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Molybdoenzymes isolated from *S. glanis* liver can produce nitric oxide from nitrates and nitrites

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Abstract: Nitric oxide (NO) plays numerous essential physiological functions in terrestrial animals. In mammals, NO production from L-arginine is catalysed by the enzyme NO synthase (NOS). In recent years, data have begun to emerge on NOS expression and the physiological significance of NO in ectothermic vertebrates such as fish. However, there are relatively fewer data compared to the mammalian system. Although it is already well known that animal molybdoenzymes can convert nitrate and nitrite into NO, there is almost no information on the content and properties of molybdoenzymes in fish organs in the scientific literature. In this regard, the objectives of the present work were to detect the activity of classical molybdoenzymes xanthine oxidase (XO) and aldehyde oxidase (AO) in the liver and to study their possible activity to reduce nitrate and nitrite to nitrogen monoxide. In this work, the intrinsic activity of XO and AO was examined by using their substrates. At the same time, their nitrate (NR) – and nitrite reductase (NiR) activity were determined. It was determined that XO and AO in the fish liver are mainly represented by the molybdenum-free forms. The presence of an additional source of molybdenum can activate both the intrinsic and the NR and NiR activities. The NiR activity of XO and AO was higher than their NR activity. The data shows that treatment at a concentration of sodium molybdate and glutathione of 1.0 mM increases all activities of XO and AO. The optimal conditions for maximum activation of exogenous molybdate for XO and AO were reached by heating at 70 °C for 5 minutes. The activity of XO increased almost 4.7 times, and the activity of AO 7.7 times compared with its intrinsic activity without heat treatment. NO is formed from nitrite by the enzymes XO and AO much more than from nitrate.

Keywords: fish liver enzymes; xanthine oxidase; aldehyde oxidase; nitrate reductase activity; nitrite reductase activity

Four molybdoenzymes, xanthine oxidase (XO), aldehyde oxidase (AO), mitochondrial amidoxime-reducing component 1 (mARC), and sulfite oxidase (SO) have been found and studied in animals (Hille et al. 2014). The last two enzymes are localised in the outer membrane of the mitochondria, thus being located inside the cell. Their functions are to catalyse sulphite oxidation to sulphate for SO

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and to reduce amidoximes to amidines for mARC (Kappler and Enemark 2015; Kubitza et al. 2018). It should be noted that mARC was discovered relatively recently and is the newest molybdenum-containing animal enzyme (Hille et al. 2014).

XO is present in almost all tissues of the animal organism, including the membranes of milk fat globules. However, the largest amount of XO and AO in terrestrial animals is found in the liver. XO in higher primates catalyses the last step in the oxidation of purines (adenine and guanine) by converting them into uric acid via hypoxanthine and xanthine. AO is an enzyme that catalyses the oxidation of aldehydes to carboxylic acids. In addition, XO and AO are homodimers similar in structure; each subunit contains an iron-sulphur centre, one FAD, one molybdenum atom, and a molybdocofactor (Maia and Mira 2002). Their molybdocofactor, like most eukaryotic molybdoenzymes, is represented by pyranopterin, which binds the molybdenum atom during the catalytic reaction through sulfhydryl (-SH) groups, forming an S–Mo–S bond (Hover et al. 2015).

In 1980, Alikulov with colleagues showed for the first time that homogeneous XO purified from cow's milk has the activity of reducing nitrates and nitrites (Alikulov et al. 1980). Later, these associated activities were found in XO isolated from the animal's other organs, with the product of nitrate and nitrite reduction by XO being nitric oxide (NO) (Millar et al. 1998). Further studies have shown that other animal molybdoenzymes, AO, SO, and mARC, also can reduce nitrate and nitrite to nitric oxide (Sparacino-Watkins et al. 2014; Wang et al. 2015; Maia and Moura 2018). Nitric oxide (NO) plays numerous physiological and pathophysiological functions in animals. In mammals, it engages in different physiological processes, including cell proliferation, differentiation, vasodilation, neurotransmission, angiogenesis, apoptosis, hormone secretion, and smooth muscle motility, and it has antimicrobial and antitumour activities (Lundberg et al. 2008). In terrestrial animals, nitric oxide formation from L-arginine is catalysed specifically by the enzyme NO-synthase (NOS) (Alderton et al. 2001; Daff 2010). In recent years, there has been increasing evidence for NOS expression and the physiological significance of NO in non-mammalian vertebrates. However, there are fewer reports of NOS activity and the physiological role of NO in ectothermic vertebrates such as fish than in the mammalian system (Choudhury and Saha 2015). The study of NOS-independent NO generation by molybdoenzymes from nitrate and nitrite is of great interest in the fish body because of the possibility of reducing stress caused by water pollution with nitrogen compounds.

Robles-Porchas et al. 2020 describe that pollution of aquatic environments with nitrogen compounds, such as ammonia (NH_3) and nitrite (NO_2^-), is an actual problem in aquaculture and aquaponics. If not removed, high levels of NH_3 can damage fish gills and reduce their ability to extract oxygen from water, leading to oxygen starvation, neurotoxicity, suffocation, and death. NO_2^-, produced as an intermediate product in the conversion of NH_3 to NO_3^-, can also reach toxic levels if nitrification is not efficient. High NO_2^- levels can reduce fish growth, and stress, and cause death by affecting their ability to transport oxygen. NO_3^-, on the other hand, is less toxic but can accumulate in water and cause stress, reduced growth, and changes in fish colour and health if denitrification is not efficient. To prevent harm to fish in an aquaponic system, it is crucial to monitor nitrogen levels and implement strategies to control them, such as water changes, adjusting stocking density, and promoting nitrification and denitrification.

Nitrification and denitrification are two important processes that occur in an aquaponic system (Robles-Porchas et al. 2020). Nitrification is the conversion of NH_3 produced by fish into NO_3^- by bacteria. It can occur through single-stage nitrification (also known as Commamox) or two-stage nitrification. In single-stage nitrification, *Nitrospira* bacteria directly convert NH_3 into NO_3^-, while in two-stage nitrification, two different groups of bacteria, *Nitrosomonas* and *Nitrobacter*, first convert NH_3 to NO_2^- and then to NO_3^-. On the other hand, denitrification is the process where NO_3^- is converted back into nitrogen gas by anaerobic bacteria in the absence of oxygen. This typically occurs in plant roots, sediment, or in low-flow, oxygen-depleted areas in an aquaponic system. Denitrification helps reduce the accumulation of NO_3^-, which can become toxic to fish if its concentration becomes too high.

At present, there is no information about the properties of fish molybdoenzymes and their ability to generate NO independently of NOS from nitrates and nitrites. However, the reduction of NO_3^- and NO_2^- by animal molybdoenzymes has been previously proven. NO is important for fish health because it plays a crucial role in many physiological

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processes, just as it does in terrestrial animals. NO acts as a powerful antioxidant, neutralizing reactive oxygen species and reducing oxidative stress (Choudhury and Saha 2015). By maintaining a healthy balance between NO and reactive oxygen species, fish can protect themselves from cellular damage and oxidative stress caused by high levels of nitrogen compounds (Choudhury and Saha 2015). Based on the physiological role of this molecule, it can be assumed that it plays an important role in fish health (Choudhury and Saha 2015). It was hypothesized that molybdoenzymes' ability to convert NO_3^- and NO_2^- to NO helps protect fish from the harmful effects of nitrogen compounds in an environment. Therefore, the study of fish molybdoenzymes and their associated nitrate and nitrite reduction activities is highly important. This work aims to study the molybdoenzymes XO and AO of the European catfish (*Silurus glanis*) and their possible associated activities to reduce nitrates and nitrites to nitric oxide.

MATERIAL AND METHODS

European catfish (*S. glanis*) was provided by the Nazarbayev Intellectual School of Physics and Mathematics in Astana. *S. glanis* was grown under aquaculture conditions under 22 and 26 °C, at a constant water pH of 6.2 ± 0.2, and the level of ammonium was approximately 0.6 mg/m^3. The traditional hypothermia method was used to mortify fish ethically (approval number 4, March 2022), based on lowering the internal body temperature – an ice-slurry (Blessing et al. 2010).

Samples preparation

Fish molybdoenzymes and their localization in internal organs have not been previously studied. Based on the results of studies on terrestrial animals, after mortification, the liver of *S. glanis* was used as a source of XO and AO (Maia and Mira 2002). Before homogenization, a thoroughly washed liver of *S. glanis* was cut into small pieces. Small pieces of *S. glanis* liver were mixed with 0.1 M potassium phosphate buffer (NaH_2PO_4, KH_2PO_4, BioChemica, AppliChem GmbH, Darmstadt, Germany) pH 7.0 (tissue to buffer ratio was 1 : 2), containing 0.1 mM ethylenediaminetetraacetic acid (EDTA, BioChemica, AppliChem GmbH, Darmstadt, Germany), 10 µM phenylmethylsulfonyl fluoride (PMSF, BioChemica, AppliChem GmbH, Darmstadt, Germany), 5.0 µM glutathione (GSH, BioChemica, AppliChem GmbH, Darmstadt, Germany), and 10 µM Na_2MoO_4 (sodium molybdate, BioChemica, AppliChem GmbH, Darmstadt, Germany) – hereinafter referred to as buffer A. To obtain a cell-free liver extract, the mixture containing *S. glanis* liver and buffer A was destroyed using an ultrasonic homogenizer (device UP200S, Hielscher UltrasonicsGmbH, Teltow, Germany). Then, to obtain an extract, the liver homogenate was centrifuged in a centrifuge Eppendorf 5804R (Sigma-Aldrich, St Louis, MO, USA) at 15 000 *g*, +4 °C for 15 minutes. To study molybdoenzymes, catfish liver extract was passed through Sephadex G-50 medium (1.5 × 20 cm, Sigma-Aldrich, St Louis, MO, USA) to remove low molecular weight substances (including substrates and natural electron donors of molybdoenzymes). Gel filtration on Sephadex was performed in the 50 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 10 µM Na_2MoO_4, 5.0 µM GSH and 10 µM PMSF. After gel filtration, the first protein fraction was used to study molybdoenzymes.

Determination of the activity of molybdoenzymes

Determination of intrinsic activities of molybdoenzymes. The intrinsic activity of two molybdenum-containing enzymes xanthine oxidase and aldehyde oxidase was determined in the catfish liver extract. XO and AO, using NAD^+ as an electron acceptor, oxidize hypoxanthine and benzaldehyde in the presence of O_2. To reduce nitrate or nitrite by XO and AO enzymes, terrestrial animals can use NADH and their substrates, i.e., hypoxanthine and benzaldehyde as electron donors (Maia and Moura 2018). XO and AO activities were determined in 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA. XO activity was determined by the reduction of NAD^+ and the formation of uric acid at room temperature (Iyer and Kadam 2007). The amount of uric acid formed was determined by measuring its ultraviolet light absorption at 295 nm (Specol-2000; Analytik Jena AG, Jena, Germany). The activity of aldehyde oxidase was determined by the reduction of $K_3[Fe(CN)_6]$ (Potassium ferrocyanide trihydrate, BioChemica, AppliChem GmbH, Darmstadt, Germany) using benzaldehyde as a substrate at room temperature (Kadam

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and Iyer 2008). The colour change of the reaction mixture after incubation was measured at 420 nm on a spectrophotometer. One unit of XO (xanthine oxidase) and AO (aldehyde oxidase) intrinsic activity was determined by the amount of protein catalysing the formation of 1 nmol of uric acid and reduction of potassium ferrocyanide trihydrate in one minute respectively.

Determination of associated activities of molybdoenzymes. The nitrate reductase (NR) activity of molybdoenzymes was determined by the amount of nitrite formed after incubation of the reaction mixture (Alikulov et al. 1980). Nitrite reductase (NiR) activity was determined by nitrite reduction, i.e., by looking at the reduction of the amount of nitrite added before the incubation of the mixture (Alikulov et al. 1980). To analyse NR and NiR activities of XO and AO possible physiological electron donors (Mukhamejanova et al. 2021) were used: hypoxanthine (for XO, BioChemica, AppliChem GmbH, Darmstadt, Germany), benzaldehyde (for AO, BioChemica, AppliChem GmbH, Darmstadt, Germany), NADH (BioChemica, AppliChem GmbH, Darmstadt, Germany), and methyl viologen (BioChemica, AppliChem GmbH, Darmstadt, Germany). NR and NiR activities were determined by the amount of nitrite in nanomoles per mg of protein and per minute.

Photometric method for the determination of nitrites

Nitrites were determined using N-(1-naphtyl) ethylenediamine dihydrochloride (NEDD, BioChemica, AppliChem GmbH, Darmstadt, Germany) and sulfanilamide (SA, BioChemica, AppliChem GmbH, Darmstadt, Germany) dissolved in hydrochloric acid (Mendel and Muller 1978). At pH 2–2.5, NO_2^- forms a diazonium compound with SA. The latter reacts with NEDD to form a purple azo dye. The colour intensity is determined on a spectrophotometer at 1 cm thickness of the liquid layer in a cuvette and at 548 nm.

Determination of molybdoenzymes activities with heat treatment

Godber et al. (2005) found that various animal tissues contain populations of molybdenum-free molecules of molybdoenzymes. This means that the active centre of these molecules does not contain the required number of molybdenum atoms. Therefore, experiments on the activation of XO and AO isolated from the liver of *S. glanis* by exogenous molybdenum were carried out. Na_2MoO_4 was used as a source of exogenous molybdenum atoms. Since animal XO and AO are known to be highly thermally stable, molybdoenzymes were activated by heat treatment of the protein fraction at various temperatures in the presence of molybdate and GSH (Iyer and Kadam 2007; Kadam and Iyer 2008). Preliminary experiments on the activation of animal molybdoenzymes showed that the optimal concentrations of sodium molybdate and GSH were 1.0 mM (Mukhamejanova et al. 2021). Therefore, these concentrations of the indicated reagents were used to treat protein fraction derived from *S. glanis* liver. Heat treatment in the presence of GSH and sodium molybdate for up to 7 min leads to partial denaturation of the enzyme molecule, and as a result, access to the active centre for exogenous molybdenum is opened. The presence of GSH protects the SH groups of pyranopterin from oxidation (Mukhamejanova et al. 2021). Under reduced conditions, the exogenous molybdenum atom is bound by these groups. To determine the optimal temperature for activation of XO and AO, catfish liver protein fraction was heated at temperatures ranging from 40 °C to 80 °C in the presence of 1.0 mM GSH and 1.0 mM sodium molybdate for 3, 5, and 7 min (followed by rapid cooling) (Mukhamejanova et al. 2021). Then the intrinsic activities of XO and AO were determined using the same method as described above (Iyer and Kadam 2007; Kadam and Iyer 2008).

Study of the effect of tungsten on the activity of molybdoenzymes

After determining, the optimal temperature for activation of XO and AO in the presence of molybdenum, the effect of tungsten on the activation of these enzymes at optimal temperature was also studied. For this, highly water-soluble sodium salt of tungsten (Sodium tungstate, Na_2WO_4, BioChemica, AppliChem GmbH, Darmstadt, Germany) was used at the same concentration as molybdenum (1 mM). It is well known that tung-

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sten is a chemical analogue of molybdenum, i.e., the atomic structures of tungsten and molybdenum are similar. Therefore, in the absence of molybdenum, tungsten is easily integrated into the active centre of the molybdoenzyme and binds with pyranopterin in place of molybdenum. However, compared with molybdenum, the redox potential of the tungsten atom is much lower, and therefore, during a catalytic reaction, it cannot transfer electrons in the active centre of molybdoenzymes in eukaryotes, i.e., their tungsten-bound molybdoenzymes become inactive (Cordas and Moura 2019). Therefore, the use of tungsten plays an important role in the identification of new molybdoenzymes in eukaryotes. Furthermore, using tungsten makes it possible to confirm the inclusions of exogenous molybdenum in the active centre of molybdoenzymes. The study of tungsten's influence compared with the influence of molybdenum was carried out to determine their influence on the activation of fish molybdoenzymes. For this, the intrinsic activities of molybdoenzymes and associated activities (NOS activity) were determined. Associated activities were determined by the formation of nitric oxide (NO) via NR and NiR activities of molybdoenzymes with different possible physiological electron donors such as hypoxanthine, benzaldehyde, NADH, and methyl viologen (as an artificial electron donor) (Alikulov et al. 1980; Mukhamejanova et al. 2021).

Determination of nitric oxide (NO) content

Nitric oxide is determined by a photometric method by measuring the optical density of a red-pink azo dye formed because of the following reactions: (1) oxidation of nitric oxide NO (II) to nitrogen dioxide (IV) at the time of sampling; (2) interaction of nitrogen dioxide with potassium iodide to form potassium nitrite (KNO_2); (3) interaction of potassium nitrite with SA and NEDD. The measurement was carried out on a spectrophotometer at 548 nm (Mendel and Muller 1978).

Data analysis

The data was statistically analysed using Microsoft Excel 365 (v2022; Microsoft Corporation, Redmond, WA, USA). The two-sample *t*-test, one-way and two-way analysis of variance (ANOVA) were used for the statistical analysis of the experimental data. A value of 0.05 was chosen to determine the significance of the *t*-test and the alpha value of the ANOVA. *P*-values lower than 0.05 (95% confidence level) were considered statistically significant.

RESULTS AND DISCUSSION

The nitrate- and nitrite-reducing (NR and NiR) activities of xanthine oxidase (XO) and aldehyde oxidase (AO) in liver protein fraction were determined using natural electron donors and an artificial electron donor, methyl viologen. The experiments began with the determination of the intrinsic (physiological) activity of XO and AO in the protein fraction of the *S. glanis* liver extract after gel filtration on Sephadex. At the same time, their possible associated activities (i.e., NR and NiR activity) were determined (Table 1). One-way ANOVA was used to analyse the molybdoenzymes' intrinsic and associated activities. ANOVA shows the *P*-value for the NR activity $P < 0.000\ 01$ and for NiR activity $P < 0.000\ 1$. Furthermore, NR and NiR activities were compared depending on electron donors by a two-sample *t*-test.

The results in Table 1 show that NADH is the electron donor for intrinsic XO and AO activities. The substrates of these enzymes, i.e., hypoxanthine and benzaldehyde can also act as electron donors for the associated NR and NiR activities. Notably, NR and NiR activities were significantly higher when associated with NADH rather than with hypoxanthine and benzaldehyde. This is consistent with the earlier report that NADH acts as a reducing substrate (Millar et al. 1998). However, compared to the results of Millar et al. (1998), replacing NADH with xanthine as a reducing substrate did not inhibit NR and NiR activities. Thereunto, the maximum activity of these enzymes is found with an artificial electron donor, reduced methyl viologen. A similar effect of reduced methyl viologen on NiR and NR activity on XO from cow's milk was described by Alikulov et al. 1980. For all electron donors, the NiR activity of XO and AO was higher than their NR activity ($P < 0.05$).

The results presented in Table 2 show that heat treatment of the molybdoenzyme fraction in the presence of molybdate and glutathione (GSH) leads to the activation of XO and AO: the optimal conditions for max-

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Table 1. Determination of intrinsic and associated activities of molybdoenzymes in the fraction obtained by gel filtration of *S. glanis* liver extract

Intrinsic activities			
XO: NAD^+ + hypoxanthine		3.7 ± 0.3	
AO: NAD^+ + benzaldehyde		1.3 ± 0.2	
Associated activities			
NR: electron donor + NO_3^-		NiR: electron donor + NO_2^-	
[ab]NADH + NO_3^-	4.7 ± 0.3	[ab]NADH + NO_2^-	5.4 ± 0.2*
[a]Hypoxanthine + NO_3^-	3.7 ± 0.1	[a]Hypoxanthine + NO_2^-	4.4 ± 0.2*
[b]Benzaldehyde + NO_3^-	1.9 ± 0.4	[b]Benzaldehyde + NO_2^-	3.8 ± 0.6*
[ab]Methyl viologen + NO_3^-	5.8 ± 0.2	[ab]Methyl viologen + NO_2^-	7.2 ± 0.5*

AO = aldehyde oxidase; NiR = nitrite reductase; NR = nitrate reductase; XO = xanthine oxidase

[a]NR or NiR activities of XO; [ab]common NR or NiR activities of first protein fraction; [b]NR or NiR activities of AO

*Statistically significant differences in NiR compared to NR ($P < 0.05$)

The table shows the mean values ± standard deviation. One unit of XO and AO intrinsic activity was determined by the amount of protein (mg) catalysing the formation of 1 nmol of uric acid and reduction of $K_3[Fe(CN)_6]$ in one minute respectively. NR and NiR activities were determined for the formation and utilization of nitrites in the reaction mixture (amount of nitrite in nanomoles per mg of protein per minute)

One-way ANOVA revealed statistically significant results for the NR ($P < 0.000\ 01$) and NiR activities ($P < 0.000\ 1$)

imum activation of exogenous molybdate for XO and AO were reached by heating at 70 °C for 5 minutes. Statistical analysis of the data was carried out using two-way ANOVA. Significances for both molybdoenzymes were for sample, columns, and interaction $P < 0.000\ 01$. The activity of XO increased almost 4.7 times and the activity of AO 7.7 times compared with its intrinsic activity without heat treatment. The presence of molybdate and GSH in the phosphate buffer used for homogenization and gel filtration through Sephadex (i.e., heat treatments) did not change the initial activity of these enzymes. These results suggest that a molybdenum-free population of these enzymes is present in the catfish liver. Previously, it was shown

Table 2. Effect of heat treatment at different temperatures in the presence of sodium molybdate and GSH on the activity of molybdoenzymes [intrinsic activities of xanthine oxidase (XO) and aldehyde oxidase (AO)]

Duration of heat treatment in minutes	Temperature (°C)				
	40	50	60	70	80
Xanthine oxidase					
Control	1.3 ± 0.3	ND	ND	ND	ND
3	4.3 ± 0.3	6.5 ± 0.3	12.4 ± 0.4	16.7 ± 0.4	15.4 ± 0.5
5	4.3 ± 0.5	7.2 ± 0.5	13.7 ± 0.3	17.4 ± 0.3	16.2 ± 0.4
7	4.6 ± 0.5	7.4 ± 0.4	15.3 ± 0.5	16.7 ± 0.6	14.2 ± 0.2
Aldehyde oxidase					
Control	0.8 ± 0.1	ND	ND	ND	ND
3	1.7 ± 0.3	2.6 ± 0.3	6.2 ± 0.5	8.8 ± 0.5	8.1 ± 0.3
5	1.9 ± 0.2	3.4 ± 0.5	8.1 ± 0.4	10.4 ± 0.8	9.1 ± 0.4
7	2.1 ± 0.4	3.7 ± 0.8	8.3 ± 0.3	8.9 ± 0.3	7.7± 0.2

Control = heat treatment for 5 min without sodium molybdate and glutathione; ND = not detected

The table shows the mean values ± standard deviation. The control values in the table are given minus the initial activity of XO and AO. The initial activities of XO and AO without heat treatment were 3.7 and 1.3 units respectively. One unit of XO and AO intrinsic activity was determined by the amount of protein (mg) catalysing the formation of 1 nmol of uric acid and reduction of $K_3[Fe(CN)_6]$ in one minute respectively

Two-way ANOVA revealed statistically significant results for the XO and AO ($P < 0.000\ 01$)

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by Godber et al. (2005) that some molybdoenzymes can occur in molybdenum-free form and have no molybdenum in cofactors.

To confirm the incorporation of exogenous molybdenum into the active centre of molybdoenzymes, during heat treatment (70 °C), sodium tungstate was used instead of molybdate at the same concentration as molybdate in the presence of glutathione. In these experiments in protein fraction, in addition to the intrinsic activities of XO and AO, the activities of NR and NiR, and the amount of NO (NOS activity) formed during the catalytic reaction of these enzymes using various electron donors were also determined (Table 3). Thus, the effect of tungsten on the activity of XO and AO was studied as well. Two-sample *t*-tests, one-way and two-way ANOVA were used for the statistical analysis of the data. One-way ANOVA shows the *P*-value for the intrinsic activity of XO and AO was $P < 0.000\,01$. As shown by two-way ANOVA, there were statistically significant differences in the effect of heat treatment in the presence of exogenous molybdate or tungstate on the formation of NO in both NR and NiR ($P < 0.01$). Intrinsic and associated activities of molybdoenzymes were compared depending on heat treatment temperature by a two-sample *t*-test and the statistically significant difference was observed ($P < 0.000\,01$).

The results which are presented in Table 3, show that protein fraction was active in reducing nitrates and nitrites to nitrogen monoxide (NO) using both natural and artificial electron donors. NO is formed from nitrite by the enzymes XO and AO much more than from nitrate, i.e., the NiR activity of XO and AO is much higher than NR. Both of these protein fraction activities also sharply increased after heat treatment in the presence of molybdate and glutathione. The highest NR and NiR activities were obtained using an artificial electron donor, reduced methyl viologen. Exogenous molybdenum after such treatment increases the nitrite-reducing activity with methyl viologen up to 5.6 times compared with the control (without heat treatment).

Heat treatment in the presence of WO_4^{2-} resulted in a complete loss of all activities of these enzymes

Table 3. Comparative study of the effect of heat treatment (70 °C for 5 min) of the fraction of molybdoenzymes in the presence of exogenous molybdate or tungstate on the activity of xanthine oxidase (XO), aldehyde oxidase (AO), their nitrate reductase (NR) and nitrite reductase (NiR), and the formation of nitric oxide (NO)

Electron donor + substrate	Pre-treatment in the presence of glutathione, MoO_4^{2-} or WO_4^{2-}		
	25 °C + MoO_4^{2-}	70 °C + MoO_4^{2-}	70 °C + WO_4^{2-}
Intrinsic activities			
XO: NAD^+ + hypoxanthine	3.8 ± 0.4	17.0 ± 1.2*	ND
AO: NAD^+ + benzaldehyde	1.6 ± 0.7	9.8 ± 0.9*	ND
Formation of NO by NR activity of XO and AO enzymes			
[ab]NADH + NO_3^-	3.8 ± 0.4	17.5 ± 3.2*	ND
[a]Hypoxanthine + NO_3^-	2.1 ± 0.3	9.3 ± 1.4*	ND
[b]Benzaldehyde + NO_3^-	1.9 ± 0.1	14.3 ± 2.7*	ND
[ab]Methyl viologen + NO_3^-	3.2 ± 0.2	21.4 ± 2.9*	ND
Formation of NO by NiR activity of XO and AO enzymes			
[ab]NADH + NO_2^-	4.3 ± 0.7	24.3 ± 3.5*	ND
[a]Hypoxanthine + NO_2^-	2.3 ± 0.3	13.5 ± 3.7*	ND
[b]Benzaldehyde + NO_2^-	2.8 ± 0.4	16.3 ± 1.7*	ND
[ab]Methyl viologen + NO_2^-	5.2 ± 0.7	29.2 ± 2.6*	ND

[a]NOS activity of XO; [ab]common NOS activities of first protein fraction; [b]NOS activity of AO

*Statistically significant differences by a two-sample *t*-test compared depending on heat treatment temperature ($P < 0.000\,01$)

The table shows the mean values ± standard deviation. One unit of XO and AO intrinsic activity was determined by the amount of protein (mg) catalysing the formation of 1 nmol of uric acid and reduction of $K_3[Fe(CN)_6]$ in one minute respectively. NR and NiR activities (NOS activity) for the formation of NO (number of nitrites in nanomoles per mg of protein per minute)

One-way ANOVA revealed statistically significant results for the intrinsic activity of XO and AO was $P < 0.000\,01$. Two-way ANOVA revealed statistically significant NO formation results in NR and NiR ($P < 0.01$)

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(Table 3). Thus, the results obtained show that during heat treatment in the presence of MoO_4^{2-} or WO_4^{2-} and GSH, the atoms of these metals are included in the active centre of these enzymes, increasing their activity, or completely inactivating them. This effect of tungsten as an inhibitor of molybdoenzymes has already been described in several works (Cordas and Moura 2019). These results convincingly show that molybdoenzymes, at least XO and AO contained in protein fraction, have the activity to reduce nitrates and nitrites to nitric oxide.

The NiR and NR activities of AO and XO already have been described in various terrestrial animals (Alikulov et al. 1980; Millar et al. 1998; Maia and Moura 2018). However, as noted by Millar et al. in 1998, these NOS-independent NO generation activities are activated under only hypoxic conditions of tissues. Hypoxia is a state in which living organisms have inadequate oxygen levels to satisfy their requirements. The main cause of hypoxia in fish is a decrease in dissolved oxygen content in the aquatic environment. In theory, under conditions of water hypoxia, nitrogen compounds, especially NO_2^- present in water, can aggravate hypoxia in fish by reducing the transported oxygen. However, studies conducted by Hansen and Jensen in 2010 on goldfish (*Carassius auratus L.*) under conditions of hypoxia with increased content of NO_2^- (0.43 ± 0.28 µmol/100 ml) showed a decrease in NO_2^- and NO_3^- in the internal organs. They also showed that tissue-specific NO values comparable to normoxic goldfish were preserved during hypoxia, with a concomitant decrease in NOS activity. After establishing the ability of XO and AO isolated from the liver of *Silurus glanis* to convert NO_2^- and NO_3^- into NO, it is suggested that the NiR and NR activities of these serve to detoxify the fish body from nitrates and nitrites. NOS-independent NO generation under hypoxic conditions by XO and AO can be considered an addition to NOS activity, which requires oxygen (Millar et al. 1998). Thereby, the NO molecules generated by AO and XO likely protect the liver cells from oxidative stress-related damage and help fish better adapt to water pollution with nitrogen compounds (Choudhury and Saha 2015).

CONCLUSION

In this work, the activities of fish liver molybdoenzymes were studied for the first time. It was found that molybdoenzymes XO and AO have the activity to convert nitrates and nitrites into NO (nitric oxide). With this, molybdoenzymes can possibly make a significant contribution to the removal of nitrates and nitrites in the fish body by NOS-independent NO generation. It has been determined that a proportion of molybdoenzymes occurs in the molybdenum-free form. However, it has been shown that the activity of molybdoenzymes can be induced in the presence of an additional source of molybdenum. As a result of these data, it can be assumed that feeding fish with molybdenum-containing feed can lead to the activation of molybdoenzymes and to the utilization of nitrates and nitrites, and protect fish from the harmful effects of nitrogen compounds in an environment. In solving this problem, searching for *in vivo* methods of activating molybdoenzymes of the internal organs of fish by exogenous molybdate is an essential direction for future research.

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Conflict of interest

The authors declare no conflict of interest.

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