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ACTIVATION AND DETERMINATION OF MOLYBDENUM OF XANTHINE OXIDASE FROM ANIMAL MILK

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Abstract

Xanthine oxidase (XO) in goat, camel and horse milk is in a molybdenum-free form, which means that it is inactive. The enzymatic activity is realized through exogenous molybdate (Mo) and thiols during milk heat-treatment at 85 °C for 10 minutes. Mo atoms bind to sulfhydryl (-SH) groups of molybdenum cofactor (Moco) in the active center of XO. However, during the enzyme heat-treatment, the SH-group is oxidized with oxygen. Therefore, different antioxidants were used for the protection of SH-groups of the cofactor. XO nitrate reductase (NR) and nitrite reductase (NiR) activity is determined only by the amount of nitrite: for NR, nitrite is the product of a catalytic reaction, and for NiR, nitrite is a substrate. Sulfanilamide and N-(1-Naphthyl)-Ethylenediamine are used to determine the amount of nitrite. We have discovered that in the presence of phosphate and such antioxidants as dithiothreitol, ascorbate and dithionite, exogenous Mo was discolored with sulfanilamide to blue (maximum absorption at 695 nm). When using cysteine or glutathione as antioxidants, the molybdate did not discolor with sulfanilamide, i.e. they do not interfere with the determination of nitrite. The use of dithiothreitol, phosphate and sulfanilamide led to the presenting of exogenous molybdate in the molecule of XO micelles of cream fat globules, thereby the enzyme was activated and a method for determining molybdenum in biological materials was found by the example of animal milk.

INTRODUCTION

Xanthine oxidase (XO; EC 1.1.3.22) is an enzyme in the xanthine oxidoreductase group (XOR) presented at the highest concentrations in mammalian tissues of liver and intestines (Borges, Fernandes & Roleira, 2002) and in milk (Beyaztaş & Arslan, 2014). Isolation of XO, as the widespread enzymes among different species, involves the extraction of the enzyme from a wide range of materials (bacteria, milk, organs of different animals, etc.) and its purification from crude extract. XO is concentrated in the milk fat/lipid globule membrane (MFGM), in which it is the second most abundant protein, after butyrophilin. Therefore, all isolation methods use cream as the starting material; the cream is washed and churned to yield a crude MFGM preparation, dissociating and reducing agents are used to liberate XO from membrane lipoproteins, and some form of chromatography is used for purification (Fox & Kelly, 2006). Spectrophotometric determination of XO activity is based on measuring uric acid production from xanthine or hypoxanthine substrate at around 295 nm. The assay mixture always contains xanthine as a substrate and sample. Reaction is initiated by adding the XO. Higher values indicate a pathological condition (Kostić et al., 2015).

XOR exists in a great variety of organisms from bacteria to higher plants and humans. The enzyme catalyzes the metabolic reactions leading from hypoxanthine to xanthine and from xanthine to urate, which are the last steps in the human purine excretion system (Okamoto, Kusano, & Nishino, 2013). Under normal conditions the mammalian XOR exists in the form of xanthine dehydrogenase (XDH; EC 1.1.1.204), but approximately 10 % of its groups presented in the form of xanthine oxidase (XO). However, when there is a lack of oxygen, XDH is converted to XO through a proteolysis process. Nishino et al. (2008) in his study took a closer look at this process. Both XDH and XO convert hypoxanthine to uric acid via xanthine. However, as shown in **Figure 1**, the difference is that the activity of XDH is mainly related to the conversion of NAD^+ to NADH, while the activity of XO is mainly related to the conversion of oxygen.

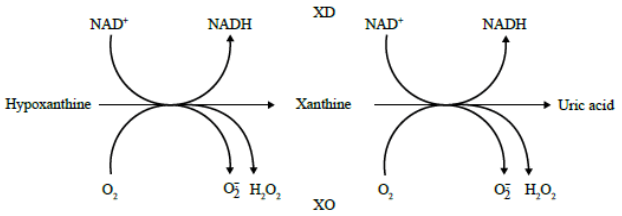


Figure 1. Two types of reaction of uric acid production XO/XDH (Shintani, 2013).

Uric acid is the main end product of the metabolism of nitrogen-containing compounds in animals and serves as an antioxidant that reduces oxidative stress (Matata, & Elahi, 2007; Alsultanee, Evadh & Mohammed, 2014). Purines and other substrates react with xanthine oxidase in the Mo-containing center, and electron acceptors react on the FAD site (Hille, Nishino, & Bittner, 2011; Harrison, 2002). The protein part of the enzyme is rich in cysteine and contains 60-62 free sulfhydryl (-SH) groups. In the structure of the enzyme, there are also centers representing the 2Fe-2S cluster (Harrison, 2006). The composition of the active enzyme includes molybdenum, being in the form of the so-called molybdenum cofactor (Moco); it is connected by two S-bonds from the side chain of the pterin of the cofactor molecule (Bryan, Bian & Murad, 2009). Two Mo atoms are involved in electron transfer in the active center of the enzyme, i.e. enzyme activity depends on the content of molybdenum in the enzyme molecule.

Molybdenum is one of the main trace elements in animal, human and plant nutrition. Molybdenum was first discovered to be present in molybdenite (MoS_2) by Scheele in 1778–79 and later isolated from molybdenite by Hjelm in 1782, who named it molybdenum (from greek molybdos, meaning lead or lead-like). Molybdenum (Mo) occurs in a wide range of metalloenzymes in bacteria, fungi, algae, plants and animals where it forms part of the active sites of these enzymes. Mo has a versatile redox-chemistry that is used by the enzymes to catalyze diverse redox reactions. This redox-chemistry is controlled both by the different ligands at the Mo atom and the enzyme environment (Mendel & Kruse, 2012). In order to gain biological activity, Mo has to be complexed by a pterin compound thereby forming the molybdenum cofactor (Moco) (Iobbi-Nivol & Leimkühler, 2013). In this cofactor, molybdenum binds with the pterin ring through two sulfur atoms. A further sulfur atom and two oxygen atoms are coordinated to the molybdenum, and are exposed to solvent (Okamoto, Kusano, & Nishino, 2013). As suggest Rajagopalan and Johnson (1992), the essential presence of molybdopterin in all the variant forms of the molybdenum cofactor suggests that the pterin ring contributes in some manner to the catalytic competence of the cofactor. Possible roles include (a) participation in the electron transfer process; (b) providing the major determinant for the widely differing oxidation-reduction potentials of the molybdenum centers of different enzymes; and (c) rate enhancement by facilitating an otherwise rate-limiting step. Because of the difficulty of obtaining the free molybdenum cofactor in a stable, homogeneous native structure, it would seem that only x-ray crystallographic data on molybdoenzymes will reveal further intimate structural features of the cofactors in native enzymes essential for a full understanding of these functional aspects.

Moco is found in animal organisms, mainly as the metalloenzyme xanthine-oxidase in the enzymes aldehyde-oxidase and sulfite-oxidase, and in plants. This element is necessary for the fixation of atmospheric nitrogen by bacteria at the start of protein synthesis (Regina de Amorim et al., 2011). Molybdenum cofactor (Moco) biosynthesis is an ancient, ubiquitous, and highly conserved pathway leading to the biochemical activation of molybdenum. Moco is the essential component of a group of redox enzymes, which are diverse in terms of their phylogenetic distribution and their architectures, both at the overall level and in their catalytic geometry. A wide variety of transformations are catalyzed by these enzymes at carbon, sulfur and nitrogen atoms, which include the transfer of an oxo group or two electrons to or from the substrate (Iobbi-Nivol & Leimkühler, 2013).

Moco is a coenzyme of four important enzymes: xanthine dehydrogenase, xanthine oxidase, sulfitoxidase and aldehyde oxidase. Xanthine dehydrogenase, along with xanthine oxidase, is involved in purine metabolism (NADH formation from NAD+). Molybdenum, entering the body with plant food, is more toxic than its inorganic compounds, and causes changes in animals similar to selenium. Molybdenosis is observed in humans and animals in certain geographical areas with an excess of Mo in the soil. Proved the role of Mo as a bioelement in tissue respiration, there has been established its effect on purine metabolism, the synthesis of ascorbic acid, carbohydrate metabolism, gonadal function and vegetative-endocrine reactions and other processes. There is a certain antagonism between Mo and Cu; Zn enhances Mo toxicity; the relationship between Mo and other trace elements has been studied, as well as Mo with P and S.

Once molybdate enters the cell it is subsequently incorporated by complex biosynthetic machineries into metal cofactors. These metal cofactors are then incorporated into different enzymes and these molybdenum enzymes are found in nearly all organisms, with *Saccharomyces cerevisiae* as a prominent eukaryotic exception (Zhang, Rump, & Gladyshev, 2011). In the large group of molybdoenzymes coordinating Moco, the molybdenum atom is coordinated to the dithiolene group on the 6-alkyl side chain of the tricyclic pyranopterin, molybdopterin (MPT) also referred to as metal-binding pterin (Schwarz, Mendel, & Ribbe**,** 2009; Iobbi-Nivol & Leimkühler, 2013). More than 50 molybdoenzymes have been characterized in different organisms (mostly bacteria), which could be divided into five families: sulfite oxidase (SO), xanthine oxidase (XO), dimethylsulfoxide reductase (DMSOR), AOR (W-specific) and MOSC (Moco sulfurase C-terminal domain)-containing protein (including YcbX and YiiM in bacteria and mitochondrial amidoxime reducing component (mARC) in eukaryotes) (Zhang, Rump, & Gladyshev, 2011).

Medical researches show that molybdoenzymes may serve as anticancer agent. For example, esophageal cancer is particularly widespread in South Africa, in the Transkei region and in Lin Xian – region in the Honan province in the North of China, which was known as the region with the highest percentage of incidence of stomach and esophageal cancer among the local population (Blot, Li, & Taylor, 1993). The conducted researches of the soil from these regions showed low content of molybdenum. Application of molybdenum fertilizers into the soil significantly reduced the percentage of morbidity. It is concluded that the lack of molybdenum in the soil leads to a decrease or loss of activity of molybdoenzymes of plants, animals and humans. As

a result, the level of nitrites and carcinogenic nitrosamines, which are not oxidized by molybdoenzymes to their harmless derivatives, increases in humans and animals. It was found that molybdenum inclusion in the diet of rats inhibits the development of stomach and esophageal cancer even after the inclusion of N-nitroso-sarcosin ethyl ester. The addition of molybdenum in drinking water of rats inhibits breast carcinogenesis caused by N-Nitroso-N-Methylurea (Brewer, Dick, & Grover 2000). According to American scientists, soil in the States of Colorado and Ohio is rich in molybdenum and the lowest incidence of esophageal cancer is found there. Thus, the content of molybdenum in food of animal and vegetable origin depends significantly on its content in soil and water. The Joint FAO/WHO Expert Committee on Food Additives included molybdenum in a number of necessary elements in the hygienic control of food products. The daily human requirement for molybdenum is 1-2 mg. At the same time, international standards do not include any methods for determining molybdenum in food products.

Animals and humans body contains three molybdoenzymes: aldehyde oxidase, xanthine oxidase and sulfite oxidase. Aldehyde oxidase and xanthine oxidase play an important role in the metabolism of heterocyclic xenobiotics, thereby neutralizing them and protecting the body from harmful nitrogen-containing heterocycles, therefore their high content is found in the liver of animals (Beedham, 2009). Numerous scientific works and studies were devoted to xanthine oxidase (Cao, Pauff, & Hille, 2010; Doonan et al., 2005); XO catalyzes the conversion of hypoxanthine purine into xanthine and then into uric acid. As is known, purine bases are components of nucleic acids, i.e. RNA and DNA. Aldehyde oxidase takes part in the reactions of biotransformation of xenobiotics – natural and foreign to the human and animal body substances generated to some extent by anthropogenic activity and not included naturally in the biotic cycle. It is the ability of aldehyde oxidase to catalyze oxidation in the body of carcinogenic xenobiotics that is associated with the supposed anti-cancer activity of molybdenum (Blot, Li, & Taylor, 1993; Beedham, 2009). Sulfite oxidase, being in the mitochondria, is involved in the metabolism of sulfur-containing amino acids – cysteine and methionine – and catalyzes the oxidation of sulfite to sulfate.

There are thoroughly studied three Mo-containing enzyme plants contain: nitrate reductase, xanthine dehydrogenase and aldehyde oxidase. Nitrate assimilation is a fundamental process in the plant kingdom and therefore the nitrate reductase enzyme, which restores nitrate, is seen as a limiting factor in plant growth, development, protein formation and final yield. In plant purine catabolism, xanthine dehydrogenase oxidizes xanthine via uric acid to ureides (allantoic acid). Ureide is easily digestible storage and transport form of nitrogen for plant tissues. It is established that aldehyde oxidase catalyzes the conversion of indole-3-acetaldehyde (IAAld) and abscisic aldehyde (ABAld) to the phytohormones abscisic acid (ABA) and indole-acetic acid (IAA), respectively.

Thus, the above information shows the important role of molybdenum in the metabolism of living organisms. Therefore, monitoring of molybdenum level is an important component in assessing the quality of food products of plant and animal origin, as well as soil and water. High concentrations of molybdenum are determined by such methods as atomic absorption spectrometry, voltammetry, spectrophotometry, combining them often with extraction. There are few methods for determining low molybdenum content in natural and biological objects and the available ones are either thresholds (mass spectrometry) or time-consuming ones and require highly qualified personnel, special reagents and operating conditions (kinetic, radiochemical methods). There are basically no methods for molybdenum determination in the field (test methods), that are simple and do not require special equipment, suitable for the needs of environmentalists. Therefore, methods for molybdenum determination in the environment and biological materials have been constantly improved.

Some researchers used the procedure of preliminary concentration and separation of the matrix using the Amberlite ion exchanger and 5% sodium hydroxide solution as an eluant to eliminate matrix effects and reduce the limit of molybdenum detection in milk (Lopez-Garcia, 2007). To improve the metrological characteristics of the determination of molybdenum in skim milk there can be used the europium additive and a complex pre-treatment of graphite furnace with a solution of niobium (Regina de Amorim et al., 2011). Despite this, the existing methods do not satisfy analysts because of the duration of the determination process, the presence of additional stages of separation of the matrix components, the use of toxic gases, insufficient detection limit and poor reproducibility. There were proposed new palladium-complex chemical modifiers (complexes of Pd (II) with chromazurol S and xylenol orange) for the determination of molybdenum in milk; after its dry mineralization there was used electrothermal atomic absorption spectroscopy, allowing to reduce Mo detection limit by 12 times (Shchepina, 2012).

MATERIALS AND METHODS

Chemicals

Sodium molybdate (M = 241.95), a sulfonamide (M = 172.21), N-(1-Naphthyl)ethylenediamine dihydrochloride (M = 259.18) of AppliChem brand (Germany), cysteine (M = 157.6), dithiothreitol (M=154.2) of Sigma brand (Germany).

Preparation of milk for the determination of various enzymatic activities

Before treatment, 10 μM of EDTA was added to animal milk to bind heavy metals. For boiling, fresh milk was poured into narrow conical 2 ml tubes. Thereafter, to further determine the enzymatic activity of the tube, it was heated in a water bath with a temperature of 85 °C and kept for 2 to 10 minutes, after which it was quickly hydrocooled.

Nitrate reductase activity of xanthine oxidase of fresh milk was determined by the formation of nitrites in the reaction medium as a result of the catalytic reaction of the enzyme. Nitrite reductase activity was determined by the disappearance of nitrites in the reaction medium. As a donor of electrons we used 25 μm of NADH (final concentration) or 1.0 μm of methyl viologen (benzyl viologen), reduced by dithionite. Since NADH and methyl viologen give comparable results, the determination of these activities was carried out using the last electron donor (a much cheaper reagent compared to NADH).

Determination of the nitrites concentration using the N-(1-Naphthyl) ethylenediamine dihydrochloride and sulphanilamide

The nitrites concentration was determined with the addition of 0.5 ml of a sulphanilamide solution and 0.5 ml of N-(1-Naphthyl) ethylenediamine dihydrochloride in 500 ml of the reaction

mixture. These reagents were prepared in the following manner: 6 g of sulphanilamide were dissolved in 1 liter of a 20% HCl and 1.23 grams of N-(1-Naphthyl) ethylenediamine dihydrochloride in 1 liter of distilled water. 48 mg of dithionite (sodium hydrosulfite, $Na_2S_2O_4$) was dissolved in 6 ml of 95 mM of sodium bicarbonate. At pH = 2-2.5 nitrous acid forms a diazonium compound with sulfanilamide. Diazonium compound undergoes coupling reaction with N-(1-Naphthyl) ethylenediamine dihydrochloride with the formation of the azo coloring agent of red color (**Figure 2**). The obtained colored solution is photometered at 548 nm spectrophotometer wavelength. Xanthine oxidase activity was determined by the formation of uric acid from hypoxanthine at pH 8.5. The concentration of uric acid was determined by its absorption at 295 nm using a spectrophotometer.

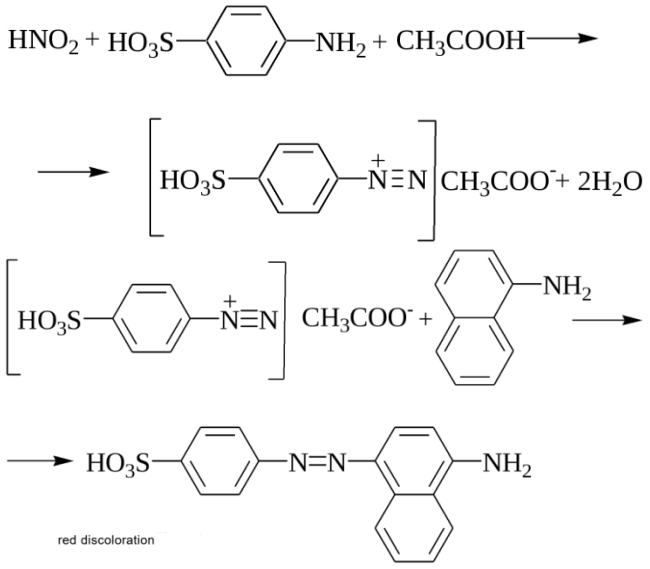


Figure 2. Formation of the azo coloring agent of red color.

Determination of molybdenum content by atomic absorption method

Molybdenum content was determined on the atomic absorption spectrometer AAS-IN (Karl Zeiss, Germany). Molybdenum was isolated by the wet mineralization method from cream, extracted from milk. Wet mineralization was performed according to the following procedure. Cream was mixed with concentrated HNO_3 and this mixture was heated in a ceramic cup under the air flow. Heating reduced the volume of the extract by evaporation – 50 ml was concentrated to 5 ml. Then the concentrated extract was again mixed with concentrated acid, centrifuged and molybdenum was determined in the supernatant.

RESULTS AND DISCUSSION

Xanthine oxidase is one of the best studied complex flavoproteins and has different reviews (Harrison, 2002; Hille, Nishino, & Bittner, 2011). XO is concentrated in the milk fat/lipid globule membrane (MFGM) and therefore the milk XO isolation were done using crude MFGM with dissociating and reducing agents to liberate XO from membrane lipoproteins, and further purified with chromatographic methods (Nile et al., 2018).

The chemical nature and reaction mechanisms of the XO molybdenum cofactor in molybdenum-dependent reactions, including nitric oxide (NO) synthesis, were previously observed (Okamoto, Kusano, & Nishino, 2013). It is also known that enzymes share a molybdenum cofactor (Moco) with a metal ion by binding a molybdopterin molecule (MPT) via dithiolene, sulfur, and oxygen (*Reschke et al., 2007)*.

When studying the activation of xanthine oxidase of animal milk (of cow, goat, camel and horse origin) through exogenous molybdate we have found that in the presence of reducing agents such as ascorbic acid and dithionite, molybdenum discolors in a mixture of sulfanilamide and N-(1-Naphthyl)ethylenediamine dihydrochloride. Typically, a mixture of these reagents is used to detect nitrites. The activity of nitrate reductase of plants and microorganisms is determined by the amount of nitrites. It is well known that the Mo-containing enzyme – nitrate reductase (NR) with nitrate nutrition of plants and microorganisms restores nitrate (NO_3-) to nitrite (NO_2-). Nitrite is further restored by nitrite reductase (NiR) to hydroxylamine (then by hydroxylamine enzyme is converted into ammonium, which is included in the newly synthesized amino acids). No NR has yet been identified in animals. However, earlier for the first time it was found that a homogeneous Mo-containing enzyme – xanthine oxidase (XO) isolated from fresh cow's milk, has high NR and NiR activities (Godber et al., 2005; Atmani, Benboubetra, & Harrison, 2004). NADH was a natural electron donor for such activities, an artificial donor was methyl viologen (or benzyl viologen), restored by dithionite (**Tables 1-3; Figures 3-8**). Since NADH and methyl viologen give comparable results, the determination of these activities was carried out using the last electron donor (a much cheaper reagent compared to NADH).

Table 1. Nitrite presence in the reaction medium depending on the initial concentration of nitrate added as a substrate of NR activity of goat milk XO.

NO_3- concentration in reaction mixture before incubation	**Electron donor**	**NO_2- content in reaction mixture after incubation**
200 nM	NADH	0.0
	BV+DT	0.0
2μM	NADH	387.6 ± 15.6 nM
	BV+DT	225.3 ± 21.7 nM
20μM	NADH	21.8 ± 1.5 μM
	BV+DT	13.4± 0.8 μM

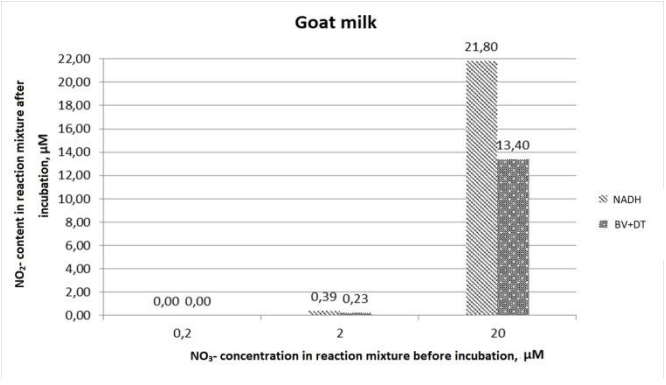
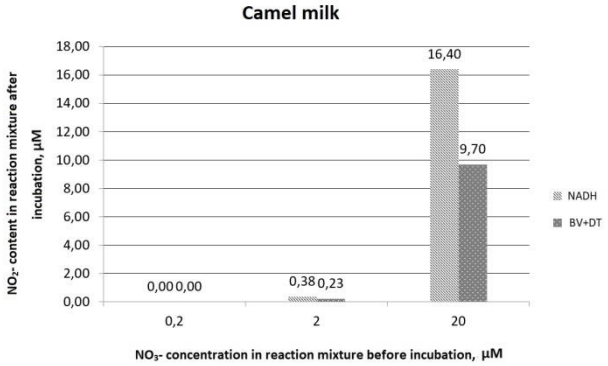


Figure 3. Nitrite presence in the reaction medium (Goat milk).

Table 2. Nitrite presence in the reaction medium depending on the initial concentration of nitrate added as a substrate of NR activity of camel milk XO.



NO_3- concentration in reaction mixture before incubation	Electron donor	NO_2- content in reaction mixture after incubation
200 nM	NADH	0.0
	BV+DT	0.0
2µM	NADH	375.7± 24.8 nM
	BV+DT	227.3 ± 18.3 nM
20 µM	NADH	16.4 ± 0.9 µM
	BV+DT	9.7 ± 0.5 µM

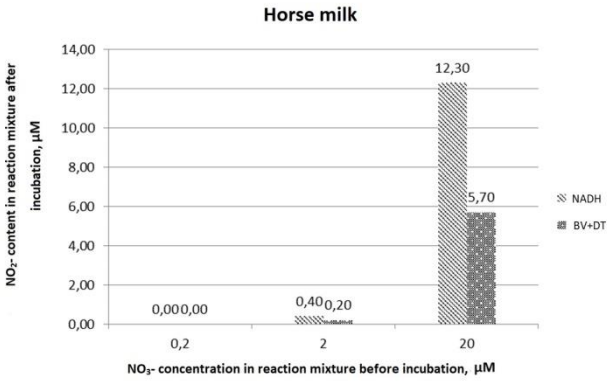
Figure 4. Nitrite presence in the reaction medium (Camel milk).

Table 3. Nitrite presence in the reaction medium depending on the initial concentration of nitrate added as a substrate of NR activity of horse milk XO.

NO_3- concentration in reaction mixture before incubation	Electron donor	NO_2- content in reaction mixture after incubation
200 nM	NADH	0.0
	BV+DT	0.0
2µM	NADH	397.2 ± 32.4 nM
	BV+DT	202.5 ± 18.3 nM
20µM	NADH	12.3 ± 0.8 µM
	BV+DT	5.7µ± 0.4 M

A new method for molybdenum determination

The reaction mixture for determination of the NR and NiR activity of animal milk XO in the absence of milk with the addition of sulfanilamide and N-(1-Naphthyl) ethylenediamine was discolored to blue. The reaction mixture contained 200 µl of 0.1 M sodium phosphate buffer (Na_2HPO_4/KH_2PO_4 1:9) pH 6.0, 10 µl of 10 µM EDTA, 100 µl of fresh milk, 100 µl of 0.1 M KNO_3 (or 0.4 мМKNO_2), 10 µl of 0.1 МNa_2MoO_4, 10 µl of 0.1 M ascorbic acid (ascorbate), 100 µl of 0.1 M methyl viologen (benzyl viologen) and 50 µl of dithionite. After incubation, 0.5 ml of sulfanilamide and 0.5 ml of N-(1-Naphthyl) ethylenediamine were being added for 15 minutes at 37 °C, nitrite discoloration to red lasted for 2-4 minutes. We found that in the absence of milk the discoloration to dark blue lasted for a minute. The maximum blue



color absorption at the visible spectral range of the spectrophotometer was 695 nm.

Figure 5. Nitrite presence in the reaction medium (Horse milk)

In the following experiments, individual constituents were excluded from the reaction mixture, and their participation was revealed due to discoloration to blue color. After mixing the components, the reaction mixture discoloration occurred immediately at room temperature without incubation. The total results of these experiments are presented in **Table 4.**

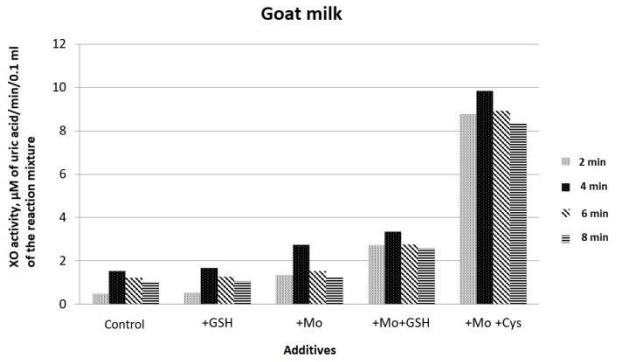


Figure 6. The effect of the goat milk heating duration (80 ºC) in the presence of thiols and molybdates on the XO activity

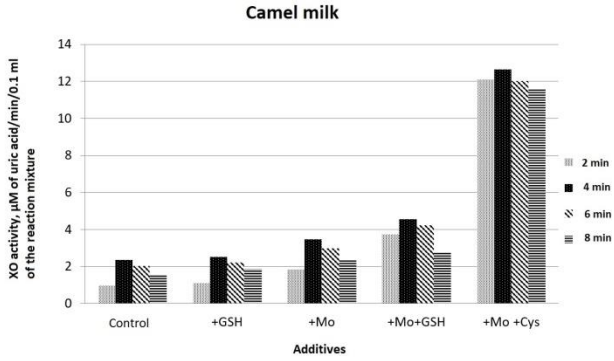


Figure 7. The effect of the camel milk heating duration (80 ºC) in the presence of thiols and molybdates on the XO activity

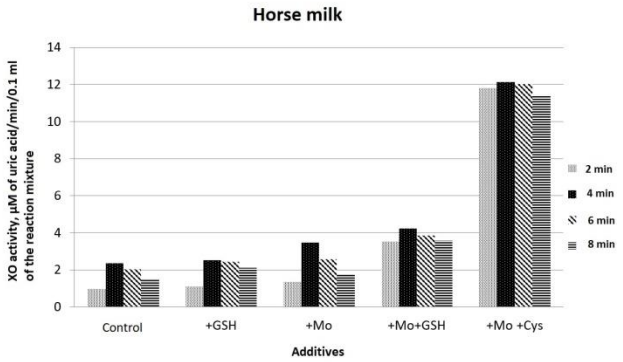


Figure 8. The effect of the horse milk heating duration (80 ºC) in the presence of thiols and molybdates on the XO activity

Table 4. Participation of individual constituents in the reaction mixture discoloration to blue (in the absence of milk in reaction mixture)

Variant no.	Reaction mixture constituents*	Absorption at 695 nm
1	All the constituents	0.650
2	Minus nitrate	0.655
3	Minus nitrite	0.653
4	Minus molybdate	0.0
5	Minus ascorbic acid	0.0
6	Minus phosphate buffer	0.0
7	Minus methyl viologen	0.645
8	Minus benzyl viologen	0.645
9	Minus dithionite	0.428
10	Minus sulfanilamide	0.0
11	Minus N-(1-Naphthyl)ethylenediamine	0.642
12	Minus EDTA	0.637
13	All the constituents, but tungsten instead of molybdenum	0.0

The results of these experiments have shown that four components are involved in the discoloration of the reaction mixture: sodium molybdate, ascorbic acid, phosphate buffer and sulfanilamide. When tungstate was used instead of molybdate there was no discoloring of the reaction mixture (variant 13). If the simultaneous presence of sulfanilamide and N-(1-Naphthyl) ethylenediamine was absolutely necessary for nitrite discoloring, then the second reagent was not essential for molybdenum discoloring. The absence of dithionite in the reaction mixture slightly reduced the mixture discoloring. Since dithionite is a strong chemical reducing agent (antioxidant), an assumption was made that the antioxidants are also necessary for the discoloring. The absence of EDTA also significantly reduced the discoloring to blue color. Therefore, in the following experiments, the effect of various antioxidants and EDTA in the discoloring of the reaction mixture was tested. The total results of these experiments are presented in **Table 5**.

The results presented in **Table 5** show that in case of discoloring of the reaction mixture of molybdate, phosphate and sulfonamide, the most effective antioxidant is dithiothreitol (and in this case, when replacing molybdate with tungstate none of the antioxidants caused discoloring (option 15). Ascorbic acid and dithionite had weaker effect.

It should be noted that dithiothreitol inhibited nitrate reductase and nitrite reductase activity of animals milk XO. As mentioned above, these activities occur only after the milk heat-treatment in the presence of exogenous molybdate and cysteine. Apparently, dithiothreitol binds to molybdenum and does not allow molybdenum to be included in the XO active center.

Table 5. Effect of various reducing agents on the discoloring of the reaction mixture of molybdenum, phosphate and sulfanilamide

Variant no.	Antioxidant (reducing agent)	Absorption at 695 nm
1	Ascorbic acid	0.656
2	Dithionite	0.324
3	Dithiothreitol	0.843
4	Glutathione	0.063
5	Cysteine	0.032
6	Unitiol	0.122
7	Mercaptoethanol	0.072
8	Tungstate+phosohate+sulfanilamide+individual antioxidants	0.0

As a result of further experiments, we have established the optimal concentration of dithiothreitol, phosphate buffer and sulfanilamide to determine the content of molybdenum in the reaction medium. The optimal final concentration of dithiothreitol for the reaction medium was 6 mM, sodium-phosphate buffer – 30 mM and sulfanilamide – 3.5 mM. The sensitivity of this determination method was 4.1 μg of molybdenum in 1 ml of the reaction mixture.

Activation of XO molybdenum-free forms by exogenous molybdenum

Thus, we have found that most of the XO molecules in cow, goat, camel and horse milk do not contain molybdenum and are inactive. Heating of the animal milk at 80 °C for 5 minutes in the presence of molybdate, sodium and cysteine (or glutathione) followed by chilling to room temperature led to a leap of the XO activity and its associated NR and NiR activities. When using sodium tungstate instead of the molybdate, the original milk XO activity and its NR and NiR completely disappeared. It is well known that to identify any Mo-containing enzyme in the growth medium in the organism's cultivation, tungstate is added instead of molybdate. Under such conditions, tungsten, as a chemical analog of molybdenum, is included in the enzyme molecule. However, the tungstate enzyme becomes inactive, since tungstate atoms are unable to transfer electrons from the donor to the acceptor during the catalytic act of the molybdoenzyme. The use of a specific XO inhibitor – allopurinol – after milk heat-treatment in the presence of molybdate and cysteine led to complete inactivation of the enzyme. All these results show that a large population of XO molecules is in a molybdenum-free form, exogenous molybdenum is included in the XO active center and all catalytic reactions occur in the molybdenum site of the enzyme.

The same data were obtained by other authors. Thus, comparison of the content of molybdenum and level of XO activity for humans and cows allows evaluating the activity of the enzyme on the basis of 100 % Mo content. XO, purified from human milk contained 0.08 Mo atoms, instead of the theoretical 2 atoms. Homogeneous human XO contained 25 times less Mo and enzymatic activity, respectively (Harrison, 2002). Purified goat

and sheep milk XO contained 0.09 and 0.18 Mo atoms (Harrison, 2006). Thus, human and cow milk also contain molybdenum-free forms of XO.

Fresh goat milk was divided into 3 variants: in the 1st variant, to facilitate separation 10 ml of 0.5 sodium molybdate (2.5 mM in the final concentration in milk) was added to 2 liter of milk; in the 2nd variant, molybdate and 10 ml of 1.0 mM cysteine (5.0 μM in final concentration) was added to the same amount of milk and the 3rd variant was the control one without any additions. Variants were divided into two parts by 1 liter. The first part was left without treatment, and the second part was heated at 80 °C for 5 minutes and then chilled to 35 °C. In all variants, NR and NIR activity was determined using reduced methyl viologene (or benzyl viologene). From all three variants, cream was excreted from milk using separator of Sibir-2 brand (it is known that in some cases the cream is excreted from the milk heated at 80-90 °C). About 20% of the cream of the total mass of milk was obtained from each variant. The above activity and the content of molybdenum in the cream using our method and the method of atomic absorption spectrometry were determined in the obtained cream and skim milk (**Table 6; Figure 9**).

Table 6. XO, NR and NiR activities and Mo content in the cream and skim milk, obtained by separation of goat skim milk after various treatment

Variants	Constituents after separation	Enzyme activities		
		*XO	**NR	***NiR
1	Cream	0.0	0.0	0.0
	Skim milk	0.0	0.0	0.0
2	Cream	25.6	48.6	78.6
	Skim milk	0.9	0.0	0.0
3	Cream	0.0	0.0	0.0
	Skim milk	0.0	0.0	0.0

*nM of obtained uric acid/ml of milk; **nM of obtained NO_2-/ml of milk; ***nM of utilized NO_3-/ml of milk.

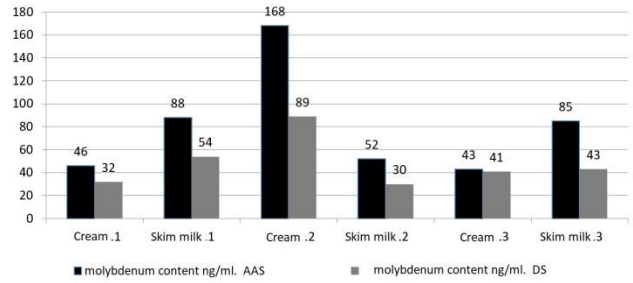


Figure 9. Mo content in cream and skim milk obtained by separation of fresh goat milk after various treatments. At the x-axis – content of molybdenum in nanograms (ng) in milliliter. AAS – molybdenum was determined by the atomic-absorption spectroscopy method, DS - molybdenum was determined by dithiothreitol and sulfanilamide.

During the heat-treatment (80 °C) and in the presence of molybdate and cysteine (or glutathione), molybdenum atoms are included in the XO molecule, which is part of the micelles of fat globules of goat milk. As a result, all activities associated with XO molecule are activated. In the absence of cysteine, molybdenum isn't included in the enzyme molecule, i.e., XO activity is not revealed.

Proposed mechanisms of milk XO activation

One of the possible ways to isolate XO from the inner membrane is the destruction of milk fat globule membrane (MFGM). It is well known that XO relates to thermostable enzymes – it does not lose its activity at 75-80 °C for several minutes. Thus, the milk heating at this temperature leads to MFGM loosening and XO molecules become slightly "exposed". On the other hand, XO molecules at high temperature are partially denatured and oxygen access to the active center of the enzyme is increased. In this case, molybdenum-free forms of molecules can be rapidly irreversibly inactivated as a result of oxidation of the active center of two sulfhydryl (-SH) groups of molybdenum cofactor (Moco). In all molybdoenzymes, molybdenum atom binds to these SH-groups cofactors (**Figure 10**).

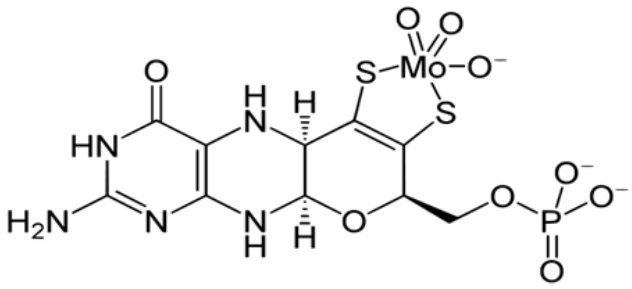


Figure 10. Moco structure and its bond to the molybdenum atom in XO active center.

Therefore, to protect them from oxidation, the presence of an antioxidant (reducing agent) is necessary. Such reducing agents include natural antioxidants – ascorbic acid, cysteine and glutathione. Thus, in the process of heating: (a) MFGM are partially denatured, exposing XO molecules, (b) enzyme molecules are partially denatured – whereby access of exogenous molybdenum and antioxidants to Moco increases sharply. Antioxidants protect the SH-groups of Moco from oxidation, the molybdenum atom easily binds to the sulfhydryl groups of the cofactor, and XO activity is restored.

CONCLUSION

It is already established that monitoring of molybdenum level is an important component in assessing the quality of food products of plant and animal origin, as well as soil and water. High concentrations of molybdenum are determined by such methods as atomic absorption spectrometry, voltammetry, spectrophotometry, combining them often with extraction. There are few methods for determining low molybdenum content in natural and biological objects and the available ones are either thresholds (mass spectrometry) or time-consuming ones and require highly qualified personnel, special reagents and operating conditions (kinetic, radiochemical methods). The developed method of activation and determination of molybdenum in biological materials is a very fast and harmless procedure. Compared with some known methods, the sensitivity of this method may be slightly lower, but the concentration of biological material and

activation before the determination of molybdenum is not difficult.

We have found that most of the XO molecules in cow, goat, camel and horse milk do not contain molybdenum and are inactive. Heating of the animal milk at 80 ℃ for 5 minutes in the presence of molybdate, sodium and cysteine (or glutathione) followed by chilling to room temperature led to a leap of the XO activity and its associated NR and NiR activities. In this study we also have established the optimal concentration of dithiothreitol, phosphate buffer and sulfanilamide to determine the content of molybdenum in the reaction medium. The study has shown that antioxidants or reducing agents are necessary for preventing the oxidation of SH-groups of the molybdenum cofactor that occur during the enzyme heat-treatment.

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