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THE EFFECT OF ANTIOXIDANTS ON XANTHINE OXIDASE ACTIVITY IN FRESH OVINE MILK

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ABSTRACT

In the present, the consequences of nitrate pollution of the environment are very pronounced. In humans and animals, microorganisms can reduce nitrates to nitrites, which cause cancer. Purified and homogeneous xanthine oxidase (XO) of cow's milk can restore these compounds, which makes the article extremely relevant. The purpose of the article is to determine the effect of antioxidants on the activity of xanthine oxidase in fresh ovine milk. Various natural and artificial antioxidants were examined for the detection of xanthine oxidase (XO) activity in ovine milk. Among the natural antioxidants, L-cysteine was more effective in the stabilization of XO in heated milk. XO of sheep milk activated by heat treatment in the presence of cysteine and molybdenum became able to convert nitrate and nitrite to nitric oxide (NO). Therefore, L-cysteine was used for double purposes: as the protector of enzyme active center against the oxidation during heat treatment of milk and as a reagent for S-nitrosothiol formation. Hypoxanthine, as a natural substrate of XO, is an effective electron donor for nitrate reductase (NR) and nitrite reductase (NiR) activities. Heat treatment of the milk in the presence of exogenous lecithin increased the activity of NR and NiR of XO and CysNO formation. Thus, during the heat treatment: a) excess of exogenous phospholipids disintegrates the structure of milk fat globule membrane (MFGM) and b) enzyme molecules denatured partially and their active center became available for exogenous cysteine, molybdenum, hypoxanthine, and nitrate or nitrite.

Keywords: antioxidant; molybdenum; tungsten; phospholipids; nitrate; nitrite

INTRODUCTION

At present time the pollution of the environment with nitrates is a big problem in the world. In the environment and in the human and animal body microorganisms may reduce nitrates to nitrites.

It is well known that nitrates (NO_3^-) and nitrites (NO_2^-) cause various diseases, including cancer. It is well known that nitrites irreversible bind to hemoglobin forming methemoglobin which losses the ability to transport oxygen **(Reynolds et al., 2007)**. Deficiency of oxygen causes asphyxiation and it is particularly hazardous to health for babies. Furthermore, nitrites easily bind to primary amines, such as cadaverine, putrescine, spermidine, and form potential carcinogens – nitrosamines **(Bryan, 2006; Gladwin et al., 2005)**.

For the first time in 1980 were observed that purified and homogeneous xanthine oxidase (XO) of cow's milk can reduce NO_3^- and NO_2^- **(Alikulov et al., 1980)**. Later, other groups of scientists have found that animal XO converts NO_3^- and NO_2^- to a physiologically important substance – nitric oxide (NO) **(Millar et al., 1998; Zhang et al., 1998; Hord et al., 2011)**. It is generally recognized that NO is one of the major biological messenger molecules, regulating blood pressure and blood flow, neurotransmission and brain function, immune system function, wound healing inhibition of platelet aggregation. NO is also involved in defense mechanisms against pathogens and some kinds of cancer cells **(Milkowski et al., 2010)**. In 1992 the nitric oxide was recognized as a molecule of the year. Scientists, studying its properties, have been awarded the Nobel Prize later in 1998 **(Bryan et al., 2009)**.

Because of the ability to form nitric oxide, the XO of milk harbors an antimicrobial activity. It is known that NO as the oxidant is a strong antibacterial agent. Antibacterial functions of XO are associated with peroxynitrite (ONOO-) which is the product of the reaction between NO and O_2 (superoxide anion). XO is also involved in protective and antiviral response by catalyzing the conversion of retinaldehyde to retinoic acid. Retinoic acid derivatives can inhibit viral replication and, thus, preventing the spread of viral disease **(Taibi et al., 2001; Taibi and Nicotra, 2007)**.

Xanthine oxidoreductase or dehydrogenase/oxidase (XO; EC 1.1.3.22) – molybdenum and iron-containing flavoprotein. Each of the two subunits of XO contains one molybdenum center, two iron-sulfur center, and one FAD. XO of the breast milk of women shows a molecular mass of

160 kDa subunit, XO of the cow's milk – 150 kDa. It is believed that the main biological function of XO is catalysis the final step of purine oxidation in eukaryotes; it catalyzes the sequence of hydroxylation that converts hypoxanthine to xanthine, then to uric acid **(Harrison, 2004)**. However, the enzyme has broad substrate specificity and is capable of reducing oxygen to generate the reactive oxygen species (ROS), superoxide and hydrogen peroxide, as well as oxidative transformation of pteridines and some aliphatic and aromatic aldehydes. Xanthine oxidase (XO) is not strongly specific to the oxidation of hypoxanthine or xanthine; it may catalyze the oxidation of about thirty nitrogen-containing heterocycles and aldehydes **(Harrison, 2004)**. Therefore, because of its multifunctional enzymatic reactions, XO is considered a potential enzyme detoxifying different xenobiotics **(Beedham, 2001)**. It is known that numerous heterocyclic xenobiotics (including pesticides) are carcinogens. Thus, in the case of contamination of milk with harmful xenobiotics, the active XO makes possible their biotransformation into harmless forms. XO reduces nitrite (and nitrate), yielding reactive nitrogen species (RNS), such as nitric oxide and peroxynitrite **(Harrison, 2006)**.

In cow's milk XO is localized in the inner membrane layer of the fat globules (XO content is about 8% of total proteins of cove milk fat globules) **(Mondy and Keenan, 1993)**. However, XO of milk fat globule membranes (MFGM) contains tenth times less molybdenum or does not contain it which results in very low enzyme activity **(Godber et al., 1997; Bray et al., 1999; Atmani et al., 2004)**. A study of the cofactor composition of XO isolated from cow's and women's milk showed that more than 85-90% of enzyme molecules don't contain molybdenum in their active center and are inactive molecules **(Bray et al., 1999)**. The occurrence of xanthine oxidase in animal milk was a matter of slight controversy for a long time because in the milk xanthine oxidation has never been observed, although the levels of XO in the milk are relatively high. Presumably, this is because, before the inclusion of Mo atoms in the active center of the enzyme, Mo-free molecules of XO already is associated with the inner membrane of milk fat globules. As a result, XO molecules become molecules without molybdenum. It is proposed that XO is involved in the secretion of milk fat globules in a process dependent on the enzyme protein rather than on its enzymatic activity **(Harrison, 2006)**.

The ability of XOR to generate a diverse range of Reactive Oxygen and Nitrogen Species (RONS) has been the driving force for the intense study of the XOR enzyme. The XOR generation of ROS and the reactive nitrogen species (RNS) have been demonstrated **(Godber et al., 2000)**. The hypothesized scheme for O_2•- and H_2O_2 generation during the re-oxidation of reduced XO with O_2 was confirmed in 1981, it was concluded that a fully reduced XO monomer could generate two molecules of H_2O_2 and O_2•-, respectively **(Porras et al., 1981)**. The discovery of XOR nitric oxide synthase and peroxynitrite synthase activity has added further interest to the XOR. The enzyme XOR is capable of reducing organic and inorganic nitrate to nitrite, and nitrite to NO•, under hypoxic conditions. According to Millar et al. **(1998)**, all redox centers need to be inserted and be active. However, in the absence of active cofactors, for example, Mo, it is feasible to suggest that the FAD redox center alone could generate RONS under XOR–NADH oxidase activity **(Abadeh et al., 1992)**.

In the presence of the molybdenum atom in the active center, XO is active but in the absence of the metal, it becomes inactive. Like other molybdoenzymes, in the active center, XO contains the so-called molybdocofactor which has a pterin nature (therefore, it is called also molybdopterin) **(Kramer et al., 1987)**. The molybdenum atom is bound to the pterin via its vicinal thiol groups (Figure 1). This pterin is synthesized in vivo independently in the presence of molybdenum, i.e. in the absence of molybdenum cellular XO contains a normal amount of the cofactor. The cofactor is buried deep within the interior of the enzyme molecule and a tunnel-like structure makes it accessible to the appropriate substrates **(Kisker et al., 1997)**.

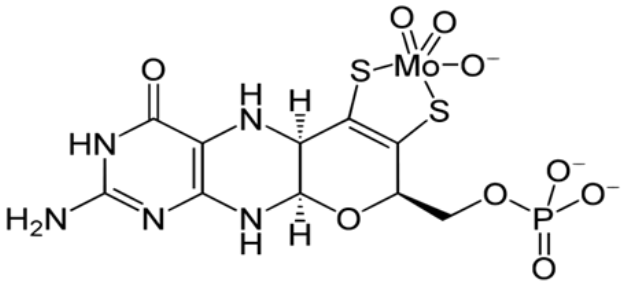

Figure 1 Structure of molybdocofactor and its bond with molybdenum atom via vicinal SH-groups in the active center of XO **(Kramer et al., 1987)**.

Scientific Hypothesis

As mentioned above, milk XO exists in molybdenum-free form and is localized in the inner layer of MFGM. Therefore, the activation of milk XO requires the incorporation of exogenous molybdenum into its active center. It is generally known that XO belongs to heat-stable enzymes – it remains active at a 75 – 80 °C temperature in several minutes **(Alikulov et al., 1980)**. However, at this temperature, the enzyme molecules undergo partially reversible denaturation. Therefore, one of the possible ways for the availability of XO molecules for exogenous molybdenum is the disintegration of milk fat globule membranes and partial denaturation of enzyme molecules. Thus, during the heat treatment:

a) excess of exogenous phospholipids disintegrate the structure of MFGM and

b) enzyme molecules denatured partially and their active center became available for exogenous cysteine, molybdenum, hypoxanthine, and nitrate or nitrite. Cysteine not only protects SH-groups of Mo-co against oxidation but also promotes the binding of molybdenum to the cofactor.

MATERIAL AND METHODOLOGY

Samples

Fresh milk samples were obtained from healthy female animals at a local farm near Astana city.

Chemicals

Chemicals were purchased from Sigma-Aldrich Chemical Co. All common chemicals and solvents used were of analytical grade.

Instruments

Ethylenediaminetetraacetic acid (EDTA) to bind heavy metals was used for binding heavy metals from milk. Milk was boiled in narrow conical tubes in a volume of 2 ml. Special reagents were used dor detecting different activities of XO. Egg yolk lecithin from AppliChem (Panreac, ITW Companies, Darmstadt, Germany) was used in the experiments.

Laboratory Methods

Method for determination of the intrinsic activity of xanthine oxidase (XO). To determine the intrinsic activity of XO 200 μL aliquot of milk was mixed with 700 μL 0.1 M sodium phosphate buffer containing 10 μM EDTA, 5 μL phenylmethylsulfonylfluor (for inhibition of protease activity). To this mixture was added 100 μM 10 mM hypoxanthine. The mixture was incubated at 30 °C for 10 min under aerobic conditions. The proteins in the mixture are precipitated with trichloroacetic acid. After centrifugation, the amount of uric acid (under the influence of XO converted hypoxanthine to uric acid) in the supernatant was determined by measuring the absorbance of the reaction mixture in a spectrophotometer at 295 nm **(Suzuki et al., 2015)**. The amount of uric acid also is determined by its chemical oxidation with potassium permanganate (K_2MnO_4) or hydrogen peroxide (H_2O_2), thereby forming allantoin **(Poje and Sokolić-Maravić, 1986)**. Allantoin in biological liquids is easy to very accurately determined by a known method **(Vogels and Van der Drift, 1970)**.

Method for the determination of nitrate- and nitrite-reducing activity of XO. The nitrate-reducing activity of XO is determined by the disappearance of the added nitrate (NO_3^-) or by the appearance of nitrite (NO_2^-) in the reaction medium **(Alikulov et al., 1980)**. The nitrite-reducing activity of XO is determined by the disappearance of the nitrite to the reaction medium or by the appearance of nitric oxide (NO) **(Kuo et al., 2003)**.

Sample preparation:

Preparation of the milk for the detection of the various enzymatic activities. Before treatment in the milk of domestic animals added 10 μM ethylenediaminetetraacetic acid (EDTA) to bind heavy metals. For boiling fresh milk is poured into the narrow conical tubes in a volume of 2 mL. Then, for further determination of the enzymatic activity the tubes are placed in the water bath at 35 °C temperature and kept for 10 min, then using the special reagents different activities of XO are determined.

Statistical Analysis

All determinations were conducted in triplicate or more and all results were calculated as mean ± standard deviation (SD). In this study Statistical analysis was performed using Student's t-test for significance and analysis of variance (ANOVA). The *p*-values less than 0.05 were considered statistically significant. The statistical analysis data were produced by Microsoft Excel and Statistica 15.

RESULTS AND DISCUSSION

Effects of different antioxidants in the stabilizing of heat-denatured milk XO

Our preliminary experiments showed that the optimal heating temperature and its duration for detection of all activities in fresh milk XO were 80 °C and 7 min (data not shown). Besides, the presence of 0.1 mM concentrations of cysteine and exogenous molybdenum was also necessary for the detection. Thus, to detect the activity of MFGM-bound enzyme is required heat treatment and the addition of cysteine as an antioxidant and molybdenum to fresh sheep milk. In the next experiments, we examined various natural and artificial antioxidants for the detection of milk XO activity. Natural antioxidants – cysteine, glutathione, lipoic acid, and ascorbic acid, and artificial antioxidants – dithiothreitol, unithiol and mercaptoethanol (2,3-dimercapto-1-propane sulfonic acid) were used in different concentrations (Figures 2, 3, 4).

Results present in the Figures 2, 3, 4 show that among the natural antioxidants L-cysteine was more effective in the stabilization of XO in heat-treated milk. In the same concentrations the effectiveness of cysteine-containing antioxidant – glutathione was considerably lower. This is because the tripeptide molecule of glutathione contains only one cysteine residue. Although both the oxidized (disulfide) and reduced (dihydric) forms of lipoic acid (dithiol) show antioxidant properties **(Milkowski et al., 2010; Rochette et al., 2013)**, its stabilizing effect for milk XO was lower than glutathione. Among the artificial antioxidants, monothiol mercaptoethanol was more effective in the stabilization of heated milk XO. Although dithiothreitol and unithiol contain two sulfhydryl groups they don't stabilize heat-treated milk XO. Recently we found that dithiothreitol in the presence of sulfanilamide forms a stable blue complex with molybdate ion (based on this finding we developed a new fast method for molybdenum determination in the biological materials).

Results in the Figures 5, 6, 7 show that the highest levels of the associated activities of milk XO observed with artificial electron donor – methyl viologen reduced by dithionite ($Na_2S_2O_4$). Among the physiological electron donors, the highest associated activities were obtained using reduced NADH. The natural substrate of XO – hypoxanthine was also effective in the donating of electrons for NR and NiR activities of the enzyme in fresh sheep milk. Thus, the electron transfer may also occur from the hypoxanthine (the reducing substrate) to nitrite and nitrate (the oxidizing substrates) **(Abadeh et al., 1992; Godber et al., 1997)**. Because in the presence of tungstate in the reaction mixture the associated activities were not detected, during nitrate reduction by hypoxanthine, the XO molybdenum center may participate directly in both the oxidative (hypoxanthine oxidation) and the reductive (nitrate and nitrite reduction) half-reactions.

Detection of the products of nitrate- and nitrite reduction by XO in heat-treated sheep fresh milk

It is found that animal XO converts NO_3^- and NO_2^- to physiologically important gas – nitric oxide (NO) **(Millar et al., 1998; Zhang et al., 1998; Hord et al., 2011)**. NO effectively reacts with L-cysteine or reduced glutathione (GSH) at pH 7.0 and 7.4, to form orange-pink products of S-nitrosocysteine (CySNO) or S-nitrosoglutathione (GSNO). These products exhibited a peak absorbance at around 340 and 540 nm **(Kuo et al., 2003)**.

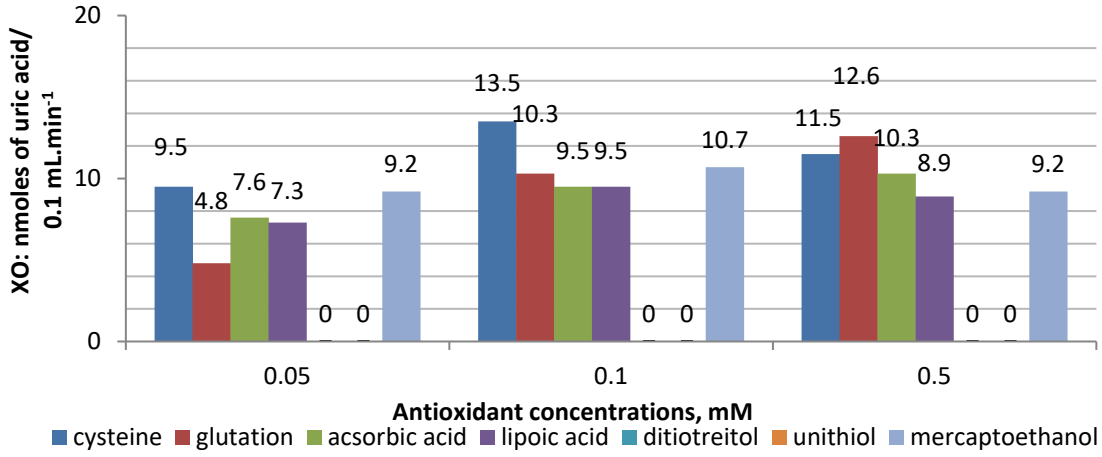

Figure 2 Effects of different antioxidants on the stability of XO during the heat treatment of fresh sheep milk in the presence of Na_2MoO_4.

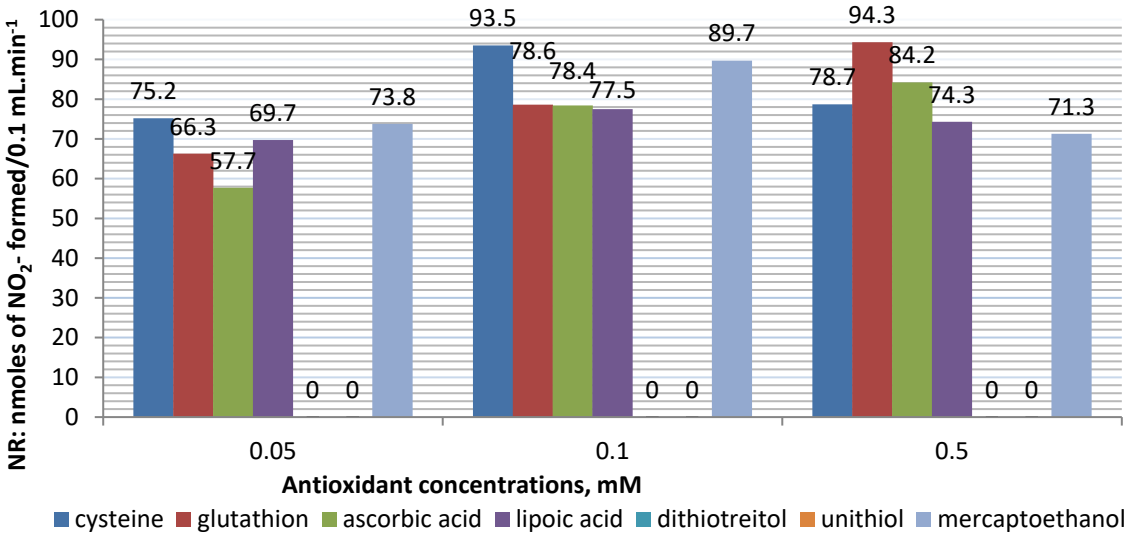

Figure 3 Effects of different antioxidants on the stability of NR activity during the heat treatment of fresh sheep milk in the presence of Na_2MoO_4.

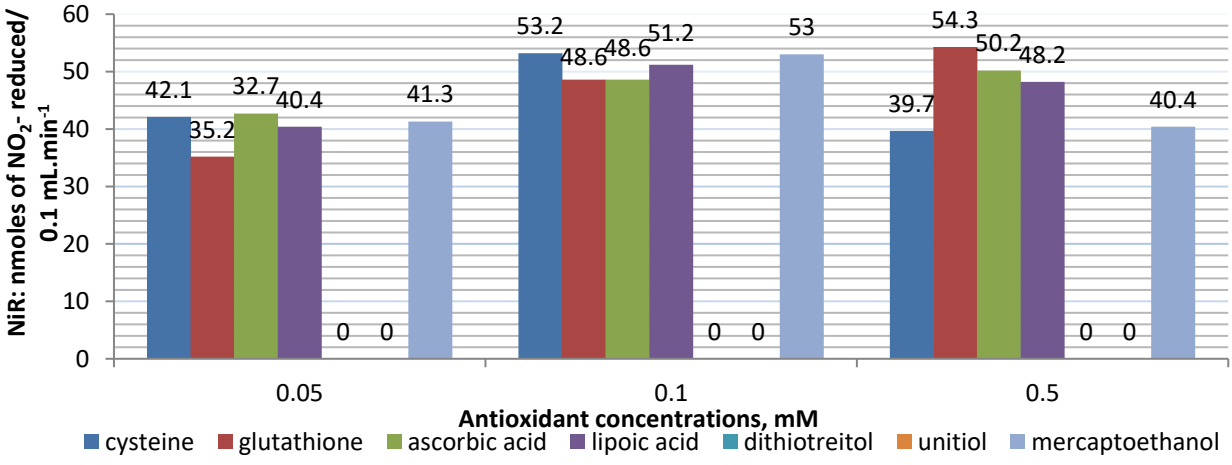

Figure 4 Effects of different antioxidants on the stability of NiR activity during the heat treatment of fresh sheep milk in the presence of Na_2MoO_4.

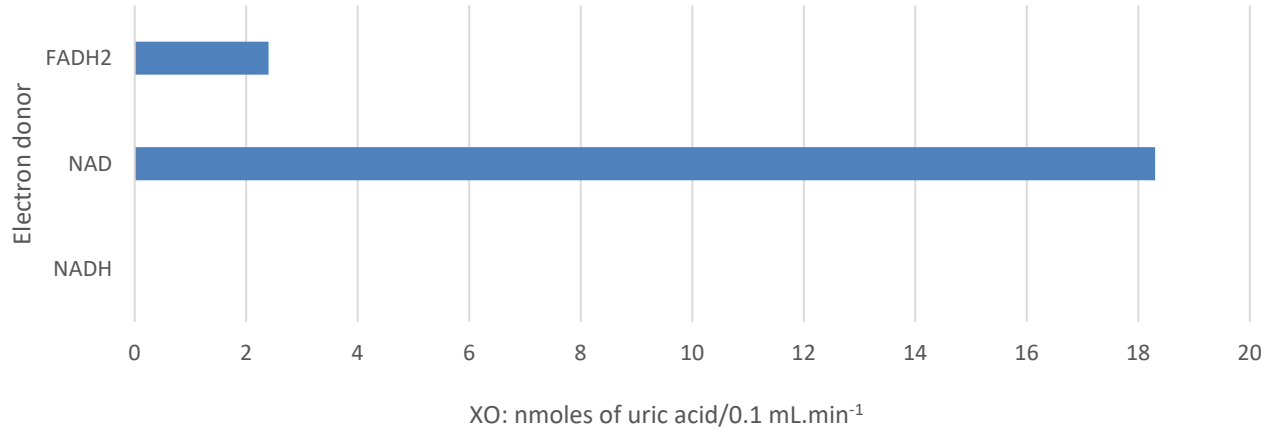

Figure 5 Effects of different electron donors on XO activity of sheep milk in the presence of cysteine.

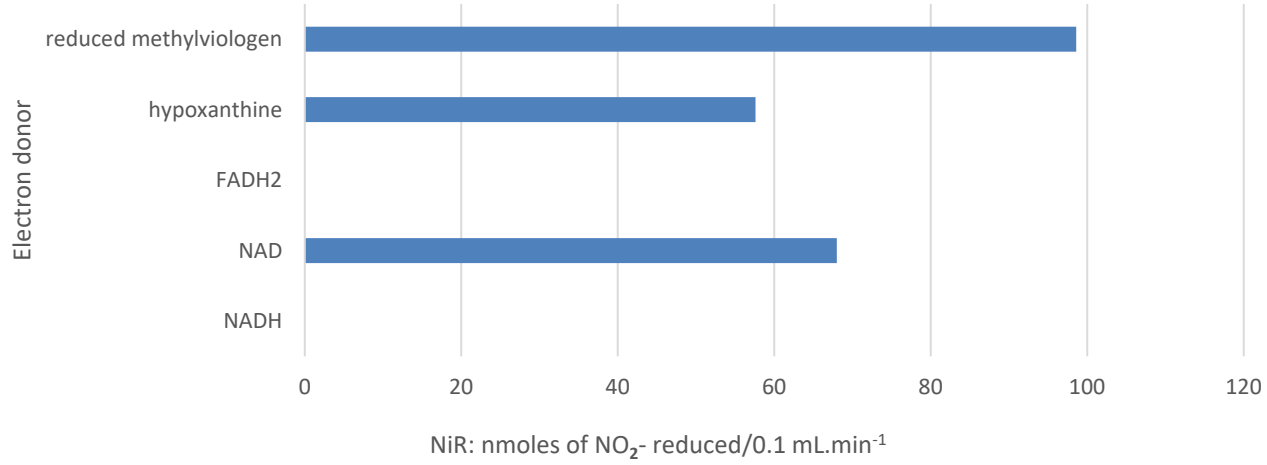

Figure 6 Effects of different electron donors on nitrite reductase activity of sheep milk in the presence of cysteine.

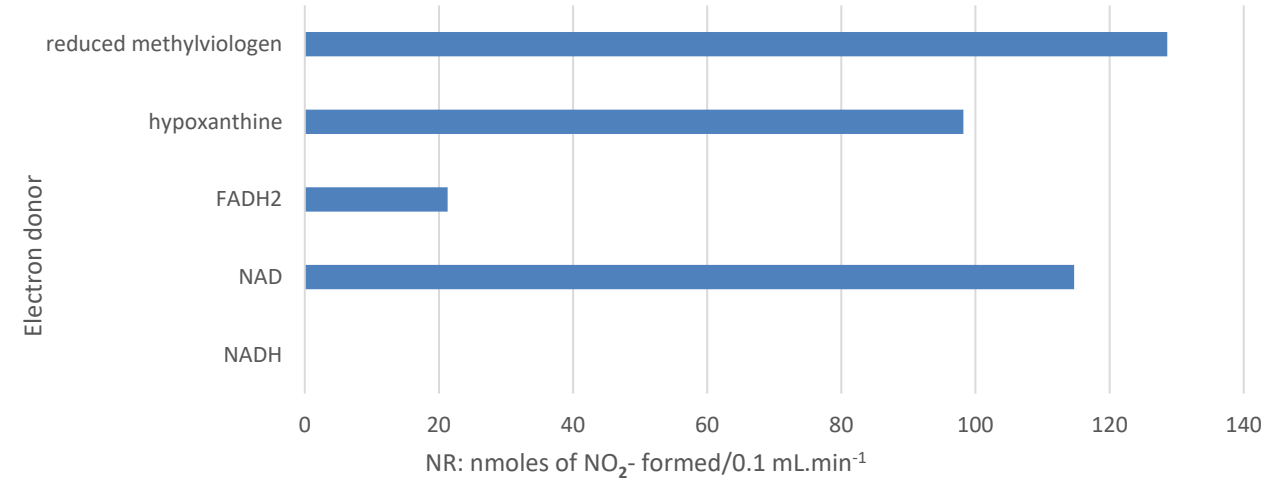

Figure 7 Effects of different electron donors on nitrate reductase activity of sheep milk in the presence of cysteine.

Table 1 Formation of CySNO in fresh sheep milk after heat treatment in the presence of cysteine and molybdenum or tungsten (n = 3, ±*SD*).

Treatments of the milk	A substrate in reaction mixture	Absorbance, nм	Amount of CySNO, mM/0.1 mL.min^{-1}
Control – PBS only without milk		340	0.0
	NO_3-	540	0.0
		340	0.0
	NO_2-	540	0.0
Control – milk without heat treatment		340	2.7 ±0.3
	NO_3-	540	0.0
		340	5.8 ±0.2
	NO_2-	540	0.0
Heating + Na_2MoO_4 at 80 °C, 7 minutes		340	12.6 ±0.8
	NO_3-	540 nм	3.2 ±0.2
		340 nм	35.5 ±3.5
	NO_2-	540 nм	7.3 ±1.3
Heating + Na_2WO_4 at 80 °C, 7 minutes		340 nм	0.0
	NO_3-	540 nм	0.0
		340 nм	0.0
	NO_2-	540 nм	0.0
Heating of milk without $MoO_4^=$ or $WO_4^=$		340 nм	0.0
	NO_3-	540 nм	0.0
		340 nм	0.0
	NO_2-	540 nм	0.0

Table 2 Effect of increasing concentrations of the lecithin on nitrate- and nitrite-reducing activities and CysNO formation in sheep milk (n = 3, ±*SD*).

Lecithin content	Associated activities		Amount of CySNO*	
	NR	NiR	From NR	From NiR
Control	92.8 ±8.5	57.6 ±7.6	13.2 ±1.2	33.7 ±5.2
0.01 mg.mL^{-1}	91.2 ±10.2	58.4 ±6.8	14.3 ±2.6	33.5 ±7.3
0.05 mg.mL^{-1}	92.7 ±9.5	57.8 ±9.3	14.4 ±1.8	34.7 ±7.1
0.1 mg.mL^{-1}	95.8 ±11.6	62.9 ±9.7	18.4 ±3.2	42.9 ±8.2
0.5 mg.mL^{-1}	122.5 ±14.7	117.6 ±12.8	33.6 ±4.5	78.9 ±12.2
1.0 mg.mL^{-1}	128.6 ±12.8	135.2 ±18.6	36.7 ±7.3	93.8 ±11.7

It is known that NADH is one of the potential physiological electron donors for XO and it also has an absorbance at 340 nm (reduced NADH exhibits strong UV absorption at 340 nm whilst the oxidized form has virtually no absorption at this wavelength).

Therefore, to avoid mutual interference between the optical density of NADH and CysNO at 340 nm, instead of NADH we used hypoxanthine as an electron donor for NR and NiR activities of milk XO. It was demonstrated that heat treatment (80°C, 10 min) of homogeny XO resulted in the release of molybdenum cofactor (Mo-co) from the active center of the denatured enzyme molecule. During the heat treatment of XO ascorbic acid was the potential protector against the oxidation of released Mo-co. However, in the absence of ascorbic acid, it is quickly inactivated by oxygen (even in anaerobic conditions) **(Alikulov et al., 1980; Vogels and Van der Drift, 1970)**. Later we showed that glutathione and cysteine were the more powerful protectors for isolated Mo-co **(Alikulov and Mendel, 1984; Poje and Sokolić-Maravić, 1986)**.

However ascorbic acid decomposes S-nitrosocysteine **(Stsiapura et al., 2018)** and, therefore, we used L-cysteine as the protector against the oxidation of the cofactor in the active center of XO localized in MFGM. Thus, in our experiments L-cysteine was used for double purposes: as the protector of enzyme Mo-co against the oxidation during heat treatment of milk and as a reagent for S-nitrosothiol formation. For the construction of the calibration curve, we used nitroprusside as a donor of NO. Increasing concentrations of nitroprusside from 10 nM to 1.0 μM mixed with constant 0.1 mM concentration of cysteine in milk serum. Micromolar concentrations of nitroprusside release Nanomolar concentrations of NO **(Porras et al., 1981; Kramer et al., 1987)**. The serum was obtained by heating fresh milk, precipitating its proteins with acetic acid, and centrifuging. To obtain a transparent milk serum, its filtration was carried out through a membrane (33 mm Millex Filter).

For NO determination sheep milk mixed in the ratio of 1:1 with 0.2 M chlorinated phosphate buffer (PBS), pH 6.5, containing 10mM NEM, 2.5 mM EDTA **(Kisker et al., 1997; Marley et al., 2001)**, 0.2 mM Na_2MoO_4 or Na_2WO_4 and 0.1 mM cysteine. After incubation in 15 minutes at a temperature of 36 °C, the milk proteins were precipitated by diluted acetic acid added to the reaction mixture until pH 4.0. After centrifugation in the supernatant absorbance at 340 nm and 540 nm were measured. For many years tungsten was considered to be a biological antagonist of molybdenum and was used for the study of the properties and functions of molybdenum in Mo-enzymes. This was because tungsten can replace molybdenum in Mo-enzymes, forming catalytically inactive analogs **(Kletzin and Adams, 1996; Bray et al., 1999; Beedham, 2001; Taibi et al., 2001)**. Therefore, to make sure that it is the molybdenum enzyme that catalyzes the formation of NO, instead of molybdenum we incubated the milk in the presence of tungsten, and the results obtained (Tables 1, 2) convincingly show that the heat treatment of fresh sheep

milk in the presence of exogenous molybdenum activates XO and the enzyme becomes capable of converting nitrate and nitrite to nitric oxide. However, when nitrates were used as a substrate NO formation was very low. At the same time, using nitrites as substrate resulted in a 10 times higher amount of formed NO (i.e. CySNO) in comparison with nitrate substrates. The levels of CysNO determined by absorbance at 340 and 540 nm were completely different. This was likely due to the difference in the sensitivity of the absorption at the ultraviolet and visible wavelengths of the spectrophotometer.

Effect of exogenous phospholipid on the associated activities of sheep milk XO

Phospholipids (PLs) are formed by glycerol, phosphoric acid, fatty acids, and a hydroxyl compound (e.g., choline, ethanolamine, serine, inositol). It is well known that milk fat globule membranes (MFGM) is composed of four main species of PLs: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS). The most abundant PLs in milk fat expressed as a percentage of the total, are PE (26.4% – 72.3% of total PLs), PC (8.0% – 45.5%), PI (1.4% – 14.1%), and PS (2.0% – 16.1%). Of the lipids, phosphatidylcholine is largely located on the outside while the neutral lipids, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol are concentrated on the inner surface **(Garcia et al., 2012; Suzuki et al., 2015)**. Amphiphilic properties of PLs derive from the presence of both a hydrophobic tail and a hydrophilic head make them natural detergents. Thus, under high-temperature phospholipids may effectively disrupt hydrophobic bonds between fatty acids, e.g., the integrity of all membrane layers of MFGM **(Godber et al., 2000; Gladwin et al., 2005)**. Therefore, the destruction of MFGM makes the enzyme molecules available for exogenous molybdenum and its substrates (hypoxanthine, nitrate, and nitrite).

In the next experiments, we examined the effect of lecithin on NR and NiR activities of heat-treated fresh sheep milk. Egg yolk lecithin was used in the experiments. The main phospholipids of this lecithin are phosphatidylcholine and phosphatidylethanolamine, i.e. they are components of the outer and inner layers of the membrane. The lecithin was dissolved in isopropyl alcohol and then added to the milk before heat treatment. The other components required for the heat treatment of milk have already been written above. Hypoxanthine was used as an electron donor for NR and NiR activities.

CONCLUSION

During the research, it was found that dithiothreitol associated with molybdenum does not show a stabilizing or antioxidant effect. The absence of such a stabilizing effect of unithiol remains unclear. During nitrate reduction by hypoxanthine, the XO molybdenum center may participate directly in both the oxidative and the reductive half-reactions. Using nitrates as a substrate did not result in high NO formation while using nitrites as substrate increased the amount of formed NO. It can be explained by the difference in the sensitivity of the absorption at the ultraviolet and visible wavelengths of the spectrophotometer.

The results show that exogenous lecithin in relatively high concentrations (0.5 – 1.0 $mg.mL^{-1}$) increases the activity of NR, NiR, and the formation of CysNO. Apparently, during heat treatment, exogenous lecithin does partially decomposes the membrane of MFGM of milk, and enzyme molecules become more accessible. Thus, the results obtained may be important in the cleaning of animal milk contaminated with nitrates or nitrites. Thus, our results suggest the possible use of XO activation by heat treatment to remove nitrates from milk. Usually fresh sheep's milk is consumed after heat treatment.

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The authors declare no conflict of interest.

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This article does not contain any studies that would require an ethical statement.

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