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RESEARCH ARTICLE

Effects of some metals on paraoxonase activity from shark *Scyliorhinus canicula*

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Abstract

Paraoxonase (PON) is an organophosphate hydrolyser enzyme which also has antioxidant properties in metabolism. Due to its crucial functions, the inhibition of the enzyme is undesirable and very dangerous. PON enzyme activity should not be altered in any case. Inhibitory investigations of this enzyme are therefore important and useful. Metal toxicology of enzymes has become popular in the recent years. Here, we report the *in vitro* inhibitory effects of some metal ions, including Ni²⁺, Cd²⁺, Cu²⁺ and Hg²⁺, on the activity of shark serum PON (SPON). For this purpose, we first purified the enzyme from shark *Scyliorhinus canicula* (LINNAEUS, 1758) serum and analysed the alterations in the enzyme activity in the presence of metal ions. The K_M and V_{max} is 0.227 mM and 454.545 U/mL, respectively. The results show that metal ions exhibit inhibitory effects on SPON1 at low concentrations with IC₅₀ values ranging from 0.29 to 2.00 mM. Copper was determined to be the most effective inhibitor with IC₅₀ of 0.29 mM.

Keywords: Paraoxonase, *Scyliorhinus canicula*, metal toxicology

Introduction

Paraoxonase (PON1; EC 3.1.8.1) is a calcium-dependent esterase that hydrolyses aromatic carboxylic acid esters, toxic organophosphate compounds, and lactones, yet the natural substrates and physiological function(s) of PON1 remain to be established¹. Human PON1 is a member of a multigene family (PON1, PON2 and PON3 genes) encoded by a single gene on chromosome 7q21–22². PON1 is synthesised in the liver and secreted into the blood, where it is associated with high density lipoproteins. PON1 activity in serum shows a wide variation among individuals³, and this variability is attributed to the presence of polymorphisms in PON1 gene⁴. Paraoxonase, a member of the A-oxonase family, breaks down acetylcholinesterase inhibitors before they bind to the cholinesterases, and it protects people from harmful effects caused by exposure to low doses of OP pesticides^{5,6}. Serum paraoxonase has been purified from several mammals, but only human, rat and rabbit PON1 proteins have been extensively characterised^{7,8}. They found that rabbits had by far the highest activity in

their sera and mice had the lowest. Birds were found to have very low serum paraoxonase activity⁹. Fish, which have very low PON1 activity, are very sensitive to OP insecticides¹⁰. Studies of PON in bovine are limited. They investigated the activity of PON in pregnant, early lactating, and late lactating dairy cows. They suggested that the observed reduction in PON activity immediately postpartum may be due to (1) fat mobilisation and triglyceride deposition in liver cells, which cause liver damage or dysfunction (2) reduction in blood cholesterol HDL, (3) an increase in oxidative stress or (4) a combination of these^{11,12}.

Inhibition by metallic ions is well known for many enzymes and has been reported for A-esterase from different sources. Other compounds, as complexation agents and thiol reagents, are also typical enzyme inhibitors. Aldridge^{13,14} proposed to do detailed studies with inhibitors as a tool for a better classification of esterase that hydrolyses OP compounds. In addition, inhibition studies can also be used to identify some components in the active centre of the enzymes.

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Scyliorhinus canicula (Linnaeus, 1758) belongs to the family Scyliorhinidae (Carcharhiniformes) and is considered to be the most abundant species of cat shark in European inshore waters¹⁵. It is found in the northeastern Atlantic from Norway and British Isles, south to Senegal, including the Mediterranean Sea, primarily over sandy, gravely or muddy bottoms at depths of a few meters down to 400 m¹⁶. Sharks do not, or rarely, get cancer. Squalamine, which is derived from stomach and liver of the dogfish shark, inhibited angiogenesis and solid tumour growth *in vivo* in phase I clinical trials that were initiated to evaluate the feasibility of this novel aminosterol for cancer treatment¹⁷.

The main goal of this study is to purify PON enzyme from shark serum using simple and cheap methods and observe kinetic alterations in the enzyme activity in the presence of metal ions, including Ni²⁺, Cd²⁺, Cu²⁺ and Hg²⁺. The rationale to perform this study is that exposure to heavy metals is an important problem of environmental toxicology. Most heavy metals are toxic to humans, animals, and plants, and man is at great risk of suffering from health hazards associated with toxic metals because of bioaccumulation.

Materials and methods

The materials used include sepharose 4B, L-tyrosine, 1-naphthylamine, paraoxon, protein assay reagents and chemicals for electrophoresis were obtained from Sigma Chem. Co. All other chemicals used were analytical grade and obtained from either Sigma or Merck.

Collection of fish samples and blood collection

S. canicula (LINNAEUS, 1758) was collected from the Edremit Bay, in the Aegean Sea, Turkey. Fish were held in aerated dechlorinated freshwater (8–15°C). Blood samples were collected by blind caudal puncture and centrifuged at 1500 rpm for 15 min and serum was isolated from fresh shark blood taken to dry tube.

Paraoxonase enzyme assay

Paraoxonase enzyme activity towards paraoxon was quantified spectrophotometrically with the method described by Adkins et al.¹⁸. The reaction was followed in 2 min at 37°C by monitoring the appearance of *p*-nitrophenol at 412 nm in Biotech automated recording spectrophotometer. A molar extinction coefficient (ϵ) of *p*-nitrophenol at pH 8.0 in 100 mM Tris-base buffer of 17,100 M⁻¹ cm⁻¹ was used for the calculation. PON activity (1 U/L) was defined as 1 μ mol of *p*-nitrophenol formed per minute.

Ammonium sulphate precipitation

Serum paraoxonase was isolated by ammonium sulphate precipitation (60–80%)¹⁹. The precipitate was collected by centrifugation at 15,000 rpm for 20 min, and redissolved in 100 mM Tris-HCl buffer (pH 8.0).

Purification of SPON by hydrophobic interaction chromatography

The pooled precipitate obtained from *S. canicula* serum by using ammonium sulphate precipitation was subjected to hydrophobic interaction chromatography. The final saline concentration of precipitate was adjusted to 1 M ammonium sulphate, prior to that it was loaded onto the hydrophobic column prepared from Sepharose-4B-L-tyrosine-1-naphthylamine. Then, we synthesised the hydrophobic gel, including Sepharose-4B-L-tyrosine- and 1-naphthylamine, for the purification of human serum paraoxonase¹⁹. The column was equilibrated with 0.1 M Na₂HPO₄ buffer pH 8.0 including 1 M ammonium sulphate. The paraoxonase was eluted with ammonium sulphate gradient using 0.1 M Na₂HPO₄ buffer with and without ammonium sulphate pH 8.0. The purified SPON enzyme was stored in the presence of 2 mM CaCl₂ at + 4°C in order to maintain activity.

Total protein determination

The absorbance at 280 nm was used for monitoring the protein in the column effluents and ammonium sulphate precipitation. Quantitative protein determination was achieved by absorbance measurements at 595 nm according to Bradford²⁰, with bovine serum albumin standard.

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed after purification of the enzyme. It was carried out in 10% and 3% acrylamide concentrations, containing 0.1% SDS, for the running and stacking gel, respectively, according to Laemmli²¹.

In vitro inhibition studies

Different concentrations of metals were added to the enzyme activity. SPON enzyme activity with metals was assayed by following the hydration of paraoxon. Activity% values of paraoxonase for five different concentrations of each metal were determined by regression analysis using Microsoft Office 2000 Excel. Paraoxonase activity without a metal was accepted as 100% activity. For the metals having an inhibition effect, the inhibitor concentration causing up to 50% inhibition (IC₅₀ values) was determined from the graphs.

Results and discussion

Purification of SPON was performed using the following methods: ammonium sulphate fractionation (60–80%) and Sepharose 4B-L-tyrosine-1-naphthylamine hydrophobic interaction chromatography. A 37-fold purification with a yield of 1.127% was found (Table 1), and the mass and purity of the enzyme was assessed by SDS-PAGE (data not showed). The purified SPON appeared as a single species with a mass of 66 kDa. The K_M and V_{max} values were calculated by the method of Lineweaver-Burk. The Lineweaver-Burk

Table 1. Summary of the purification and IC₅₀ values of SPON.

Fraction	Volume (mL)	Activity (U/mL)	Total activity (U/mL)	Protein amount (mg/mL)	Total protein (mg/mL)	Specific activity (mU/mg)	Overall yield (%)	Overall purification (fold)
Serum	30	392.960	11788.8	1.1400	34.200	344.701	100	1.0
Ammonium sulphate precipitation	23	421.818	9701.81	0.2605	5.9915	1619.26	82.29	4.69
Hydrophobic interaction chromatography	1.5	88.600	132.900	0.0069	0.01035	12840.6	1.127	37.3
Metals	Ni ²⁺		Cd ²⁺		Hg ²⁺		Cu ²⁺	
IC ₅₀ (mM)	2.00		0.73		0.49		0.29	

double-reciprocal plot was analysed with a range of paraoxon concentration (0.01–1 mM). The K_M and V_{max} values of the purified enzyme calculated for paraoxon were 0.227 and 454.545 U/mg for SPON. Ekinci and Beydemir reported that human PON1 was purified 314-fold with a yield of 25%²². Our purification fold was lower than their fold. This might be lower in fish than the human because of the activity of PON1.

IC₅₀ values were calculated as 2.00, 0.73, 0.49 and 0.29 mM for Ni²⁺, Cd²⁺, Hg²⁺ and Cu²⁺, respectively (Table 1). The metal ions inhibited SPON at millimolar levels. Cu²⁺ was the most powerful inhibitor among others. Our groups reported that metals were more effective inhibitors on human serum PON1 activity. Similarly, Cu²⁺ was also the most powerful inhibitor among others on human PON1²³.

Hernández et al. aimed to investigate whether environmental exposure to metal compounds has any influence on PON1 and cholinesterase. They conducted the research in a representative sample of the general population of Andalusia, South of Spain. They determined that blood lead levels were significantly associated with increased PON1 in serum. Mercury also showed a significant and direct association with PON1 towards paraoxon and phenylacetate. In turn, cadmium and zinc levels were significantly associated with a decreased PON1. Arsenic, nickel, and manganese failed to be significantly associated with any of the PON1 activities assayed²⁴. Ekinci and Beydemir investigated the *in vitro* effects of some heavy metal ions (Pb²⁺, Cr²⁺, Fe²⁺, and Zn²⁺) on the purified human serum PON1. In that study, it was reported that metal ions exhibit inhibitory effects on hPON1 at low concentrations with IC₅₀ values ranging from 0.838 to 7.410 mM⁵.

In addition to the studies above, there are investigations regarding the inhibitory effects of metals on PON activity as well. In a research study, it was reported that metal ions, such as Co (II), Cu (II), Mn (II), Hg (II), and *p*-hydroxymercuribenzoate (pOHMB) change PON1 and PON3 activity in rat liver, indicating that their active sites may contain lysine, histidine, phenylalanine, cysteine, tryptophan, aspartic acid, glutamic acid, and asparagine residues, which can bind metals²⁵. The rank order of inhibitors were different: for PON1, Hg²⁺>pOHMB>Co²⁺>Mn²⁺>Cu²⁺, and for PON3, Hg²⁺>Cu²⁺>pOHMB>Mn²⁺>Co²⁺, suggesting

that more work is necessary to determine the protective role of PONs against the toxic effects of xenobiotics, including environmental heavy metals and oxidative stress by-products.

This research was undertaken to purify PON1 from human serum and to address whether concentrations of Ni²⁺, Cd²⁺, Hg²⁺, and Cu²⁺ have any association with the activity of pure enzyme. It was found that metal ions were associated with low PON activity. This study provides supportive information for further investigations regarding the inhibitory effects of metal toxicity on PON enzyme, which takes an important place in environmental toxicology researches. Our findings suggest that PON activity is negatively modulated by exposure to metal compounds, which may have implications in public health given the defensive role played by this bio-scavenger enzyme against environmental toxicants.

Durrington et al reported that PON1 is highly conserved in mammals but it is absent in fish²⁶. On the other hand, Bastos et al reported that paraoxonase activity is determined of four neotropical fish²⁷. We have also determined paraoxonase activity in shark.

Declaration of interest

The authors report no conflicts of interest.

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