Materials of Conferences

NITRATE- AND NITRITE REDUCING ACTIVITY OF XANTHINE OXIDASE IN GOAT, CAMEL AND HORSE MILK

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This study was designed to examine the reduction of nitrite to NO by milk xanthine oxidase (XO) of domestic animals such as camel, horse and goat. We found that XO in fresh milk of these animals catalyzes the reduction of nitrate and nitrite. This redox reaction requires NADH as a natural electron donor and is oxygen independent. Heat treatment of fresh milk in the presence of thiols such as cysteine and molybdenum led to an increase in the catalysis of nitrate and nitrite reduction. The inhibitory profiles by tungsten suggest that reduction of nitrate and nitrite takes place at the molybdenum center of XO.

Nitrate isa natural material in soils. It is primary source of nitrogen for plants and microorganisms. Probably more than 90 percent of the nitrogen absorbed by plants is in the nitrate form. Nitrate- and nitrite-nitrogen is soluble in water and moves with soil moisture. A potential cancer risk from nitrate (and nitrite) in water and food has been reported. Recent human epidemiology studies have shown that nitrate ingestion may be linked to gastric or bladder cancer. The most likely mechanism for human cancer related to nitrite is the body's formation of N-nitrosamines [1]. Nitrosamines have been shown to cause tumors at multiple organ sites in every animal species tested [1, 2]. The nitrite oxidizes iron in the hemoglobin of the red blood cells to form met hemoglobin, which lacks the oxygen-carrying ability of hemoglobin. This creates the condition known as methaemoglobinemia (sometimes referred to as "blue baby syndrome") [3, 4]. Nitrate contamination in groundwater from fertilizer and animal manure is severe and getting worse for hundreds of thousands of residents in Kazakhstan

Thirty years ago we observed that milk xanthine oxidase is able to catalyze the disappearance of nitrates and nitrites in the reaction mixture [5]. More later, it was found that both purified and tissue containing XO catalyzes the reduction of nitrate and nitrite to NO [6, 7].

XO (EC 1.17.3.2) catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid. The enzyme protein is large, having a molecular weight of 270 kDa, and has 2 FAD molecules, 2 molybdenum atoms, and 8 iron atoms bound per enzymatic unit [8]. Comparison of the Mo contents and XO activities of human and bovine XO allowed estimation of activities corresponding to 100% Mo content. This gave estimates of 59% and 55% content of inactive Mo-containing enzyme for human and bovine XOR respectively [9]. XO purified from human milk was shown to contain 0.04 atoms Mo per subunit. Thus, it seems clear that bovine and human XOs contain similar demolybdo-forms of the enzyme [10]. It is likely that the human milk samples usedare usual in that the donors came from Mo-deficient area. Preparation of XO from goat and sheep milk contain only 0,09 and 0,18 atoms Mo per subunit respectively and, although purified bovine milk XOR is clearly much richer in Mo, it is still 40% deficient [11]. Thus, in human and bovine milk XO also exists in enzymatic inactive demolybdo form. However, in goat and camel milk only a limited amount of information has been published concerning this enzyme. Therefore the purpose of this paper is to discuss the results related to nitrate- and nitrite-reducing activity of XO from goat, camel and horse milk.

Materials and methods of research. Fresh milk from goat, camel and horse were used in our experiments.0,2 M phosphate buffer containing 20 μM was added to fresh milk in 1:1 ratio. XO activity was determined spectrophotometric ally by measuring uric acid formation at 293 nm with xanthine as substrate. One enzyme unit is defined as amount of enzyme required to produce 1 μM uric acid per min per 0,1 ml reaction mixture from 10 μM of xanthine at 37°C, pH 7.4. The mixture is allowed to incubate for 15 min at 37°C. Reaction mixture for NR activity of milk XO contained 0,3 ml of 0,2 M sodium phosphate buffer (pH 6,5) containing 10 μM EDTA, 0,1 ml of fresh milk, 0,1 ml of 0,1 M KNO_3, 0,1 ml of 10 μM natural electron donor (NADPH, NADH, $FADH_2$) or 0,1 ml of 50 μM methylviologen. Reaction mixture for NiR was the same as that for NR activity, but instead of KNO3 was added 0,1 ml of 4 mM $NaNO_2$. Reaction was started by addition of 20 μM of 0,1 M dithionite ($Na_2S_2O_4$) for reduction of artificial electron donor methylviologen. The reaction mixture was incubated for 15 min at 37°C. NR and NiR activities estimated by the amount of nitrite (NO_2^-). NR activity of milk XO converted nitrate to nitrite, i.e. this activity estimated by nitrite content formed after incubation. NiR activity of the enzyme converted nitrite to NO [6, 7], i.e. after incubation the content of nitrite in reaction mixture is decreased or disappeared. Nitrite in reaction mixture after incubation was colored by adding an equal volume (0,5 ml) of sulfanilamide and N-(1-naphtyl) ethylene diamine hydrochloride. Absorption of the colour was

measured at 548 nm of the spectrophotometer. The optical measurements were performed on Spekol 1300 dual beam spectrophotometer (Germany).

Results of research and their discussion. In the earliest experiments we showed that incubation of xanthine dehydrogenase (XDH) from wheat embryo with molybdate and glutathione at high temperature (80°C) resulted in significant increase of enzyme activity. This fact led us to following interpretations. It is known that soils in all regions of Kazakhstan contain molybdenum 3–5 times less than that concentrationsneeded for normal growth and development of plants. Such a deficiency of soil molybdenum causes in the formation of molybdenum-free molecules of XDH. Incubation of the wheat embryo extract containing molybdenum-free molecules with molybdenum in the presence of glutathione made possible the incorporation of molybdenum into the active center of the enzyme and its activation. Glutathione as a strong reductant protects molybdenum-coordinating SH-groups in the active center against oxidation by oxygen.

We carried out the same experiments to activate milk xanthine oxidase of animals. 10mM sodium molybdate (Na_2MoO_4) and 10 μM cysteine (Cys) or glutathione (GSH) in final concentrations were added to the milk. The milk was heated in 2, 4, 6 and 8 minutes at 80°C. The variants were cooled to room temperature and the activity of XO (oxidation of hypoxanthine to uric acid) was determined in their aliquots. Results obtained are present in Table 1.

As shown in Table 1, heating of goat, camel and horse milk for 5–10 min in the presence of molybdate and cysteine resulted in dramatically increase of XO activity in all the milk. Whilst heat treatment in the presence of GSH increased XO activity about 3 times less than cysteine. Enzyme activity after heat treatment in the absence of thiols was negligible.

Study on nitrate- and nitrite-reducing activities of milk XO

In the cells of microorganisms and plants nitrate is reduced by the enzyme – nitrate reductase (NR). The enzyme converts nitrate (NO_3^-) to nitrite (NO_2^-). Another enzyme – nitrite reductase (NiR) converts nitrite further to ammonia (NH_4^{+}). Further ammonia is incorporated directly into organic acids converting them to amino acids.

Since heat treatment at 80°C strongly increased XO activity of fresh milk, we used such pretreatment of the milk for determination of NR and NiR activity of milk XO. In our first experiments for determination of NR and NiR activity of milk XO we used reduced methylviologen as an electron donor (methylviologen was reduced by dithionite) [5]. Therefore first of all we examined different natural electron donors for NR and NiR activities of fresh milk XO. Possible electron donors were tested for their effects on NO_3^- and NO_2^- reducing activity of milk XO and the results are presented in Table 2.

In order to study such a nitrate reducing activity (NR) of XO the milk was heated at 80°C in 2, 4, 6, 8 an 10 min in the presence of optimal concentration of 20 μM molybdate and cysteine. After cooling to room temperature in the milk the appearance of nitrite, a reduced product of NR and disappearance of nitrite as a substrate by NiR activity of milk XO. The activities were determined by using reduced methylviologen.

Table 1

Effect of heat treatment (80°C) of the milk of different animals in the presence of molybdate and glutathione on the activity of *XO

Milk source	Additions	Heating time, min			
		2	4	6	8
Goat	No addition	0,48 ± 0,03	1,53 ± 0,21	1,23 ± 0,34	1,04 ± 0,16
	+GSH	0,52 ± 0,08	1,68 ± 0,27	1,28 ± 0,5	1,06 ± 0,18
	+Mo	1,35 ± 0,12	2,75 ± 0,15	1,54 ± 0,2	1,23 ± 0,16
	+Mo + GSH	2,73 ± 0,32	3,35 ± 0,52	2,76 ± 0,7	2,58 ± 0,2
	+Mo + Cys	8,77 ± 0,83	9,85 ± 1,2	8,93 ± 0,32	8,37 ± 0,53
Camel	No addition	0,98 ± 0,12	2,37 ± 0,32	2,03 ± 0,18	1,53 ± 0,3
	+GSH	1,12 ± 0,12	2,53 ± 0,16	2,23 ± 0,3	1,87 ± 0,2
	+Mo	1,85 ± 0,27	3,47 ± 0,19	2,98 ± 0,21	2,34 ± 0,12
	+Mo + GSH	3,73 ± 0,42	4,56 ± 0,9	4,24 ± 0,2	2,75 ± 0,21
	+Mo + Cys	12,1 ± 1,27	12,64 ± 1,3	12,03 ± 0,6	11,58 ± 0,3
Horse	No addition	0,98 ± 0,72	2,37 ± 0,17	2,13 ± 0,2	1,43 ± 0,3
	+GSH	1,12 ± 0,16	2,53 ± 0,21	2,43 ± 0,22	2,12 ± 0,17
	+Mo	1,35 ± 0,15	3,47 ± 0,63	2,58 ± 0,2	1,74 ± 0,4
	+Mo + GSH	3,52 ± 0,31	4,24 ± 0,28	3,86 ± 0,7	3,57 ± 0,52
	+Mo + Cys	11,8 ± 1,3	12,14 ± 0,13	12,02 ± 1,5	11,43 ± 1,7

N o t e . * XO activity: μmoles uric acid/ min/0,1 ml reaction mixture.

Table 2

Effects of different natural electron donors on the NR and NiR activity of molybdenum-treated milk XO

Milk source	Electron donor	*NR activity	**NiR activity
Goat	NADPH	0,0	100
	NADH	203 ± 16,3	27
	FADH2	83 ± 8,6	60
	Dithionite + MV	215 ± 18,6	0,0
Camel	NADPH	0,0	100
	NADH	114 ± 18,7	27
	FADH2	62 ± 8,6	68
	Dithionite + MV	142 ± 18,9	0,0
Horse	NADPH	0,0	100
	NADH	107 ± 18,6	25
	FADH2	56 ± 13,2	62
	Dithionite + MV	514 ± 58,2	0,0

Note. *NR activity in nmoles NO_2^- formed/min/0,5 ml, **NiR per cent of remained nitrite in reaction mixture after incubation (see "Materials and methods").

Table 3

Dependence of *nitrate reduction (nitrite formed in nanomoles in the reaction mixture) and **nitrite reduction (nitrite in nanomoles disappeared in the reaction mixture) by XO on preheating time of fresh milk

Milk of:	Substrate	Heating time, min				
		2	4	6	8	10
Goat	NO^{3-}	132 ± 28,7	1,1	198 ± 17,6	157 ± 21,8	122 ± 13,2
	NO_2^-	12	0,0	0,0	10	25
Horse	NO^{3-}	141 ± 9,6	1,3	244 ± 19,4	184 ± 42,6	148 ± 13,4
	NO_2^-	8	0,0	0,0	7	13
Camel	NO^{3-}	124 ± 22,3	0,9	163 ± 12,3	122 ± 8,75	82 ± 12,5
	NO_2^-	7	0,0	0,0	5	15

Notes: * NR activity in nmoles NO_2^- formed/min/0,5 ml; **NiR per cent of remained nitrite in reaction mixture after incubation.

At room temperature fresh milk of these animals was notable to reduce nitrate and nitrite or the activities were negligible. However, after heating the milk in 2 min we observed the formation of nitrite (NR activity) and its disappearance (NiR activity, Table). Maximal activity of reduction of nitrate and nitrite observed between 4 and 6 min heating at 80 °C. Further incubation at this temperature led to gradually decrease of these activities of milk XO (Table 3).

In the next experiments we studied the effects of molybdate, cysteine and glutathione on NR and NiR activities of milk XO. Fresh milk was heated at 80 °C in the presence of these compounds in 5 min. The results are presented in Table 4.

Thus, it is relevant that cysteine thiol is generally more effective in the incorporation of the molybdenum into active center of milk XO. All the results present in these Table 4 show that simultaneous presence of cysteine and molybdenum during heat treatment dramatically increased nitrate (NR)- and nitrite-reducing (NiR) activities of milk XO. First, these results confirm that molybdenum really incorporates into XO molecule. Second, reduction of nitrate as well as nitrite is occurred in molybdenum containing center of the enzyme.

It is well known that tungsten is close chemical analog of molybdenum. The atomic and ionic radii and the chemical properties of tungsten are very similar to those of molybdenum [12]. Therefore growing various organisms in the presence of tungstate resulted in the production of W-substituted molybdoenzymes with little or no catalytic activity. W-substituted molybdoenzymes are generally inactive because of the lower reduction potential of the tungsten site with respect to the molybdenum site [12]. Tungsten containing enzymes are inactive because of inability of tungsten to transfer electrons from donor to acceptor in the active center. Therefore, tungsten is widely used for identification

of a new molybdoenzymes. In our experiments the substitution of tungsten for molybdenum under the same conditions gave no detectable NR and NiR activity of milk XO. Tableshows that the nitrate- and nitrite-reducing activity of W-XO decreased drastically and about 97–100 % of its activity was lost in the presence of 0,02 mM tungstate.

Table 4

Effect of heat treatment of fresh milk in the presence of molybdate (Na_2MoO_4) or tungstate (Na_2WO_4) and thiols on NR and NiR activity of XO

Animal	Additions	Associated activities	
		*NR	**NiR
Goat	No addition	> 0,5	98
	+GSH	> 0,5	91
	+Mo	14 ± 1,3	83
	+Mo + GSH	78 ± 8,2	2–4
	+Mo + Cys	241 ± 21,6	0,0
	+W	> 0,1	100
	+W + GSH	0,0	100
	+W + Cys	0,0	100
Camel	No addition	> 0,3	97
	+GSH	> 0,4	90
	+Mo	17 ± 0,8	84
	+Mo + GSH	68 ± 9,6	2–3
	+Mo + Cys	197 ± 28,5	0,0
	+W	0,0	100
	+W + GSH	0,0	100
	+W + Cys	0,0	100
Horse	No addition	> 0,4	98
	+GSH	> 0,5	87
	+Mo	13 ± 0,9	74
	+Mo + GSH	86 ± 12,2	2–4
	+Mo + Cys	245 ± 19,7	0,0
	+W	0,0	100
	+W + GSH	0,0	100
	+W + Cys	0,0	100

Notes: *NR activity in nmoles NO_2^- formed/min/0,5 ml; **NiR per cent of remained nitrite in reaction mixture after incubation.

We believe that these findings suggest natural molybdenum deficiency in milk XO. It has been claimed that molybdenum status influences susceptibility to certain forms of cancer and that the high incidence of esophageal cancer among the Bantu in Transkei (South Africa) is associated with a deficiency of this element in locally available food. Studies in Henan province, China, suggest that a high incidence of esophageal cancer is associated with lower than normal contents of molybdenum in drinking water and food as well as in serum, hair and urine. Esophageal cancer tissue also had lower molybdenum content than normal. It may well be relevant that inclusion of 2 or 20 μg of molybdenum/g in the diet of rats has been found to inhibit esophageal and stomach cancer following the administration of N-nitrososarcosine ethyl ester. Molybdenum in the drinking water of rats at a concentration of 10 mg/l inhibited mammary carcinogenesis induced by N-nitroso-N-methylurea [13, 14].

Molybdenum deficiency has not been identified in free-living animal species. Molybdenum deficiency has also been produced experimentally in goats by feeding them purified diets, very low in molybdenum. In goats, a molybdenum deficient diet was associated with reduced fertility and increased mortality in both the mothers and the offspring. The high dietary Mo contents did not reduce the growth of animals and after Mo-administration the highest Mo levels were found in liver and kidney [15]. However, molybdenum levels in milk of Mo-administrated animals was not yet studied.

To our knowledge, our present report is the first to show the presence of high NO_3^- and NO_2^- reducing activity in milk from goat, camel and horse**.** These results suggested that XO was probably involved in presumably NO production in animal milk. Thus, our results for the first time show nitrate- and nitrite reductase activity of milk XO from horse, camel and goat.

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INFLUENCE OF THE PHYSICOCHEMICAL CONDITIONS ON HYDROTERMAL SYNTHESIS OF Co–Cu AND Co-Cu-Al NANOSYSTEMS

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Hydrothermal synthesis has several advantages (single stage and a high degree of mixing of the reactants, relatively mild conditions of synthesis), and is increasingly used for the synthesis of inorganic materials in a highly dispersed state . In recent years number of publications on the use of such materials as catalysts has increased.

Catalysts containing cobalt oxide or reduced state are well known and are used in many reactions: hydrogen production and the reactions with his participation, such as hydrogenation and obtaining of hydrocarbons according to Fischer – Tropsch synthesis. Intermetallic Co–Ni highly dispersed powders with a particle size of 40–80 nm were prepared earlier [1] by hydrothermal synthesis in hydroalcoholic solutions.

The aim of this investigation is the influence of physico-chemical conditions (temperature, nature of the reductant, etc.) on the synthesis of Co–Cu and Co–Cu–Al systems under hydrothermal conditions. Syntheses were performed in steel autoclaves preferably from stoichiometric mixture of nitrates of these metals in aqueous solutions of formaldehyde and polyols. Ethylenglycol and glycerol were used as polyols. The products of the reaction were characterized using X-ray diffractometer and electron microscope, UV, visible and IR spectroscope.

It is shown that in the system of Co–Cu–Al at low temperatures in the products of reaction regardless of the nature of the reductant presents metallic copper and cobalt oxalate. With a slight increase in temperature in the case of glycerol in the products of reaction aluminium oxide phase is observed, whereas with the ethylenglycol gibbsite is formed.

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