ТОРАЙҒЫРОВ УНИВЕРСИТЕТІНІҢ
ҒЫЛЫМИ ЖУРНАЛЫ

НАУЧНЫЙ ЖУРНАЛ
ТОРАЙГЫРОВ УНИВЕРСИТЕТА

ҚАЗАҚСТАН ҒЫЛЫМЫ МЕН ТЕХНИКАСЫ

2001 ЖЫЛДАН БАСТАП ШЫҒАДЫ

TORAIGHYROV UNIVERSITY

НАУКА И ТЕХНИКА КАЗАХСТАНА

ИЗДАЕТСЯ С 2001 ГОДА

ISSN 2788-8770

№ 2 (2022)

ПАВЛОДАР

НАУЧНЫЙ ЖУРНАЛ
ТОРАЙГЫРОВ УНИВЕРСИТЕТ
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СВИДЕТЕЛЬСТВО
о постановке на переучет периодического печатного издания,
информационного агенства и сетевого издания
№ KZ51VPY00036165

выдано
Министерством информации и общественного развития
Республики Казахстан

Тематическая направленность
Публикация научных исследований по широкому спектру проблем
в области металлургии, машиностроения, транспорта, строительства,
химической и нефтегазовой инженерии, производства продуктов питания

Подписной индекс – 76129

https://doi.org/10.48081/UIQR5237

Импакт-фактор РИНЦ – 0,342

SRSTI 65.63.03

https://doi.org/10.48081/PYRD2567

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THE IMPACT OF MOLYBDENUM ON XANTHINE OXIDASE AND ITS ASSOCIATED ACTIVITIES IN OVINE MILK

Xanthine oxidase is a molybdenum and iron containing flavoprotein, catalyzing the final oxidation stage of purines and oxidative transformation of pterins and some aliphatic and aromatic aldehydes.

The results show that in fresh sheep milk xanthine oxidase does not contain molybdenum. Apparently, XO located in the inner membrane of the fat globule micelles (MFGM) is not available for exogenous molybdenum. During heat treatment at 80 °C, the milk globules are destroyed, then the molecule is denatured. As a result, the access of molybdenum to the MPT ('molybdopterin' or metal-binding Pterin ene-1,2-dithiolate)-containing active center increases. Our and other numerous studies have shown that MPT is extremely sensitive to oxygen.

Despite the importance of this enzyme, the distribution of xanthine oxidase in traditional household animal tissues is unknown. Formerly, we have found most of the xanthine oxidase molecules in animal milk are inactive because of lack of molybdenum. Ovine milk was processed by inserting in vivo molybdenum in drinking water. Heating the milk of animals at 80 °C for 5 minutes in the presence of molybdenum and cysteine led to a sharp increase of xanthine oxidase and it's associated - nitrate reductase and nitrite reductase activities.

Keywords: sheep, milk, molybdenum, xanthine oxidase, nitrate reductase, nitrite reductase, activity.

Introduction

Xanthine oxidase (XO) is the enzyme is responsible for the synthesis of uric acid in mammalian. Uric acid is the major final product of the metabolism of nitrogen-containing compounds in animals and it functions as an antioxidant to reduce oxidative stress [1]. Purines and other substrates react with xanthine oxidase at the site containing molybdenum and the electron acceptors react at the FAD site [2]. The protein part of the enzyme is rich in cysteine and contains 60–62 free sulfhydryl (-SH) groups. In the structure XO there are also centers that represent 2Fe-2S complex [3].

Molybdenum (Mo) is one of the important microelements in animals' organism and its concentrations varying depending on tissue type. Molybdenum is an essential cofactor of animal molybdenum-containing enzymes (Mo-enzymes) such as xanthine oxidase, aldehyde oxidase, sulfite oxidase, and recently discovered mitochondrial amidexime-

reducing protein (mARC) [Hille et al. 2011 [4]. It is connected by two S-bonds with the side chain of the pterin of the cofactor molecule [5].

It was found earlier that homogenous xanthine oxidase purified from cow's milk reduces the nitrate (NO−3) to nitrite (NO−2) [6]. However, it was unclear in what substances nitrite reduce. Later scientists had established that the xanthine oxidase isolated from cow's liver reduce nitrite under anaerobic conditions and converts it into nitric oxide (NO) [7]. Thus, xanthine oxidase, contained in the tissue and liquids in animal body is a unique tool not only for decontamination of nitrates and nitrites, but also the formation of important substances for the body – nitric oxide. Consequently, the data for the study on nitrates and nitrites reduction activity mechanisms has both scientific and practical value. So, knowledge base will increase by research ovine milk xanthine oxidase in this area.

Materials and methods

Ovine milk was obtained from healthy six sheep in mid-lactation period (from May to June) based on a farm in Almaty region (Kazakhstan).

The experiments carried out using molybdenum (M = 241.95), L-cysteine (M = 157.6), sulfanilamide (M = 172.21) from Sigma-Aldrich Chemical Co., N-(1-naphthyl)-ethylenediamindigidrochloride (M = 259.18) from AppliChem (Germany).

Obtaining and preparing milk and liver samples from animals

Live weight of animals at the beginning of the experiments was 40–45 kg. Animals were fed with freshly cut green plants (Ad lib feeding). The drinking water of animals was added to study the effect of exogenous ammonium molybdenum ((NH4)2MoO4) per 10 mg per kg of animal weight in experiments. The animals were watered with molybdenum (about 3 L) after feeding at 01.00 PM. The second portion of water without molybdenum animals have received at 06.00 PM during the data period. Animals were weighed weekly for correcting the dose of molybdenum. Every four days 100 ml portions of fresh milk were immediately frozen at a temperature of -20 °C.

Activation of the milk and liver samples using metal ions and thiols

The solution of sodium phosphate buffer was added to the milk in final concentrations of 100 mM, pH 6,5; 10 μM EDTA solution, 2 mM sodium molybdate (Na2MoO4) or sodium tungstate (Na2WO4) and 2 mM cysteine solution. Milk supernatant warmed up at a temperature of 80 °C for 5 min. After cooling, 100 mL aliquots of milk were used to determine enzyme activities. Determination of each activity was carried out in three replications (n=3, SD±).

Detection xanthine oxidase and its associated activity of animal milk.

To determine steps carried out the xanthine oxidase and its associated activity activity is described in developed method [8].

The absorbance obtained color solution was measured photometrically at a wavelength of 548 nm in spectrophotometer («Specol-2000», Germany) The calibration curve is constructed by using increasing concentrations of nitrite (NaNO2) colored with sulfanilamide and NEDA.

Determination of the amount of free molybdenum in milk.

The amount of molybdenum was determined using the method developed by our research group. The heat treatment of molybdenum led to determine the total amount of molybdenum [9].

Statistically analysis

All determinations were carried out three times (n=3). All results were calculated as mean ± standard deviation (SD) by BioStat.

Results and discussing

Determination of xanthine oxidase and its associated activities (NR and NiR activity) in fresh milk after storage at minus 20 °C showed that storing milk frozen preserves the activities of the milk. Results show that in fresh sheep milk, obtained during the month, none of XO activities were detected. However, after heat treatment of milk at 80 °C for 5 min (our previous experiment) in the presence of exogenous molybdenum and cysteine (in the table show as Milk +Mo +Cys +to) all associated activities of this enzyme appear (table 1).

Table 1 – Dynamics of changes in activities XO of fresh ovine milk during the month (n=3, SD±)

Day	Activities					
	XO activity*		NO3-reductase activity**		NO2-reductase activity***	
	Milk +Mo	Milk +Mo+Cys+to	Milk +Mo	Milk +Mo+Cys+to	Milk +Mo	Milk +Mo+Cys+to
0	>0.2	~3.2	~1.4	136.8±13.7	~2.3	243.7±28.3
4	>0.2	~3.2	~1.4	136.7±20.1	~2.3	243.7±24.9
8	>0.2	~3.0	~1.4	136.3±18.6	~2.2	243.1±19.8
12	>0.2	~2.8	~1.3	132.6±12.3	~2.0	240.2±22.3
16	>0.2	~2.8	~1.3	132.5±14.8	~1.8	240.2±27.6
20	>0.2	~2.8	~1.2	130.7±21.6	~1.8	238.3±21.4
24	>0.2	~2.6	~1.1	128.8±23.5	~1.8	236.4±18.7
28	>0.2	~2.6	~1.1	128.6±18.9	~1.7	236.4±21.8
32	>0.2	~2.6	~1.0	128.4±10.3	~1.7	236.2±22.4

*XO-activity**: nanomoles of uric acid formed/100 µl milk/min; NR** activity: nanomoles of NO2- formed/100 µl milk/min); NiR*** activity: nanomoles of NO2- disappeared/100 µl milk/min).

The results presented in the table show that the associated XO activity up to the 8 th day in milk. It slightly increased first and decreased then. There is an assumption that the relatively high activity of XO at the beginning of lactation is associated with the anti-pathogenic property of this enzyme. However, our results show that milk XO does not contain molybdenum. Accordingly, XO is inactive. Perhaps there are another explanation that the superoxide-producing center does not contain molybdenum, but contains FAD [10].

The results show that in fresh sheep milk xanthine oxidase does not contain molybdenum. Apparently, XO located in the inner membrane of the fat globule micelles (MFGM) is not available for exogenous molybdenum. During heat treatment at 80 °C, the milk globules are destroyed, then the molecule is denatured. As a result, the access of

molybdenum to the MPT (‘molybdopterin′ or metal-binding Pterin ene-1,2-dithiolate)-containing active center increases. Our and other numerous studies have shown that MPT is extremely sensitive to oxygen. Therefore, the presence of antioxidant-cysteine protects sulfhydryl groups of MPT from oxygen. Apparently, cysteine forming temporary disulfide bonds with MPT protects it from oxidation. Then from the active MPT in exogenous molybdenum is easily displaced by cysteine and associated with him in the XO active site.

Sheep had watered by molybdenum-containing water for a month had accumulated molybdenum in milk. The concentration of which reached a maximum (51 nanograms / ml) on the 20th day. Just such an amount of molybdenum in milk did not lead to the demonstration of all associated XO activities after heat treatment in the presence of cysteine (but without exogenous molybdenum). In this case, only exogenous molybdenum activated XO activity after heat treatment in the presence of cysteine (table 2). It can be assumed that before embedding molybdenum in the active center (or before binding to the MPT in the active center) newly synthesized XO molecules are involved in the formation of the inner membrane of the milk fat globule membrane (MFGM). XO is located in the inner MFGM as indicated in list of research. It is no longer available for molybdenum in vivo. Thus, the active XO is included in the inner MFGM, regardless of the presence of molybdenum in milk.

Table 2 – Influence of exogenous Mo on the dynamics of changes in the associated activities of sheep milk (n=3, SD±)

Day	Activities						Mo*
	XO activity		NO3-reductase		NO2-reductase activity		
	Milk+Mo	Milk +Mo+Cys+to	Milk+Mo	Milk +Mo+Cys +to	Milk+Mo	Milk +Mo+Cys +to	
0	>0.2	3.2±0.4	~1.4	136.8±24.6	~2.3	243.7±41.6	>2
4	>0.2	3.2±0.3	~1.4	136.8±22.4	~2.3	243.7±42.3	>2
8	~0.3	3.2±0.4	~1.5	142.7±28.6	~2.5	249.7±43.7	12.6±2.1
12	~0.4	3.4±0.5	~1.5	149.8±19.4	~2.6	249.5±51.6	42.7±7.2
16	~0.4	3.4±0.4	~1.6	152.9±12.6	~2.5	252.3±32.4	48.5±6.3
20	~0.4	3.2±0.5	~1.5	150.7±13.2	~2.5	252.2±28.6	51.3±8.4
24	~0.4	3.2±0.3	~1.4	148.9±24.3	~2.4	248.5±35.4	51.3±7.8
28	~0.4	3.0±0.3	~1.4	148.6±13.8	~2.3	246.7±28.3	51.4±9.4
32	~0.4	2.9±0.4	~1.3	148.3±12.8	~2.2	246.8±32.6	51.5±11.3

*molybdenum content in milk in nanograms in milliliter

Our previous results obtained from experiments with fresh sheep, goat, camel and mare milk associated activities showed that the milk xanthine oxidase did not show its own activity and also nitrate and nitrite reductase activity. However, heat treatment (at 80–85 °C) of fresh milk for 5 minutes in the presence of exogenous sodium molybdate and thiols (cysteine or glutathione) resulted in the appearance of the assiciated activities of xanthine oxidase [11].

One of the ways to study of in vivo effect of exogenous molybdenum on the activity of xanthine oxidase is an addition of a salt of the metal in drinking water of domestic

animals [12]. It was found previously that molybdenum added in liquid feed was less toxic than that in fresh feedstuff [13].

An experiment was conducted to determine the effect of elevated dietary Mo and duration of feeding on its concentrations in internal organs [14].

The molybdenum absorption in the gastrointestinal tract depends on its chemical nature. Molybdenum and its compounds penetrate directly to gastrointestinal tract. For instance, water-soluble molybdate, thiomolybdate and oxothiomolybdate are absorbed from 75 % to 90 % in the gastrointestinal tract. Thereafter hen completely excreted in the form of molybdenum, mainly by urine. Molybdenum can also accumulate in milk [15].

Conclusion

There are several results according to research: 1) fresh milk after storage at -20 °C showed that storing milk frozen preserves the activities of the milk; 2) fresh sheep milk xanthine oxidase does not contain molybdenum; 3) heat treatment of milk at 80 °C during 5 min in the presence of exogenous molybdenum and cysteine led to activate xanthine oxidase in sheep milk; 4) molybdenum-containing water for a month had accumulated molybdenum in milk (max51 nanograms / ml).

Acknowledgement

The work was performed within the project No. 1253/GF4: «The study of the stimulation of xanthine oxidase for the conversion of toxic nitrates and nitrites to useful nitric oxide in the fresh camel, mare, sheep and goat milk» of Ministry of Education and science of the Republic of Kazakhstan.

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Material received on 06.06.22.

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Қазақстан Республикасы, Нұр-Сұлтан қ.
Материал 06.06.22 баспаға түсті.

МОЛИБДЕННІҢ КСАНТИНОКСИДАЗАҒА ӘСЕРІ ЖӘНЕ СИЫР СҮТІМЕН БАЙЛАНЫСТЫ БЕЛСЕНДІЛІГІ

Ксантиноксидаза – бұл молибден мен темірден тұратын флавопротеин, бастапқы заттардың соңғы тотығу күйін және птериндер мен кейбір алифатты және хош иісті альдегидтердің тотығу трансформациясын катализдейді.

Жұиыс барысында нәтижелер көрсеткендей, жаңа қой сүтінде ксантин оксидазасында молибден жоқ. Майлы глобул мицелласының (MFGM) ішкі мембранасында орналасқан ХО экзогендік молибден үшін қол жетімді емес сияқты. 80 °С температурада термиялық өңдеу кезінде сүт шарлары ыдырайды, содан кейін молекула денатурацияланады. Нәтижесінде молибденнің МРТ («молибдоптерин» немесе металл байланыстыратын птеринен-1,2-дитиолат) бар белсенді орталыққа қол жетімділігі артады. Біздің және басқа да көптеген зерттеулеріміз МРТ оттегіге өте сезімтал екенін көрсетті.

Бұл ферменттің маңыздылығына қарамастан, дәстүрлі үй жануарларының тіндерінде ксантин оксидазасының таралуы белгісіз. Бұрын біз жануарлардың сүтіндегі ксантиноксидаза молекулаларының көпшілігі молибденнің болмауына байланысты белсенді емес екенін анықтадық. Сиыр сүті ауыз суға молибден in vivo қосу арқылы өңделді. Молибден мен цистеин болған кезде жануарлардың сүтін 80 °C температурада 5 минут қыздыру ксантин оксидазасының және онымен байланысты нитратредуктаза мен нитритредуктазаның белсенділігінің күрт артуына әкелді.

Кілтті сөздер: қой, сүт, молибден, ксантиноксидаза, нитратредуктаза, нитритредуктаза, белсенділік.

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Материал поступил в редакцию 06.06.22.

ВЛИЯНИЕ МОЛИБДЕНА НА КСАНТИНОКСИДАЗУ И СВЯЗАННУЮ С НЕЙ АКТИВНОСТЬ В КОРОВЬЕМ МОЛОКЕ

Ксантиноксидаза представляет собой флавопротеин, содержащий молибден и железо, катализирующий конечную степень окисления исходных веществ и окислительную трансформацию птеринов и некоторых алифатических и ароматических альдегидов.

Результаты показывают, что в свежем овечьем молоке ксантиноксидаза не содержит молибдена. По-видимому, XO, расположенный во внутренней мембране мицелл жировых глобул (MFGM), недоступен для экзогенного молибдена. Во время термической обработки при температуре 80 °C молочные шарики разрушаются, затем молекула денатурируется. В результате увеличивается доступ молибдена к активному центру, содержащему MPT («молибдоптерин» или металлсвязывающий птеринен-1,2-дитиолат). Наши и другие многочисленные исследования показали, что MPT чрезвычайно чувствителен к кислороду.

Несмотря на важность этого фермента, распределение ксантиноксидазы в тканях традиционных домашних животных неизвестно. Ранее мы обнаружили, что большинство молекул ксантиноксидазы в животном молоке неактивны из-за недостатка молибдена. Коровье молоко обрабатывали путем добавления молибдена in vivo в питьевую воду. Нагревание молока животных при 80 °C в течение 5 минут в присутствии молибдена и цистеина привело к резкому увеличению активности ксантиноксидазы и связанной с ней нитратредуктазы и нитритредуктазы.

Ключевые слова: овцы, молоко, молибден, ксантиноксидаза, нитратредуктаза, нитритредуктаза, активность.

Теруге 06.06.22 ж. жіберілді. Басуға 30.06.22 ж. қол қойылды.
Электрондық баспа
8,9 Mb RAM
Шартты баспа табағы 12,4. Таралымы 300 дана.
Бағасы келісім бойынша.
Компьютерде беттеген: З. С. Искакова
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Тапсырыс № 3964

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