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In vitro effects of peroxynitrite treatment on fish liver catalase activity

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Abstract
The effect of peroxynitrite (PN), a highly toxic agent, on catalase (CAT) activity in fish liver microsomal homogenates was determined. PN was synthesized by mixing acidic hydrogen peroxide solution with sodium nitrite solution and then adding sodium hydroxide solution into the mixture in order to stabilize the highly labile compound peroxynitrous acid (ONOOH) in peroxynitrite anion form ($ONOO^-$). The effect of PN and decomposed peroxynitrite (DPN), prepared by preincubation with HCl, was monitored by using a constant amount of homogenate containing the CAT enzyme. Significant losses were observed in the CAT activity of fish liver enzyme after treatment with PN and also with DPN products, the inhibitory effect of PN being slightly more pronounced than that of DPN. IC_{50} values were 5.5 and 8.5 μM for PN and DPN, respectively. The PN inhibition of CAT activity is due to both the effects of the secondary and decomposition products of PN and its nitration and oxidation effects on the amino acid residues of the enzyme.

Keywords: *Fish liver, catalase activity, peroxynitrite, decomposed peroxynitrite, inhibition*

Introduction

Peroxynitrite (PN) is physiologically synthesized from superoxide and nitric oxide. Despite its very short half-life, PN is a very toxic compound and can oxidize or nitrate most biological molecules including proteins, lipids, carbohydrates and nucleic acids, by which it changes their structure, function and biological activity [1,2]. The pKa of PN is 6.8, and it is stable at basic pHs though its acidic form peroxynitrous acid is unstable. Decomposition products of PN may be converted to nitrite or nitrate and oxygen [2]. Nitrite and nitrate are important for antimicrobial and flavoring/coloring purposes in meat and fish products. However, they may cause methemoglobinemia and other illnesses, and may react with certain amines to form carcinogenic nitrosamines [1]. PN has also been shown to inhibit/inactivate ornithine decarboxylase [3], catalase [4], glutathione peroxidase [5], and glutathione reductase [6]. In recent years, several inhibition/inactivation mechanisms have been proposed to explain the effects of PN on proteins [7,8]. The most pronounced effect of PN over proteins is transformation of tyrosines into nitrotyrosines and the nitrosylation of thiols [9,10]. Several studies have been reported on the scavenging potential of various compounds on PN. Diet-derived phenolics [11], vitamin E and seleno-compounds [1,12], flavonoids [13], and anthocyanins [14] have been reported to be powerful PN scavengers.

Catalase (CAT) (EC 1.11.1.6) is an antioxidant enzyme ubiquitously present in various aerobic cells, and the highest catalase activity in mammals is found in liver, kidney and erythrocytes. In hepatic and renal cells, this enzyme is located mainly in peroxisomes, serving as the marker enzyme of this organelle; CAT is one of the most popular natural antioxidant enzymes. The enzyme is capable of eliminating reactive oxygen species, thereby protecting cells and tissues from oxidative damage by decomposing hydrogen peroxide (H_2O_2) to molecular oxygen (O_2) and water (H_2O) [15,16]. CAT has been shown to catalyze peroxynitrite-mediated nitration

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of phenolic compounds [17]. CAT from various sources is also a good candidate for oxidative modification by peroxynitrite since it has many amino acid residues that are target sites for PN, especially tyrosines [18].

Hepatic oxidation in fish has been receiving increasing attention as a mechanism of toxicity for a variety of organic and inorganic environmental pollutants. Several fish liver enzymes have been extensively used in the studies of oxidative stress and the effects of pollutants on antioxidant defenses because fish possesses an antioxidative defense system that utilizes enzymatic and nonenzymatic mechanisms [19,20].

The purpose of the study was to investigate the changes that occur in the activity of fish liver catalase enzyme at physiological pH upon reaction with PN and DPN at different concentrations.

Materials and methods

Preparation of fish liver homogenate

Sarda sarda were supplied freshly by a fisherman in September 2006 (Trabzon, Turkey). Fish livers were washed with 0.15 M KCI and surface-dried with filter paper, weighed, thoroughly washed in ice-cold saline solution, and suspended in 0.15 M KCl. Homogenization was performed on ice, employing approximately 25 strokes in a Potter-Elvehjem homogenizer, followed by centrifugation at 4000 × g for 15 min at 4 °C. The supernatant was taken and centrifuged at 40.000 × g for 10 min at 4 °C. The supernatant was stored at 4°C until use.

Measurement of CAT activity

CAT activity was measured in the microsomal fraction according to *Aebi* [21]. The method is based on direct determination of H_2O_2 consumption. The decomposition of H_2O_2 was measured by monitoring the H_2O_2 concentration for 30 s, reading the absorbance at room temperature and 240 nm. The reaction volume was 3 mL and contained 1000 μL of the sample homogenate, 1500 μL of potassium phosphate buffer 50 mM, pH 6.5 and 500 μL of 30 mM H_2O_2. A control was assayed without liver homogenate. The data were expressed as absorbance change, percentage absorbance change, percent inhibition of CAT activity, and IC_{50} values, the concentration of PN or DPN causing 50% inhibition.

Synthesis of PN ($ONOO^-$)

PN was synthesized according to the method of *Koppenol et al.* [22] and quantified spectrophotometrically at 302 nm prior to use. The synthesis was accomplished by mixing sodium nitrite and acidified hydrogen peroxide solutions and then stopping the decomposition of the product peroxynitrous acid (ONOOH) by adding NaOH solution to convert it to basic form ($ONOO^-$) in a stop-flow tubing system. The method is widely used in the literature to determine the effect of peroxynitrite on proteins [23].

Effect of PN and DPN on CAT activity

The effect of PN on CAT activity was monitored in two models. First, the homogenate containing CAT enzyme at constant concentration was treated with PN at various concentrations (10, 20, 40, 50 and 100 μM), and the CAT activity was then measured. For blank, untreated enzyme was used. In order to avoid the effect of pH change due to the use of basic PN solutions, equivalent concentrations of HCl solutions were added before activity measurements. Secondly, the effect of PN decomposition products was tested by adding equivalent amount of HCl to PN solutions of equivalent hydroxide ions before treatment with the enzyme to prepare DPN, as PN is known to be stable only in basic media. The treatment with PN was done very quickly while vortexing. The results are expressed as absorbance changes and percent absorbance changes versus time (s) and percent inhibition of catalase activity with respect to increasing PN concentration as well as IC_{50} values, the concentrations of PN that causes 50% inhibition of CAT activity.

Statistical analysis

The differences in the CAT activities for the samples containing PN and DPN at the same concentrations were tested with independent t test, and the differences in those containing different PN or DPN concentrations were tested with one-way ANOVA. Differences at $p < 0.05$ level were considered to be statistically significant.

Results and discussion

Reactive oxygen species (ROS) are normal by-products of cellular metabolism. Overproduction of ROS and their derivatives occurs in a number of diseases. CAT enzyme decomposes H_2O_2, thereby preventing the formation of highly aggressive compounds such as hydroxyl (HO·), superoxide ($O_2^{\cdot -}$), and peroxynitrite ($ONOO^-$) [24]. Higher levels of oxidative stress as a result of increase in ROS may cause irreversible inactivation of several enzymes [3].

Peroxynitrite has been used in many investigations, added to the test medium as previously synthesized from nitrite ions and H_2O_2 under acidic conditions [22,23]. Physiologically it is a product of nitric oxide and superoxide [25]. These two ROS are known to produce PN in the living systems resulting in the physiological or pathophysiological consequences

in infections and many diseases. PN is believed to exert most of its oxidation effects through formation of highly reactive hydroxyl radicals.

H_2O_2 gives maximum absorbance at 240 nm. In the presence of CAT, absorbance is decreased with the decomposition of H_2O_2. CAT activity is proportional to the amount of decrease of absorbance. The more absorbance decreases, the higher the activity is. To study the effect of PN and DPN on CAT enzyme, five different concentrations (10, 20, 40, 50 and 100 μM) of PN and DPN were used. Absorbance change as a result of PN addition to the medium was

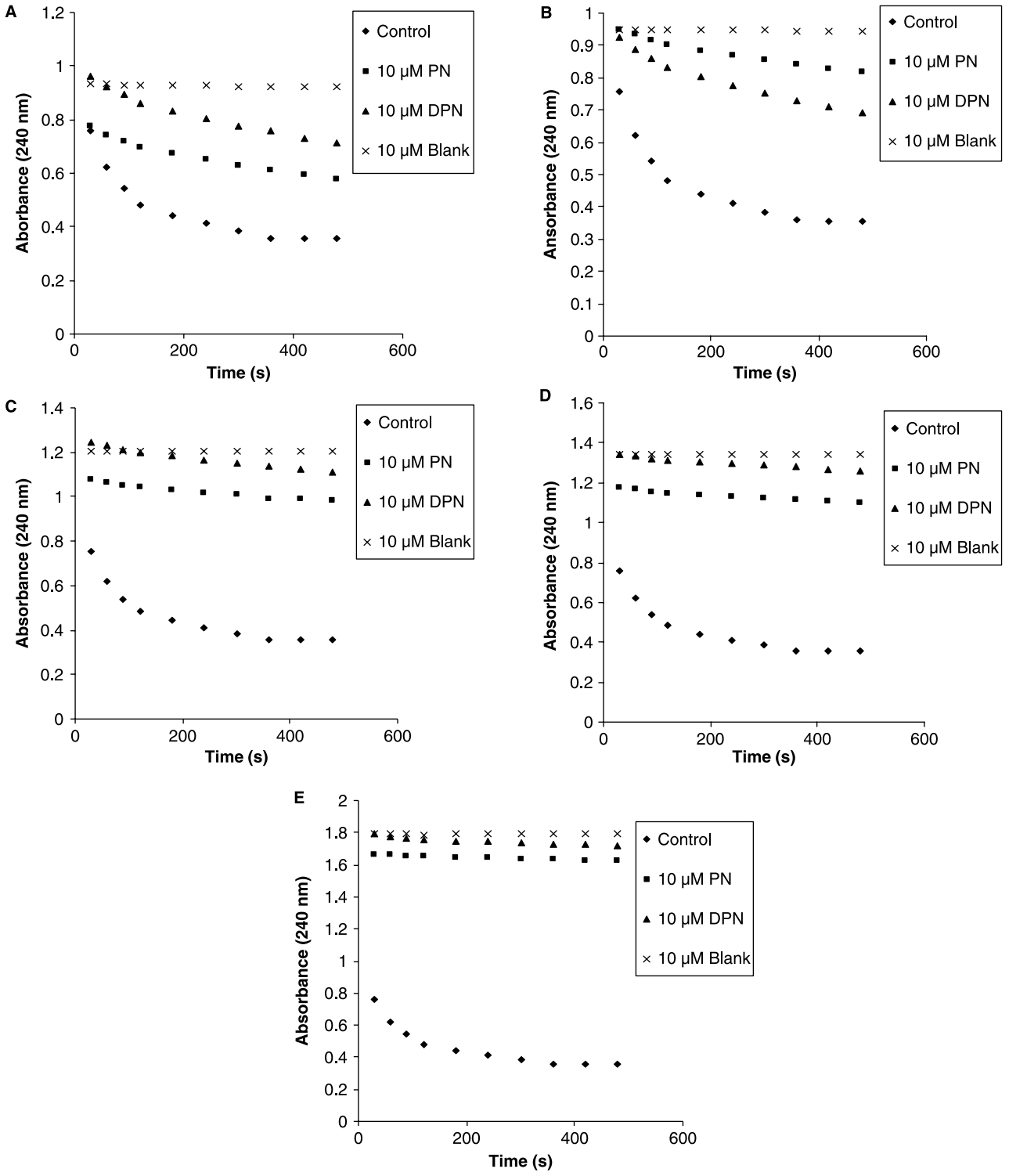

Figure 1. The effect of PN (peroxynitrite) and DPN (decomposed peroxynitrite) on CAT activity. (A) 10 μmol/L PN and DPN; (B) 20 μmol/L PN and DPN; (C) 40 μmol/L PN and DPN; (D) 50 μmol/L PN and DPN; (E) 100 μmol/L PN and DPN. Data are presented as absorbance at 240 nm versus time (s).

shown in the graphics in Figure 1. Addition of PN and DPN slows down the absorbance drop by time. Actual enzyme activity is shown by change in absorbance values in comparison of control and PN and DPN treatments. To observe the effect of addition of PN and DPN on the enzyme, we plotted percentage of absorbance change by time at all concentrations of PN (Figure 2) and DPN (Figure 3). A linear decrease was observed in the absorbance changes for both treatments with respect to increase in the concentrations of PN and DPN. CAT activity was decreased by PN or DPN. This inactivation may be due to the nitration and oxidation of the enzyme active domains by PN or DPN. The differences between the inhibitory activity of PN and DPN at the same concentrations as well as between the inhibitory activity of differing concentrations of both PN and DPN were found statistically significant ($p < 0.05$). The differences between the inhibitory effects of PN and DPN was evident from the graphs in Figure 1 and from statistical evaluation. This difference was also displayed in Figure 4 as percentage inhibition of CAT at the time point of 300 s with respect to concentration change of PN and DPN, and the IC_{50} values were calculated from this graph as 5.5 and 8.5 μM for PN and DPN, respectively. IC_{50} is the concentration of the oxidizing species resulting in 50% inhibition of the enzyme. Smaller IC_{50} value indicates higher inhibitory activity.

Aspergillus niger CAT has been studied for its regulation by PN, reduced glutathione and glutathione S-transferase and shown to undergo a reversible activity reduction by PN, which was restored by reduced glutathione [18]. This is an indication of the fact that the major effector on the activity reduction of the enzyme is not nitration of tyrosines, which cannot be reversed by reduced glutathione.

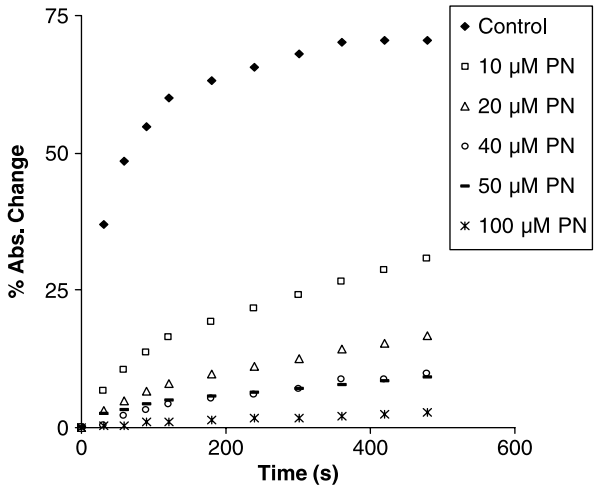

Figure 2. The effect of PN at various concentrations on CAT activity. Data are presented as percent absorbance change with respect to that of blank CAT activity, which was not incubated with PN.

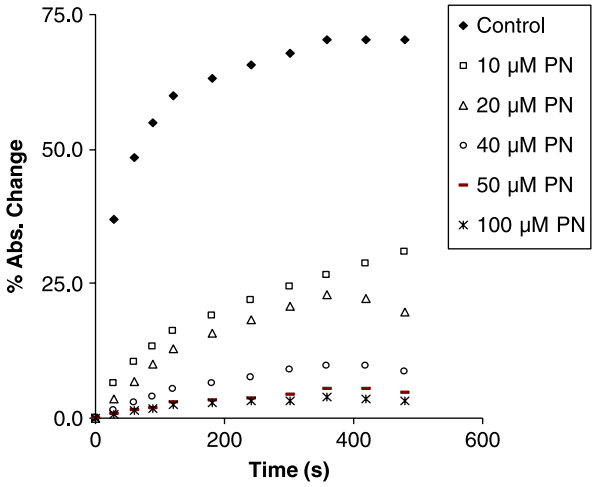

Figure 3. The effect of DPN at various concentrations on CAT activity. Data are presented as percent absorbance change with respect to that of blank CAT activity, which was not incubated with DPN. DPN was obtained by preincubating PN solutions with the amounts of HCl equivalent to the basicity of PN solutions.

Sarojini et al. [26] showed that PN causes the inactivation of the antioxidant enzyme glutathione peroxidase, and this is due to the oxidation of the active domain of the enzyme. In their study Görg et al. [27] reported that PN reversibly inhibited glutamine synthetase enzyme and this mechanism is due to the nitration of the tyrosine residues on the active domain of the enzyme. Similarly, Kucuk et al. [28] showed that PN inhibits carbonic anhydrase enzyme and the tyrosine residues of the enzyme was shown to be nitrated. They also reported that an increase was observed in the activity of carbonic anhydrase as a result of its treatment with DPN.

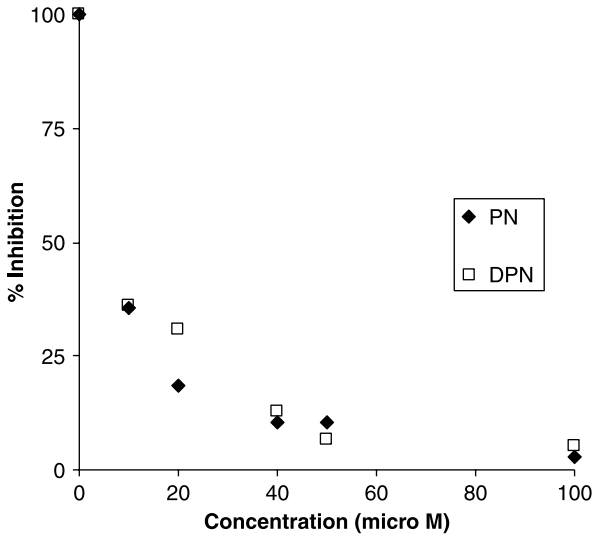

Figure 4. Percent inhibition of catalase activity with respect to increasing PN and DPN concentration based on measurements at 300 s. IC_{50} values were 5.5 and 8.5 μM for PN and DPN, respectively.

PN inhibition of CAT activity may be due to the nitration of tyrosine residues as well as oxidation of tyrosine and a number of other amino acid side chains in CAT. In vitro study presented here shows that PN and DPN can inactivate fish liver CAT. PN was shown to have a higher inhibitory activity with a smaller IC_{50} value, probably because of its higher oxidizing potential, than its decomposition products. The PN decomposition products and their individual effects on the enzymes obviously deserve further investigations, as both inhibiting and activating types of behavior has been observed.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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