurinol, aminotriazole, chlorpromazine, β-estradiol, hydrogen peroxide solution (30%), 2-pyrimidinone, indole-3-aldehyde, menadione, scopoletin, sodium azide, catalase (from bovine liver), horseradish peroxidase (HRP, type VI) and borax®, were obtained from Sigma Chemical Company Ltd., Poole, Dorset, UK. Xanthine was obtained from Koch-Light Laboratories Ltd., Colnbrook-Bucks, UK. Phenanthridine and phthalazine were obtained from Aldrich Chemical Co., Poole, Dorset, UK. Potassium hexacyanoferrate (III) was obtained from May and Baker, Dagenham, UK. All reagents and solvents were of analytical grade. Borate buffer 150 mM pH 10, acetate buffer 80 mM pH 4.7, Sorenson phosphate buffer 67 mM, pH 7 containing 100 mM EDTA were prepared.

Preparation of aldehyde oxidase: Aldehyde oxidase was partially purified from liver homogenate of male/female Dunkin-Hartley guinea pigs (600-700 g) following a published methodology[15].

Preparation of scopoletin solutions: A stock solution of 4 mM scopoletin was prepared by dissolving 3.84 mg in 5 mL dimethylsulphoxide. The stock solution was stored in dark at room temperature and was stable for at least one month under these conditions. Lower concentrations were prepared from the stock solution by dilution with distilled water as required.

Apparatus

Spectrophotometer: Shimadzu 2101 UV/VIS spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, USA), linked to Pye-Unicam cell temperature control unit (Pye-Unicam Ltd., Cambridge, UK). The spectrophotometer was computer-controlled by Shimadzu UV-210 spectroscopy software package with additional kinetics software.

Spectrofluorimeter: In an MPF-3 Spectrofluorimeter with the excitation wavelength set at 395 nm and the emission wavelength set at 470 nm. The slit width was fixed at 6 mm for both excitation and emission, using a 150-watt Xenon Lamp as the light source.

Procedure

Enzyme activity: Oxidation of 100 μM 2-pyrimidinone to uracil by partially purified guinea pig liver aldehyde

oxidase was monitored by following the decrease in absorbance of 2-pyrimidinone at 300 nm using oxygen as the electron acceptor. The molar absorptivity of 2-pyrimidinone at 300 nm is 4,750 Mol^{-1} cm^{-1}[16]. Similarly, oxidation of 50 μM phenanthridine to 6(5*H*)-phenanthridone and 100 μM indole-3-aldehyde to indole-3-acetic acid by partially purified aldehyde oxidase, was monitored directly by following decreases in absorbance at 322 and 300 nm, respectively. Molar absorptivities of 9,000 Mol^{-1} cm^{-1} at 322 nm[17] and 15,000 Mol^{-1} cm^{-1} at 300 nm were used to calculate the initial activities of phenanthridine and indole-3-aldehyde oxidation, respectively. The initial velocity for substrate oxidation was determined by measuring the change in absorbance/minute and calculating enzyme activities in μmol/min/mg protein. The following equation was used to calculate the enzyme activity:

$$\text{Initial rate of substrate oxidation} = \frac{[\text{Absorbance change/min}] \times 1000}{[\text{Molar absorptivity}] \times [\text{Protein concentration in cuvette (mg mL}^{-1})]}$$

The oxidation of 100 μM phthalazine was measured in the presence of the artificial electron acceptor, potassium hexacyanoferrate (III). Reactions were monitored by following the decrease in absorbance at 420 nm resulting from the reduction of 1 mM potassium hexacyanoferrate (III) to potassium hexacyanoferrate (II) The molar absorptivity of potassium hexacyanoferrate (III) is 1,040 Mol^{-1} cm^{-1}, but it should be noted that for each molecule of substrate oxidised two molecules of potassium hexacyanoferrate (III) are reduced and therefore, double the molar absorptivity must be used when calculating enzyme activity (2×1040 Mol^{-1} cm^{-1})[12]. The kinetic constants (K_m and V_{max} values) were calculated as previously described[18].

Measurement of initial rates of hydrogen peroxide produced by aldehyde oxidase using the sequential incubation method: Initial velocities of hydrogen peroxide formation were determined by incubating 100 μM phthalazine, 100 μM indole-3-aldehyde, 100 μM 2-pyrimidinone or 50 μM phenanthridine with partially purified molybdenum hydroxylase fractions in the presence of 1 mM sodium azide. The reactions were initiated by addition of enzyme fractions and aliquots (100 μL) were withdrawn after 20 sec and incubated with 2 μM scopoletin and 12 μg mL^{-1} horseradish peroxidase solution for 5 min in 1 mL acetate buffer pH 4.7 at room temperature. Samples (200 μL) of the scopoletin incubations were added to 1.6 mL borate buffer pH 10 and the fluorescence of each solution was measured. At least two appropriate standards ranging from 1.5 to 6 μM were prepared by dilution of 100 μM standard hydrogen peroxide solution in distilled water at 4°C. Aliquot (100 μL) of standard hydrogen peroxide was incubated with 2 μM scopoletin and 12 μg mL^{-1} horseradish peroxidase for 5 min in 1 mL 80 μM acetate buffer pH 4.7 at room temperature. Aliquot (200 μL) was then added to 1.6 mL borate buffer pH 10 to terminate the reaction and fluorescence of each solution was measured. Initial velocities of hydrogen peroxide formation with molybdenum hydroxylase fractions were calculated by comparison with standard hydrogen peroxide solutions using the following equation. It should be noted that these procedures would result in 1:90 dilution for hydrogen peroxide content.

$$\text{Initial rate of hydrogen peroxide formation} = \frac{[\text{Hydrogen peroxide formed (μM/min]} \times 90}{[1000] \times [\text{Protein concentration in vial (mg mL}^{-1})]}$$

Concomitant incubation of aldehyde oxidase-generated hydrogen peroxide with scopoletin and horseradish peroxidase: Phthalazine (100 μM) was incubated with 0.2 mL partially purified aldehyde oxidase fractions (1:10 dilution) in the presence of 120 μM scopoletin and 30 μg mL^{-1} horseradish peroxidase in 6 mL Sorenson's phosphate buffer at 37°C. The reaction was followed for up to 20 min by withdrawing 100 μL aliquots at different time intervals which were added to 2.9 mL of borate buffer pH 10 to terminate the reaction for reading the fluorescence. From the last mixture, an aliquot of 1 mL was added to 2 mL of borate buffer pH 10 and mixed thoroughly to give the appropriate scopoletin concentration. Fluorescence was measured as described above. Concentration of hydrogen peroxide in each solution was determined by comparison with standard solutions, which are described below. Hydrogen peroxide standards (30 and 60 μM) were incubated with 120 μM scopoletin in the presence of 30 μg mL^{-1} horseradish peroxidase in 6 mL Sorenson's phosphate buffer, at 37°C for 5 min. Aliquots (100 μL) were added to 2.9 mL borate buffer pH 10 and mixed thoroughly. An aliquot (1 mL), from the last mixture, was added to 2 mL of borate buffer pH 10 to give the appropriate scopoletin concentration and the fluorescence measured directly.

Effect of scopoletin, horseradish peroxidase and sodium azide on oxidation catalysed by guinea pig liver aldehyde oxidase: The oxidation of 100 μM phthalazine, 100 μM indole-3-aldehyde and 100 μM 2-pyrimidinone by partially purified molybdenum hydroxylase fractions was monitored spectrophotometrically in the presence of

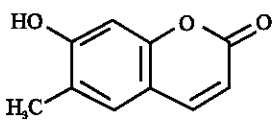
varying concentrations of scopoletin (12, 24, 60 and 120 μM) and 30 μg mL^{-1} horseradish peroxidase. The effect of varying concentrations of sodium azide (100, 200, 500, 1000 and 5000 μM) on the oxidation of 100 μM phthalazine and 100 μM 2-pyrimidinone by guinea pig liver aldehyde oxidase was also determined.

Determination of kinetic constants for the oxidation of phthalazine and indole-3-aldehyde by guinea pig liver aldehyde oxidase: Stock solutions of 6 mM phthalazine and indole-3-aldehyde were prepared and diluted with Sorenson's phosphate buffer, to appropriate concentrations. At least six different dilutions of phthalazine were prepared to give final concentrations of 10 to 500 μM and eight different dilutions of indole-3-aldehyde were prepared to give concentrations ranging from 2.5 to 50 μM. The initial velocities, v, associated with each substrate concentration [S] were measured. The Michaelis-Menten constants, K_m and maximum velocities, V_{max}, were determined from Lineweaver-Burk double reciprocal plots of 1/V versus 1/[S] using the Shimadzu kinetics software.

Calibration curves for standard hydrogen peroxide solutions determined from scopoletin fluorescence: Calibration curves were prepared for two sets of hydrogen peroxide concentrations ranging from 0.1-2 and 4-40 μM. varying concentrations of hydrogen peroxide were prepared by dilution with distilled water from standard solutions (30% H_2O_2). For hydrogen peroxide concentrations of 0.1-2 μM, 100 μL appropriately diluted standard was incubated with 4 μM scopoletin and 12 μg mL^{-1} horseradish peroxidase in 1 mL acetate buffer pH 4.7 at room temperature for 5 min. For hydrogen peroxide concentrations ranging from 4-40 μM, each 100 μL of the standard was incubated with 50 μM scopoletin and 12 μg mL^{-1} horseradish peroxidase in 1 mL acetate buffer pH 4.7 at room temperature for 5 min. Aliquots (200 μL) of the previous incubations were added to 1.6 mL borate buffer pH 10 and the fluorescence was measured. The fluorescence intensity was plotted *versus* the concentration of hydrogen peroxide to get the calibration graph.

RESULTS AND DISCUSSION

Scopoletin, 7-hydroxy-6-methoxycoumarin (Fig. 1), a naturally occurring component in cotton leaf and citrus peel, is a fluorescent substrate for peroxidase. Andreae[8] found that scopoletin fluorescence decreases in the presence of hydrogen peroxide and peroxidase. In the present study, scopoletin fluorescence was found to be



Scopoletin

Fig. 1: Structure of scopoletin

Table 1: Intra and inter-assay variation for scopoletin oxidation by standard hydrogen peroxide solutions using the fluorimetric assay based on scopoletin-horseradish peroxidase (Mean±SD)

Hydrogen peroxide concentration	Intra-assay		Inter-assay	
	Response	CV (%)	Response	CV (%)
30 μM	21.7±0.25 (n = 5)	1.15	22.2±0.41 (n = 7)	1.85
15 μM	10.9±0.086 (n = 5)	0.78	11.5±0.26 (n = 5)	2.26
7.5 μM	5.4± 0.053 (n = 5)	0.98	5.9±0.12 (n = 5)	2.03

proportional to concentration in the range 0.025-1 μM in borate buffer pH 10 (n = 3, r = 0.999). In agreement with previous observations, the hydrogen peroxide assay used in this study yields a linear fluorimetric response for hydrogen peroxide concentrations ranging from 0.1 to 40 μM[19,20]. Hydrogen peroxide concentrations were calibrated against scopoletin fluorescence using two different scopoletin standards. The lower hydrogen peroxide concentrations ranging from 0.1-2 μM were determined using a scopoletin concentration of 4 μM and corresponded to hydrogen peroxide concentrations produced in kinetic studies (n = 3, r = 0.996). Higher hydrogen peroxide concentrations ranging from 4-40 μM were determined using a scopoletin concentration of 50 μM; these were similar to hydrogen peroxide concentrations formed during reaction progress curves (n = 3, r= 0.997). The overall linear correlation coefficient (r) for hydrogen peroxide concentrations ranging from 0.1-40 μM against fluorescent response is 0.998 (n = 3). When scopoletin was incubated with either hydrogen peroxide or horseradish peroxidase alone there was no change in scopoletin fluorescence. This indicates that scopoletin was oxidised only in the presence of both hydrogen peroxide and horseradish peroxidase at pH 4.7 in acetate buffer. The reproducibility of the system for hydrogen peroxide measurement was calculated using coefficient of variation (CV%) as shown in Table 1. The inter-assay variation found with the present method are lower than other reports[9,11], de Sandro *et al.*[11] reported much higher variation values of approximately 5% (n = 3). Even with the electrode assay, the CV% for hydrogen peroxide measurements was >4.3% (n = 3)[7]. This variation in the CV% may be due to the liability of hydrogen peroxide.

Preliminary study to measure hydrogen peroxide product during aldehyde oxidase-catalysed oxidation: In a preliminary study hydrogen peroxide production by partially purified guinea pig liver aldehyde oxidase was monitored using phthalazine as a substrate. The formation of hydrogen peroxide was followed fluorimetrically during either concomitant or sequential incubations as described in Experimental Section. With the concomitant method, when 100 μM phthalazine was incubated directly with guinea pig liver aldehyde oxidase, 120 μM scopoletin and 30 μg mL^{-1} horseradish peroxidase in 6 mL Sorenson's phosphate buffer, pH 7.0, approximately 30% substrate turnover to hydrogen peroxide was obtained in 20 min (Fig. 2). However, using the sequential method, where 100 μM phthalazine was incubated initially with guinea pig liver aldehyde oxidase prior to a second incubation with scopoletin and horseradish peroxidase, no hydrogen peroxide was detected over 20 min. This was thought to be due to the presence of scavenging enzymes in the guinea pig liver enzyme preparation. Optimisation of the incubation conditions and the effect of scopoletin and sodium azide on aldehyde oxidase catalysed oxidation and hydrogen peroxide formation are described in the following section.

Effect of catalase and enzyme inhibitors on the stability of hydrogen peroxide: Approximately 98% breakdown of hydrogen peroxide occurred in 1 min of incubation of 10 μM hydrogen peroxide with enzyme preparation (Table 2, Incubations A and B). This indicates that guinea pig liver fractions contain a scavenging enzyme or chemical antioxidant which rapidly inactivates hydrogen peroxide. Guinea pig liver has been shown to contain high amounts of both catalase and glutathione peroxidase in the cytoplasm as well as peroxisomes[21]. Himeno *et al.*[22] have reported that catalase activity in guinea pig is 2-3 times higher than that of mice and rats and more than 90% of catalase activity was found in the soluble fraction of guinea pig liver. A similar effect on hydrogen peroxide was found when hydrogen peroxide (10 μM) was incubated with catalase (200 units) for up to 5 min. In the presence of 200 units-catalase approximately 95% hydrogen peroxide disappeared in one minute (Table 2, Incubation C). The decomposition of hydrogen peroxide by catalase (200 units) was inhibited in the presence of 1 mM sodium azide (an inhibitor of scavenging enzymes) by more than 78% after one minute of incubation (Table 2, Incubation D). In an attempt to investigate the reason(s) for hydrogen peroxide breakdown with the addition of molybdenum hydroxylase fractions, hydrogen peroxide (10 μM) was incubated with partially purified aldehyde oxidase in presence of 1 mM sodium azide or 100 μM

Table 2: Effect of catalase (200 units), sodium azide (1 mM), mercuriacetate (100 μM), aminotriazole (20 mM) and partially purified guinea pig liver aldehyde oxidase on the decomposition of hydrogen peroxide (10 μM) in one minute in 67 mM Sorenson's phosphate buffer, pH 7.0, containing 100 μM EDTA at 37°C (n = 3)

Incubation mixture	Contents of the incubation mixture			Hydrogen peroxide concentration (μM) ±SEM
	Catalase	Enzyme preparation	Additives	
A	-	-	-	9.70±0.53
B	-	√	-	0.20±0.50
C	√	-	-	0.47±0.22
D	√	-	Sodium azide	7.60±0.72
E	-	√	Sodium azide	9.40±0.63
F	-	√	Mercuriacetate	0.25±0.75
G	-	√	Aminotriazole	0.68±0.39
H	√	-	Aminotriazole	9.60±0.81

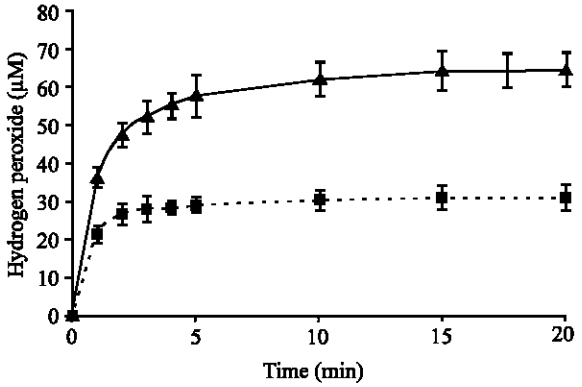


Fig. 2: Reaction progress of hydrogen peroxide production during the oxidation of 100 μM phthalazine with partially purified guinea pig liver aldehyde oxidase (-■- Concomitant incubation; -▲- Sequential incubation in the presence of 5 mM sodium azide, mean±SEM, n = 3)

mercuric acetate, an indirect inhibitor of glutathione peroxidase which reacts with reduced glutathione. Mercuric acetate failed to restore the recovery of hydrogen peroxide (Table 2, Incubations F). In contrast, sodium azide (1 mM) almost completely restored the recovery of hydrogen peroxide (10 μM) in the presence of 0.2 mL partially purified aldehyde oxidase fractions (97%) (Table 2, Incubation E). The addition of sodium azide was also effective at reducing hydrogen peroxide breakdown by catalase (Table 2, Incubation D). This may indicate that sodium azide protects hydrogen peroxide in the aldehyde oxidase incubation *via* the inhibition of catalase. Interestingly, 20 mM aminotriazole, a specific catalase inhibitor[23], did not protect hydrogen peroxide from breakdown when incubated with partially purified molybdenum hydroxylase fractions (Table 2, Incubation G) whereas the decomposition of hydrogen peroxide by catalase (200 units) was significantly inhibited by aminotriazole (~99%) (Table 2, Incubation H).

This may indicate that enzymes or other antioxidants other than catalase may participate in hydrogen peroxide decomposition in the aldehyde oxidase incubation. However, Williams *et al.*[24] reported that aminotriazole requires metabolic transformation to an active metabolite prior to reaction with catalase. This reaction would not occur in partially purified enzyme, which may lack enzyme(s) that catalyse aminotriazole transformation. Alternatively, while aminotriazole is a specific catalase inhibitor, sodium azide has a non-specific action and inhibits other enzymes such as peroxidases as well as catalase[25]. Sodium azide inhibits the hydrogen peroxide-activated of scavenging enzymes intermediate by reaction with the iron residue to form an inactive azidyl complex and preventing reduction to Fe (II)[26]. The inhibitor constants (K_i) for the reaction of sodium azide with catalases and peroxidases are 1-2 μM and 0.1-1.5 mM, respectively[25,26]. In this study, sodium azide protected hydrogen peroxide from breakdown during aldehyde oxidase catalysed reactions. Optimisation of sodium azide concentration is described in the following section.

Determination of optimum conditions

Effect of varying sodium azide concentrations on hydrogen peroxide formation: Hydrogen peroxide production during the oxidation of 100 μM phthalazine by guinea pig liver aldehyde oxidase was followed for 15 min in the presence of sodium azide concentrations ranging from 0-1 mM using the sequential method. In the presence of 1 mM sodium azide, hydrogen peroxide concentrations increased rapidly up to 5 min after which they remained constant for 15 min (Fig. 3). However, initial rates of hydrogen peroxide production were lower with sodium azide concentrations less than 1 mM.

Effect of varying sodium azide concentrations on substrate oxidation: As shown in Table 3, sodium azide had an inhibitory effect on substrate oxidation catalysed by guinea pig liver aldehyde oxidase which was more pronounced with phthalazine than 2-pyrimidinone as a substrate. However, it should be noted that phthalazine oxidation was monitored indirectly using the electron acceptor, potassium hexacyanoferrate (III), whereas 2-pyrimidinone oxidation was followed directly by following the decrease in absorbance of 2-pyrimidinone at 300 nm. Inhibition of aldehyde oxidase-catalysed oxidation by sodium azide could be due to enzyme inhibition or enzyme inactivation due to the presence of hydrogen peroxide. Aldehyde oxidase contains two Fe/S redox group which could react with sodium azide[27]. However, Rajagopalan *et al.*[13] and Terada *et al.*[28] have suggested that hydrogen peroxide accumulation results in

Table 3: Effect of different sodium azide concentrations on the oxidation rates of 100 ∝M phthalazine and 2-pyrimidinone by partially purified guinea pig liver aldehyde oxidase monitored spectrophotometrically (Mean±SD, n = 3)

Sodium azide concentration (μM)	100 μM phthalazine		100 μM 2-pyrimidinone	
	Activity (μmol/min/mg)	% Inhibition*	Activity (μmol/min/mg)	% Inhibition*
Control	0.357±0.041	-	0.263±0.032	-
100	0.334±0.062	6.4	0.261±0.036	0.8
200	0.321±0.053	10.1	0.255±0.041	3.0
500	0.312±0.056	12.6	0.249±0.030	5.6
1000	0.283±0.065	20.7	0.241±0.042	8.3
5000	0.252±0.057	29.4	0.232±0.037	11.7

*All results are presented as percentage inhibition of control values in absence of sodium azide

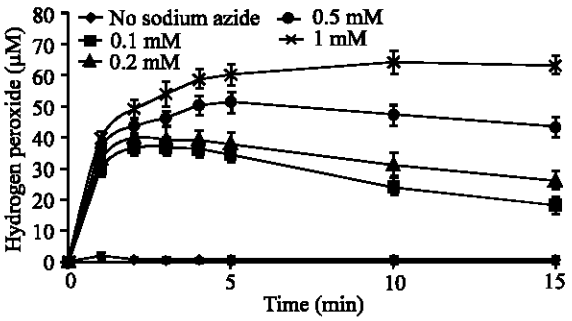


Fig. 3: Reaction progress of hydrogen peroxide production by the oxidation of 100 μM phthalazine with partially purified guinea pig liver aldehyde oxidase in the presence of sodium azide (0.1, 0.2, 0.5, 1 mM) in 67 mM Sorenson's phosphate buffer, pH 7.0, containing 100 μM EDTA at 37°C, (mean±SEM, n = 3)

enzyme autoinactivation via catalase inhibition. In either case, similar effects would be expected on the oxidation of phthalazine and 2-pyrimidinone. As can be seen from Fig. 3, the highest hydrogen peroxide levels are formed in the presence of 1 mM sodium azide and thus the inhibition of substrate oxidation is likely to be due to a direct effect on aldehyde oxidase. In view of the differences between the results obtained with phthalazine and 2-pyrimidinone, it is thought that sodium azide reacts with Fe/S group. This is the site of interaction with potassium hexacyanoferrate (III), whereas the enzyme is re-oxidised by oxygen via flavin during the oxidation of 2-pyrimidinone. Sodium azide reacts with the iron-containing heme groups in scavenging enzymes[25,26]. This would be consistent with the observation that sodium azide inhibits phthalazine oxidation to a greater extent than that of 2-pyrimidinone by preventing electron transfer from enzyme to the electron acceptor, potassium hexacyanoferrate (III). These results indicate that 1 mM sodium azide is the optimum concentration which

Table 4: Effect of different scopoletin concentrations on the oxidation of 100 μM phthalazine, indole-3-aldehyde or 2-pyrimidinone catalysed by partially purified guinea pig liver aldehyde oxidase monitored spectrophotometrically (Mean±SD, n = 3)

		% Inhibition in the substrate oxidation rates			
		Scopoletin concentration (μM)			
Substrates	Control μmol/min./mg protein	12 (p<0.1)	24 (p<0.05)	60 (p<0.02)	120 (p<0.01)
Phthalazine	0.382±0.051	17±2	28±3	37±4	56±3
Indole-3-aldehyde	0.168±0.023	30±4	32±4	39±4	49±3
2-Pyrimidinone	0.276±0.026	13±2	25±3	41±5	61±5

Table 5: Comparison between hydrogen peroxide formation in one minute of incubation using sequential- and concomitant-incubations catalysed by partially purified guinea pig liver aldehyde oxidase

Concentration of hydrogen peroxide formed in one minute by aldehyde oxidase during the oxidation of: (μM)							
Indole-3-aldehyde (100 μM) (n = 7, Mean±SD)				2-Pyrimidinone (100 μM) (n = 4, Mean±SD)			
Sequential incubation		Concomitant incubation		Sequential incubation		Concomitant incubation	
Control	Sodium azide + (1 mM)	+ Scopoletin + HRP	+ Sodium azide + scopoletin + HRP	Control	+Sodium azide (1 mM)	+ Scopoletin + HRP	+ Sodium azide + scopoletin + HRP
3.3±0.75	31.2±2.9***	21.9±3.3**	10.5±3.0*	2.4±1.0	29.1±2.6***	19.8±2.6**	14.7±2.3*

Using a 1-tailed paired students t-test: *p<0.05, **p<0.02, ***p<0.01.

maintains hydrogen peroxide concentrations with minimum effect on the enzyme activity, particularly using 2-pyri.

Effect of scopoletin on substrate oxidation catalysed by guinea pig liver aldehyde oxidase: The lower rates of hydrogen peroxide formation in concomitant incubations (Fig. 2) could be due to interaction between incubation components with either horseradish peroxidase or with aldehyde oxidase. The effect of substrates and inhibitors on horseradish peroxidase activity was found to be negligible. This was investigated by addition of 10 μM hydrogen peroxide to horseradish peroxidase-scopoletin incubation in the presence and absence of different substrates/inhibitors. Consequently, 100 μM phthalazine, indole-3-aldehyde or 2-pyrimidinone was incubated separately with aldehyde oxidase and monitored spectrophotometrically in the presence of varying concentrations of scopoletin. In this study, scopoletin inhibited the oxidation of phthalazine, indole-3-aldehyde and 2-pyrimidinone catalysed by aldehyde oxidase (Table 4). Initial oxidation rates were decreased by up to 60% with 120 μM scopoletin; however, it would appear that scopoletin is a progressive inhibitor of aldehyde oxidase as inhibition was more marked as the reaction proceeded. With all scopoletin concentrations >95% inhibition of reaction rates were obtained after 2 min of incubation. These results indicate that aldehyde oxidase activity, rather than horseradish peroxidase, is inhibited in the presence of scopoletin in concomitant incubations. Interestingly, scopoletin is structurally related to menadione, a potent and selective inhibitor for aldehyde oxidase[29].

Table 6: Initial rates of hydrogen peroxide production during oxidation of phthalazine and indole-3-aldehyde by guinea pig liver aldehyde oxidase measured up to 90 sec, Mean±SD (n = 3)

	Initial rates of hydrogen peroxide production by guinea pig liver aldehyde oxidase with (μmol/min/mg protein):	
Time interval (sec)	Phthalazine (100 μM)	Indole-3-aldehyde (100 μM)
20	0.273±0.038	0.319±0.044
40	0.256±0.022	0.286±0.031
60	0.191±0.032	0.251±0.025
90	0.138±0.041	0.203±0.023

Selection of optimum incubation conditions for hydrogen peroxide assay: Table 5 summaries the effects of sodium azide and scopoletin on hydrogen peroxide formation during the oxidation of indole-3-aldehyde and 2-pyrimidinone by aldehyde oxidase using sequential and concomitant incubations. Maximal yields of hydrogen peroxide were observed in sequential incubations containing 1 mM sodium azide with only 10% hydrogen peroxide formation occurring in control sequential incubations (without sodium azide). Hydrogen peroxide formation was lower in concomitant incubations, which contained 120 μM scopoletin and the presence of 1 mM sodium azide further reduced hydrogen peroxide concentrations. This is consistent with inhibition of aldehyde oxidase in concomitant incubations by both scopoletin and sodium azide (Table 3 and 4). Consequently, all kinetic assays to measure hydrogen peroxide concentrations were carried out using sequential incubations containing 1 mM sodium azide, which provide protection for hydrogen peroxide with minimal effect on aldehyde oxidase activity.

Optimisation of sampling time for the measurement of initial rates of hydrogen peroxide production by guinea pig liver aldehyde oxidase: The initial rates of hydrogen

Table 7: Effects of potent aldehyde oxidase and xanthine oxidase inhibitors on hydrogen peroxide production by partially purified guinea pig liver molybdenum hydroxylase fractions using phthalazine, indole-3-aldehyde, 2-pyrimidinone and phenanthridine as substrates, mean±SD (n = 4)

	Hydrogen peroxide production (μmol/min/mg protein) with oxidation of (percentage inhibition)			
Inhibitors	Phthalazine (100 μM)	Indole-3-aldehyde (100 μM)	2-Pyrimidinone (100 μM)	Phenanthridine (50 μM)
Control	0.252±0.020	0.311±0.024	0.245±0.022	0.185±0.016
Chlorpromazine (100 μM)	0.008±0.001 (97)	0.006±0.002 (98)	0.010±0.001 (96)	0.009±0.003 (95)
Menadione (100 μM)	0.035±0.003 (86)	0.083±0.003 (73)	0.041±0.005 (83)	0.035±0.003 (81)
β-Estradiol (10 μM)	0.003±0.007 (99)	0.003±0.001 (99)	0.005±0.001 (98)	0.004±0.001 (98)
Allopurinol (100 μM)	0.252±0.036 (0.0)	0.296±0.025 (5)	0.233±0.031 (5)	0.178±0.018 (3.5)

Table 8: Calculated kinetic parameters for hydrogen peroxide formation during substrate oxidation, catalysed by guinea pig liver aldehyde oxidase, Mean±SD (n = 4)

	Substrate	
	Indole-3-aldehyde (100 μM) n = 6	Phthalazine (100 μM) n = 4
K_m (μM)	11.300±1.4	32.200±2.8
V_{max} (μmol/min/mg protein)	0.358±0.055	0.333±0.052
V_{max} / K_m (mL/min/mg protein)	29.5	11.1

peroxide production with the oxidation of either 100 μM phthalazine or 100 μM indole-3-aldehyde by guinea pig liver aldehyde oxidase were determined at different time intervals (20, 40, 60 and 90 sec) using the sequential assay. The reaction was started with addition of the diluted enzyme to the incubation mixture and aliquots were withdrawn at different time intervals. The results are summarised in Table 6. It would appear that the activity of guinea pig liver aldehyde oxidase decreased with time. The initial rates of hydrogen peroxide production with either phthalazine or indole-3-aldehyde were 2-3 folds higher at 20 sec than those measured at 90 sec (Table 6). This could be a result of enzyme autoinactivation by hydrogen peroxide as suggested previously[13,28]. Consequently, 20 sec was chosen as the sampling time for measurement of initial rates of hydrogen peroxide production catalysed by guinea pig liver aldehyde oxidase.

Dependence of scopoletin oxidation on aldehyde oxidase concentration: Hydrogen peroxide production during the oxidation of 100 μM phthalazine by guinea pig liver aldehyde oxidase using the sequential assay was determined with different aldehyde oxidase concentrations ranging form 0.045 to 0.36 mg mL^{-1} (n = 3, r = 0.9997). It was found that the initial activity of hydrogen peroxide production was proportional to the amount of the enzyme added to the incubation mixture. Hence, increasing enzyme concentration leads to increase in the hydrogen peroxide formation in the presence of 100 μM phthalazine as a substrate. The concentrations of enzyme used to determine the initial rates of hydrogen peroxide production in this study ranged from 0.085 to 0.30 mg mL^{-1}.

Characterisation of aldehyde oxidase involvement in hydrogen peroxide formation in partially purified guinea pig enzyme fractions: All substrates and inhibitors were tested prior to measurement of scopoletin fluorescence for any interaction and found to be inert. Initial velocities of hydrogen peroxide production were determined by incubating phthalazine, indole-3-aldehyde, 2-pyrimidinone or phenanthridine separately with partially purified molybdenum hydroxylase fractions in the presence of the potent aldehyde oxidase inhibitors, chlorpromazine, menadione and β-estradiol and the specific xanthine oxidase inhibitor, allopurinol. Table 7 summarises the results obtained with the various inhibitors. All specific aldehyde oxidase inhibitors, chlorpromazine ($p<0.001$), menadione ($p<0.05$) and β-estradiol ($p<0.001$), caused highly significant inhibition of hydrogen peroxide production during the oxidation of phthalazine, indole-3-aldehyde, 2-pyrimidinone and phenanthridine by guinea pig liver aldehyde oxidase. On the basis of previous studies on substrate oxidation using similar inhibitor concentrations, 98-100% inhibition was obtained, which indicates that substrates are oxidised by aldehyde oxidase alone[16]. Accordingly, almost 100% inhibition of hydrogen peroxide production was observed with chlorpromazine and β-estradiol. Menadione is one of the most widely used inhibitors to characterise aldehyde oxidase from different species[13,29], nevertheless, it serves as an electron acceptor for xanthine oxidase and may enhance the oxidation rates[30]. Inhibition of hydrogen peroxide production by guinea pig liver molybdenum hydroxylase fractions by menadione was only 70-80% of control values. It has been suggested that menadione exerts its inhibitory effect through a mechanism different to that of chlorpromazine and β-estradiol, which may explain why menadione is less effective than chlorpromazine and β-estradiol in inhibiting hydrogen peroxide formation[31]. Allopurinol had very little effect on hydrogen peroxide production. Although reaction rates were reduced slightly with indole-3-aldehyde, 2-pyrimidinone and phenanthridine as substrates, hydrogen peroxide production during xanthine oxidation by partially purified guinea pig liver molybdenum

Table 9: Initial rates of hydrogen peroxide formation and substrate oxidation, catalysed by guinea pig liver aldehyde oxidase, measured by fluorimetric and spectrophotometric methods in the presence of 1 mM sodium azide, respectively

	Substrate:			
	Indole-3-aldehyde (100 μM) n = 7		2-Pyrimidinone (100 μM) n = 4	
	Hydrogen peroxide formation	Indole-3-acetic acid formation	Hydrogen peroxide formation	Uracil formation
Initial rates (μmol/min/mg protein)	0.335±0.021	0.392±0.018	0.248±0.015	0.279±0.012
(Initial rate of hydrogen peroxide/initial rate of product formation) x 100	86%		89%	

hydroxylase was inhibited in the presence of allopurinol by 97%. Even though allopurinol is a potent inhibitor of xanthine oxidase, it is relatively weak substrate for aldehyde oxidase with K_m= 0.38 mM[32]. Thus the reductions in hydrogen peroxide production observed in Table 7 are thought to arise from allopurinol competing as a substrate for aldehyde oxidase rather than inhibitory activity. However, incubation of 100 μM allopurinol alone with partially purified guinea pig liver molybdenum hydroxylase demonstrated no evidence of hydrogen peroxide production. Furthermore, compared to the rates of hydrogen peroxide formation with aldehyde oxidase substrates, hydrogen peroxide production with xanthine (50 μM) turnover was very slow (0.0288±0.0053 μmol/min/mg protein, n = 3). Therefore, it is unlikely that xanthine oxidase contributes to hydrogen peroxide formation in partially purified molybdenum hydroxylase fractions.

Determination of kinetic constants for hydrogen peroxide production during the oxidation of phthalazine or indole-3-aldehyde catalysed by guinea pig liver aldehyde oxidase: The kinetic constants for hydrogen peroxide production by aldehyde oxidase were determined using the sequential incubation method as described in section 2.5.2. In this study, hydrogen peroxide production during phthalazine or indole-3-aldehyde oxidation follows Michaelis-Menten kinetics (Table 8). The catalytic efficiency (V_{max}/K_m) for hydrogen peroxide formation during indole-3-aldehyde oxidation is approximately 2.5-fold higher than that during phthalazine oxidation with guinea pig liver aldehyde oxidase. These results are in very close agreement with the results for substrate oxidation reported previously by Peet[33] as well as our results for substrate oxidation. The kinetic parameters for the oxidation of indole-3-aldehyde and phthalazine by aldehyde oxidase are compared with kinetic constants calculated for both hydrogen peroxide and superoxide anion production[34].

A scopoletin-based fluorimetric assay for the noncontinuous, sequential measurement of hydrogen peroxide formation by aldehyde oxidase has been successfully developed. The assay involves the coupled oxidation of scopoletin by hydrogen peroxide, catalysed by horseradish peroxidase and subsequent measurement of the change in scopoletin fluorescence. The superior sensitivity and stability of scopoletin may make this hydrogen peroxide probe a useful tool for detecting hydrogen peroxide formation in biological systems[19]. In addition it is relatively simple, accurate and inexpensive compared to other fluorimetric methods[10,35]. The assay is suitable for use when only limited amounts of enzyme are available, for instance in aldehyde oxidase preparations from brain, or to study low oxidation rates. In this novel method, sodium azide was found to protect hydrogen peroxide by inhibiting scavenging enzymes. However, sodium azide was also shown to be a weak inhibitor of aldehyde oxidase. An optimum concentration of 1 mM sodium azide was found to maximise hydrogen peroxide measurement with a minimal effect on aldehyde oxidase activity. By using this method, it was possible to determine the initial rates of hydrogen peroxide formation catalysed by aldehyde oxidase at varying substrate concentrations. In addition, the initial rates for hydrogen peroxide formation have been determined in the presence of potent aldehyde oxidase and xanthine oxidase inhibitors. Almost complete inhibition of initial rates of hydrogen peroxide formation by aldehyde oxidase was obtained in the presence of chlorpromazine, β-estradiol or menadione (Table 7). Initial rates of hydrogen peroxide formation during the oxidation of 100 μM substrates, indole-3-aldehyde or 2-pyrimidinone by aldehyde oxidase in the presence of 1 mM sodium azide were found to be 86-89% of substrate oxidation (Table 9). Hydrogen peroxide production during the oxidation of phthalazine and indole-3-aldehyde was found to follow Michaelis-Menten kinetics. Although hydrogen peroxide formation from aldehyde oxidase-catalysed oxidation has been followed using indirect assay, kinetic constants for the reaction have not previously been reported[13,36]. The availability of this method now facilitates a direct comparison between substrate oxidation, hydrogen peroxide formation and superoxide anion production catalysed by aldehyde oxidase[34]. It is now possible to compare the kinetic parameters, K_m and V_{max} values, for all three processes simultaneously.

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