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NITRATE AND NITRITE REDUCTION BY XANTHINE OXIDASE OF FRESH MILK

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

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). 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. Heat treatment of the milk in the presence of exogenous lecithin increased the activity of NR and NiR of XO and CysNO formation. In result 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.

Keywords: milk, xanthine oxidase, NO, NO_3^-, NO_2^-, CysNO, molybdenum, tungsten.

Introduction

It generally known that nitrates (NO_3^-) and nitrites (NO_2^-) cause various diseases, including cancer and nitrites irreversible bind to hemoglobin forming methemoglobin which losses the ability to transport oxygen [1]. 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 [2].

For the first time were observed that purified and homogeneous xanthine oxidase (XO) of cow's milk has the ability to reduce NO_3^- and NO_2^-[4] in 1980. Later, other groups of scientists have found that animal XO converts NO_3^- and NO_2^- to physiologically important substance – nitric oxide (NO) [3]. 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 [4]. NO was recognized as a molecule of the year in 1992. Scientists had awarded the Nobel Prize later in 1998 [5].

Because of the ability to form nitric oxide the XO of milk harbors an antimicrobial activity. It 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 $\cdot O_2$ (superoxide anion). XO is also involved in protective an antiviral responses by catalyzing the conversion of retinaldehyde to retinoic acid. Retinoic acid derivatives can inhibit viral replication and, thus, preventing the spread of viral disease [6].

Xanthine oxidoreductase or dehydrogenase/oxidase (XO; EC 1.1.3.22) – molybdenum and iron-containing flavoprotein. It is believed that the main biological function of XO is the catalysis the final step of purine oxidation in eukaryotes; it catalyzes the sequence of hydroxylation that convert hypoxanthine to xanthine, then to uric acid. 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 [7]. Therefore, because of its multifunctional enzymatic reactions XO is considered as a potential enzyme detoxifying different xenobiotics [8]. 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 [5].

Materials and мethods

Fresh milk samples were obtained from healthy female animals at local farm near the Astana. Chemicals were purchased from Sigma-Aldrich Chemical Co. All common chemicals and solvents used were of analytical grade.

Preparation of the milk to 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 determine of the enzymatic activity the tubes are placed in water bath with 35 °C

temperature and kept for 10 min, then using the special reagents different activities of XO are determined.

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 is 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 [9].

Method for the determination of nitrate- and nitrite-reducing activity of XO. Nitrate-reducing activity of XO determined by the disappearance of the added nitrate (NO_3^-) or by the appearance of nitrite (NO_2^-) in the reaction medium. 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) [9, 10].

Detection of the products of nitrate- and nitrite reduction. 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 [11].

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 [12], 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.

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 BioStat.

Results and discussion

Proposed mechanisms of milk XO activation. As mentioned above, milk XO in exists in molybdenum-free form and it 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 generally known that XO belongs to heat-stable enzymes – it remain active at 75–80 ºC temperature in several minutes [9]. However, at this temperature, the enzyme molecules undergo partial reversible denaturation. Therefore, one of 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.

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). It is known that NADH is one of potential physiological electron donors for XO and it also has 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 NaR and NiR activities of milk XO.

It was demonstrated that heat treatment (80^{o}C, 10 min) of homogeny XO resulted in the release of molybdenum cofactor (Mo-co) from the active center of 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 quickly inactivated by oxygen (even in anaerobic conditions) [9]. Later we showed that glutathione and cysteine were the more powerful protectors for isolated Mo-co [13]. However ascorbic acid decomposes S-nitrosocysteine [14] 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 construction of calibration curve we used nitroprusside as a donor of NO. Increasing concentrations of nitroprusside from 10 nM to 1.0 өM mixed with constant 0.1 mM concentration of cysteine in milk serum. Micromolar concentrations of nitroprusside release Nano molar concentrations of NO [15].

For many years tungsten was considered to be a biological antagonist of molybdenum and was used for study of the properties and functions of molybdenum in Mo-enzymes. This was due to the fact that tungsten is able to replace molybdenum in Mo-enzymes, forming catalytically inactive analogs [3]. 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.

Table 1 – Formation of CySNO in fresh sheep milk after heat treatment in the presence of cysteine and molybdenum or tungsten (n=3, ±SD) [9]

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

Thus, the results obtained (Table 1) convincingly show that the heat treatment of fresh sheep milk in the presence of exogenous molybdenum actually 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 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. It is likely that this was due to the difference in the sensitivity of the absorption at the ultraviolet and visible wavelengths of the spectrophotometer.

Conclusion

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. Fresh sheep's milk is consumed after heat treatment.

The work was performed within the project № 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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СҮТТІҢ КСАНТИНОКСИДАЗАСЫМЕН НИТРАТТАР МЕН НИТРИТТЕРДІ ҚАЛПЫНА КЕЛТІРУ

Қазіргі уақытта қоршаған ортаны нитраттармен ластау әлемдегі үлкен проблема болып табылады. Қоршаған ортада, сондай-ақ адам мен жануарлар ағзасында микроорганизмдер нитраттарды нитриттерге айналдыра алады.

Цистеин мен молибденнің қатысуымен термиялық өңдеумен белсендірілген қой сүтінің ксантиноксидазасы нитратты пен

нитритті, азот оксидіне (NO) азайтуға қабілетті. L-цистеин қос мақсатта қолданылды: сүтті термиялық өңдеу кезінде ферменттің тотығудан белсенді орталығының қорғаушысы және S-нитрозотиол түзуге реагент ретінде. Экзогендік лецитиннің қатысуымен сүтті термиялық өңдеу NR және NIR хо және CysNO түзілу белсенділігін арттырды. Нәтижесінде термиялық өңдеу кезінде: а) экзогендік фосфолипидтердің артық мөлшері сүт мембраналарының май глобулаларын бұзады, б) фермент молекулалары ішінара денатурацияланады және олардың белсенді орталығы экзогендік цистеин, молибден, гипоксантин және нитрат/ нитрит үшін қол жетімді болады. Цистеин МО-СО SH тобын тотығудан қорғап қана қоймай, сонымен қатар молибденнің кофакторымен байланысуына ықпал етеді.

Кілтті сөздер: сүт, ксантиноксидаза, NO, NO^{3-}, NO^{2-}, CysNO, молибден, вольфрам.

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ВОССТАНОВЛЕНИЕ НИТРАТОВ И НИТРИТОВ КСАНТИНОКСИДАЗОЙ СВЕЖЕГО МОЛОКА

В настоящее время загрязнение окружающей среды нитратами является большой проблемой в мире. В окружающей среде, а также в организме человека и животных микроорганизмы могут превращать нитраты в нитриты.

Ксантиноксидаза овечьего молока, активированного термической обработкой в присутствии цистеина и молибдена способна восстанавливаться нитрат и нитрит, а далее в оксид азота (NO). L-цистеин использовался для следующих: в качестве защитника активного центра фермента от окисления при термической обработке молока и в качестве реагента для образования S-нитрозотиола. Термическая обработка молока в присутствии экзогенного лецитина увеличивала активность NR и NIR образования XO и CysNO. Результаты показали, что во время термической обработки: а) избыток экзогенных фосфолипидов разрушает структуру ММЖГ, б) молекулы фермента частично

денатурируются, и их активный центр становится доступным для экзогенного цистеина, молибдена, гипоксантина и нитрата нитрита. Цистеин не только защищает SH-группы Mo-co от окисления, но также способствует связыванию молибдена с кофактором.

Ключевые слова: молоко, ксантиноксидаза, NO, NO^{3-}, NO^{2-}, CysNO, молибден, вольфрам.

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