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Role of Fish Hepatic Aldehyde Oxidase in Oxidative *in vitro*Metabolism of Phenanthridine Heterocyclic Aromatic Compound

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***Abstract*—**Aldehyde oxidase is molybdo-flavoenzyme involved in the oxidation of hundreds of endogenous and exogenous and N-heterocyclic compounds and environmental pollutants. Uncharged N-heterocyclic aromatic compounds such phenanthridine are commonly distributed pollutants in soil, air, sediments, surface water and groundwater, and in animal and plant tissues. Phenanthridine as uncharged N-heterocyclic aromatic compound was incubated with partially purified aldehyde oxidase from rainbow trout fish liver. Reversed-phase HLPC method was used to separate the oxidation products from phenanthridine and the metabolite was identified. The 6(5H)-phenanthridinone was identified the major metabolite by partially purified aldehyde oxidase from fish liver. Kinetic constant for the oxidation reactions were determined spectrophotometrically and showed that this substrate has a good affinity (K_m = 78 ± 7.6μM) for hepatic aldehyde oxidase, will be a significant pathway. This study confirms that partially purified aldehyde oxidase from fish liver is indeed the enzyme responsible for the *in vitro* production 6(5H)-phenanthridinone metabolite as it is a major metabolite by mammalian aldehyde oxidase, coupled with a relatively high oxidation rate (0.77± 0.03 nmol/min/mg protein). In addition, the kinetic parameters of hepatic fish aldehyde oxidase towards the phenanthridine substrate indicate that *in vitro* biotransformation by hepatic fish aldehyde oxidase will be a significant pathway. This study confirms that partially purified aldehyde oxidase from fish liver is indeed the enzyme responsible for the *in vitro* production 6(5H)-phenanthridinone metabolite as it is a major metabolite by mammalian aldehyde oxidase.

***Keywords*—**Aldehyde oxidase, Fish, Phenanthridine, Specificity.

I. Introduction

ALDEHYDE oxidase (AO; EC 1.2.3.1) is molybdo-flavoenzyme involved in the oxidation of hundreds of endogenous and exogenous aldehydes and N-heterocycliccompounds many of which are drugs, vitamins and environmental pollutants[1]-[5]. AO is cytosolic enzyme belonging to a class of enzymes termed molybdenum hydroxylases [1]-[5]. Molybdenum hydroxylases are found in nearly every organism from humans to bacteria and have been determined to be present in a number of various tissues [1]-[5].

The examinations of AO from various different fish species have been studied [6], [7]. The roles of AO in fish have been examined in the metabolism of pollutants and the use of AO as biomarkers in response to pollution [8]-[10].The AO in fish is responsible for metabolism of AO substrates such as endogenous vitamins [11].

Heterocyclic aromatic compounds are commonly distributed pollutants in soil, air, sediments, surface water and groundwater, and in animal and plant tissues [12]. The higher polarity and water solubility of the heterocyclic compounds is developed by the substitution of one carbon atom by nitrogen, sulfur or oxygen heteroatoms(NSO-HET)[13]. As well as a consequence, many heterocyclic compounds are mentioned to be included in the priority list of the European Water Framework Directive [14]. Heterocyclic aromatic compounds are known to show a large range of ecotoxic effects, e.g. acute toxicity, developmental and reproductive toxicity, cytotoxicity, photo-induced toxicity, mutagenicity, and carcinogenicity [12], [15]. Furthermore, some studies have shown that NSO-HET bioaccumulate in aquatic organisms, and acute toxicity has been reported for Daphnia, midge, and algae [16]-[18]. Almost all studies on heterocyclic compounds have been concentrated on heterocycles with an incorporated nitrogen atom that belong to the class of azaarenes such phenanthridine[16], [17], [19]. Azaarenes belonging to the new identified classes of anthropogenic pollutants. Anthropogenic reaction of azaarenes is probably the main source of marine and lake sediments [20], [21]. Street dust is animportant source of sedimentary azaarenes [22]. Similar material can be washed from streets and roads by rainfall, carried by rivers and streams, and ultimately accumulated in the sediments. Outcome of atmospheric particulate matter containing azaarenes from fossil fuel combustion is another source [22]. Bleekeret al. (2001) examined the metabolism of phenanthridine, as azaarene, by cytochrome P450 in Carp (*Cyprium carpio*) into its metabolite 6(5H)-phenanthridinone (phenanthridone) [23]. The oxidative product, 6(5H)-phenanthridinone is a cyclic lactam substituted adjacent to a heterocyclic nitrogen atom (Fig. 1) and it is a major product of phenanthridine *invitro* metabolism by aldehyde oxidase[1], [2], [24]. Because of there is only work on the biotransformation of phenanthridine remains that of Bleekeret al. examined the metabolism of phenanthridine by cytochrome P450 in Carp fish (*Cyprium carpio*) into its metabolite 6(5H)-phenanthridinone [23]. So, present study aims to identify the major metabolites of phenanthridine by partially purified aldehyde oxidase from rainbow trout fish liver. The specificity of liver rainbow trout fish (*Oncorhynchus mykiss*) aldehyde oxidase for this substrate was quantitatively explored by

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determining kinetic constants for phenanthridine as N-heterocyclic aromatic compound.

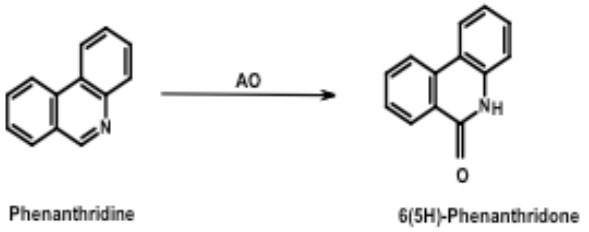

Fig. 1 Oxidation of phenanthridine to 6(5H)-phenanthridone by AO Based on [1]

II. Materials and Methods

A. Chemicals

Phenanthridine, 6(5H)-phenanthridone, absolute ethanol and acetonitrile were purchased from Fisher Scientific and Sigma/Aldrich Chemical Company Ltd, Poole, UK. Solvents forchromatography were all of HPLC grade purity.Stock standard solutions of the phenanthridine and 6(5H)-phenanthridone (1mM) were made by dissolving in absolute ethanol.Solutions of lower concentrations were prepared by appropriate dilution of the stock solution with 50mM phosphate buffer saline (PBS) (pH 7.4).

B. Preparation of Aldehyde Oxidase Fractions

The liver rapidly excised from rainbow trout fish and weighted, chopped and placed immediately in 3-4 volumes of ice-cold isotonic potassium chloride solution (0.154M) containing 0.1mM EDTA and homogenized on ice in a homogenizer fitted with a Teflon pestle for 1-2min at 4°C.The homogenate was then centrifuged at 4^0C for 15 minutes at 10,000 xg, to pellet out the tissue and nuclear/mitochondrial fractions of the homogenate. The supernatant was then removed and centrifuged for a further 60 minutes at 4^0C at 105,000 xg to obtain the cytosolic fraction. This fraction was then collected separated into 0.5ml aliquots and stored at -80^0C. Partially purified aldehyde oxidase was prepared from liver supernatant by heat treatment and ammonium sulfate precipitation, as described by [25].The fractionswerestored in liquid nitrogen until used for spectrophotometric and HPLC analyses.

C. Spectrophotometric Measurement of Enzyme Activity

All spectrophotometric determinations were carried at 37°C using Gen5™ software on a Windows XP PC connected to the microplate reader spectrophotometer (BioTek, UK). Enzyme activity of partially purified was monitored using phenanthridine [25]. The oxidation of phenanthridine was assayed by monitored the increase in absorbance at 322nm (ϵ = 6,400$M^{-1}cm^{-1}$) using molecular oxygen as the electron acceptor. All reactions were carried out in 50 mMPBS buffer pH 7.4. Phenanthridine substrate was tested for non-enzymatic interaction with PBS buffer prior to the addition of enzyme to wells (cuvettes).

D. DeterminationofKinetic Constants for Oxidation of Phenanthridine by Aldehyde Oxidase

Aldehyde oxidase activity was determined spectrometrically in PBS buffer pH 7.4 at 37°C, using phenanthridine as the substrate. Six concentrations of phenanthridine, ranging from 25 to 100µM, were prepared in 30µl PBS buffer and the reaction started by adding 50µl of the enzyme fraction. All assays were carried out in triplicate in 100µl reaction volumes. All samples were frozen and thawed only once, and the spectrophotometric data were collected at 5-second intervals for 1 to 3 minutes using the microplate reader spectrophotometer (BioTek, UK). The activity of the aldehyde oxidase enzyme was tested by monitoring the oxidation of phenanthridine by an increase in absorbance at 322nm(ϵ = 6,400 $M^{-1}cm^{-1}$) [25]. These values were used for calculating initial velocities and then K_m and V_{max} values from an Eadie Hofstee plot for the phenanthridine oxidation catalyzed by hepatic fish aldehyde oxidase. The line of the best fit through the points on the plot was calculated using linear regression by the least squares method using Microsoft Excel (Microsoft Office).

E. Protein Determination

Protein concentrations of partially purified enzyme fractions were determined using a Pierce Bicinchoninic acid (BCA) Protein assay kit with bovine serum albumin as the protein standard [26].

F. Incubations with Partially Purified Aldehyde Oxidase Fractions

Phenanthridine (50µM) were incubated with 200µl partially purified fish liver fractions at 37°C in a total volume of 1000µl of 700µl 50mM phosphate buffer saline, pH 7.4. Incubations were performed in 1000µl-closedvials, which were placed in a Dri-Block heater (DB-2D, Techne, UK). Aliquots (200µl) were removed at 5 and 10min and added to either 100µl 20% trichloroacetic acid to terminate the reaction. Samples were centrifuged in a Microfuge 16 Centrifuge (Beckman Coulter, UK) at 10,000rpm for 2min and the supernatants were subsequently analysed by HPLC. In all *in vitro* assays no spontaneous oxidation of any substrate was observed when control incubations were carried out without enzyme fraction.

G. HPLC Analysis of Phenanthridine Oxidation

The reversed-phase high performance liquid chromatography (HPLC) system used was the Beckman system gold™ 127 solvent modules (dual pump) and a programmable UV detector (module 166). The system was fitted with a 20µl injection loop and the column used was a stainless steel Kromasil (25cm X 4.6mm, 5µm; RP- C18) with guard column (5µm) to avoid contamination of the column with any biological materials at ambient temperature. At the beginning of each working day the system was started and left to run for approximately 30 minutes. Oxidized metabolites were identified by comparison of their HPLC retention times and UV spectra with those of authentic standards. The method used for the phenanthridine study by HPLC was a gradient

system, which incorporated a triethylamine and acetonitrile as the solvents. Peaks for phenanthridine and 6(5H)-phenanthridone metabolites were detected at 254nm. The gradient elution system was comprised of an aqueous buffer (7.0ml triethylamine in 1000ml of water adjusted to pH 7.56 with 80% phosphoric acid) and acetonitrile [24]. Metabolites of phenanthridine were profiled using Kromasil RP-18 column with an elution program of 10 to 100 % acetonitrile in aqueous buffer for 20min [24]. The gradient system programmed with the variation in the solvents at a flow rate of 1.5ml/min.

III. Results and Discussion

Phenanthridine is a good example of an exogenous toxic pollutant, which is a prototypical uncharged xenobiotic substrate of AO. The oxidative product, 6(5H)-phenanthridone is a cyclic lactam substituted adjacent to a heterocyclic nitrogen atom (Fig. 1) [1], [2], [24]. Once the assays were optimized, AO activities were measured in the partially purified liver fish aldehyde oxidase. The result phenanthridine oxidase assay is summarized in the following Table I. The result demonstrates phenanthridine oxidase activity, which is present in liver fish (Table I). The oxidation activity of phenanthridine was calculated to reflect the relative the aldehyde oxidase activity in the fish species (Table I).

TABLE I
Hepatic Aldehyde Oxidase Activity in Liver Fish

Enzyme activity towards phenanthridine (nmol/min/mg protein)	
Species	Phenanthridine(50 μM)
Rainbow trout fish	0.77 ± 0.03

Activity was determined as described in materials and methods. The activity is shown as nmol substrate hydroxylated/min/mg protein and are expressed as means ± S.D., n = 3.

TABLE II
Kinetic Constants for 6(5H)-Phenanthridone Metabolite Formation by Partially Purified Rainbow Trout Fish Liver Aldehyde Oxidase Determined by Spectrophotometer

Specie	Substrate	K_m(μM)	V_{max} nmol/min/mg protein
Rainbow trout fish liver aldehyde oxidase (n = 3)	Phenanthridine	78 ± 7.6	1.58 ± 0.32

The oxidation rate of phenanthridine was measured spectrophotometrically at 37˚C with molecular oxygen as an acceptor. The value shown are the mean ± S.D., n= 3.Phenanthridine concentration = 50 μM.

Phenanthridine oxidase was monitored by following product formation of 6(5H)-phenanthridone at 322nm. The assay showed linear reaction kinetic under the conditions used (Fig. 2). Kinetic constants for phenanthridine with partially purified liver fish aldehyde oxidase are shown in Table II. K_m and V_{max} values were calculated from linear oxidation rates using Eadie-Hofstee plot (Fig. 3).TheK_m and V_{max} values for oxidation of phenanthridine with partially purified fish liver aldehyde oxidase was also determined spectrophotometrically as described in Methods. The kinetic parameters obtained was K_m = 78 ± 7.6μM and V_{max} = 1.58 ± 0.32nmol/min/mg protein (Mean ± SD, N = 3) for hepatic fish aldehyde oxidase.The values of K_m and V_{max} of AO were determined using Eadie Hofstee (Fig. 3), due to this plot being superior to other methods of plotting data [27]. Eadie Hofstee plots of the data obtained gave good correlation coefficients (r^2)> 0.97 (Fig. 3). The result obtained in the present study shows the phenanthridine used was found to be good substrate of hepatic fish AO (Table II). Phenanthridine has been shown to be a good specific substrate for monitoring mammalian AO activity in many studies [1], [3], [28]-[31]. Phenanthridine as a specific substrate of AO has been used in several papers and K_m previously has been estimated were < 1μM with rabbit and guinea pig liver AO enzyme and 6μM and 14μM with rat and human liver AO, respectively [32], [33]. The present result is indicated that phenanthridine a good substrate of hepatic fish AO, in terms of a K_m of phenanthridine (K_m = 78 ± 7.6μM) (mean ± SD) but this value is considerably greater than the previously studies of mammalian AO. This might be due to a polymorphism in the fish AO gene. Further time consuming and costly molecular genetic studies would be required to clarify this however. In addition, phenanthridine was transformed by AOH1, which was responsible for N-heterocyclic oxidase activity in mouse liver [29] and fish are endowed with a single functional aldehyde oxidase gene [34], [35].

In order to determine that there was definitely activity present in liver fish for phenanthridine as substrates a sensitiveHPLC assay was performed with this substrate and was used to confirm the product formation. This is shown in Fig.4, where product was detected even after extended incubation periods.One metabolite was observed in chromatograms arising from the incubation of phenanthridine with partially purified fish liver aldehyde oxidase (Fig. 4). In the present study, biotransformation of phenanthridine to6(5H)-phenanthridone has been found, which is agreement with previous studies [3], [24], [29], [33], [36], [37].6(5H)-phenanthridone (Phenanthridone) has been shown to be a major metabolite in mammalian studies [1], [3], [24], [28]-[31], [38].

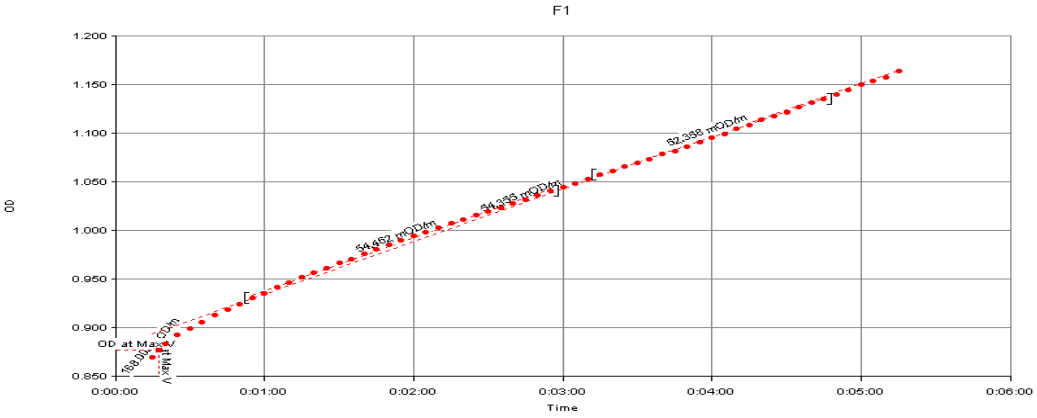

Fig. 2 Spectrophotometric assay of aldehyde oxidase activity using partially purified liver fish aldehyde oxidase at 37˚C with 50µM phenanthridine substrate

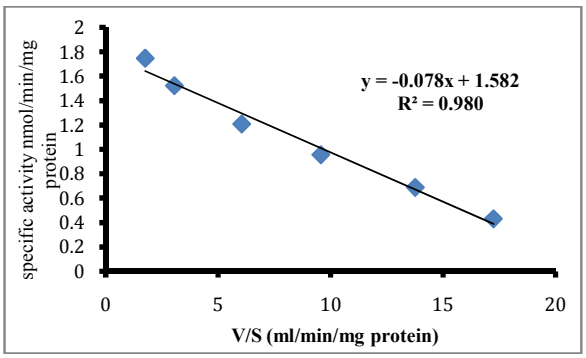

Fig. 3 Eadie- Hofstee plot for oxidation phenanthridine by partially purified rainbow trout fish liver aldehyde oxidase at 37˚C with molecular oxygen as an acceptor. The typical results are from three independent experiments

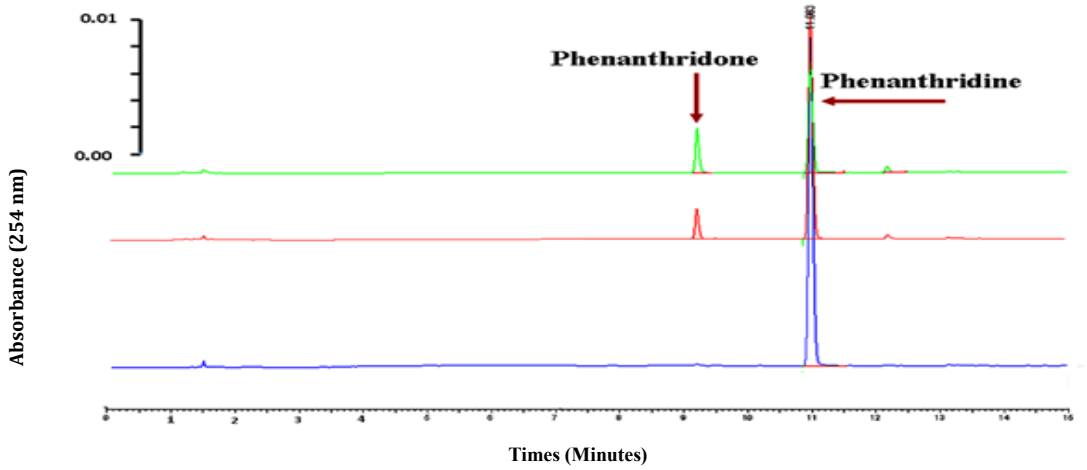

Fig. 4 HPLC analysis of the *in vitro* biotransformation of phenanthridine by partially purified rainbow trout fish liver aldehyde oxidase

HPLC chromatograms are offset on the vertical axis to allow comparison between extended incubation periods. The Lower HPLC chromatogram is *in vitro* oxidation of phenanthridine to phenanthridone at zero time. The middle chromatogram is in vitro oxidation of phenanthridine to phenanthridone after 5 minutes incubation time at 37°C. The upper chromatogram shows oxidation of phenanthridine to phenanthridone after 10 minutes incubation time at 37°C. Analytes were injected onto a RP-18 column (Kromasil 5µm) and eluted with triethylamine: acetonitrile gradient system as described in method as mobile phase. The wavelength of the detection was 254nm.

IV. Conclusion

In conclusion, Initial study using spectrophotometric and HPLC assays indicated that molybdo-flavoenzyme aldehyde oxidase in liver fish were able to catalyse the biotransformation of phenanthridine substrate of the well-characterized mammalian AO. This identification of major metabolite of phenanthridine by partially purified liver fish aldehyde oxidase has already brought novel information about aldehyde oxidase activity in fish species and it is expected that fish are better capable of transforming phenanthridine into its metabolite, due to their activity of aldehyde oxidase.

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