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Influence of zootechnical and technological factors on angiogenin content in raw milk and secondary milk raw material

[1]Titov, E.I., [1]Tikhomirova, N.A., [1]Ionova, I.I., [2,3,]Gorlov, I.F., [2]Mosolova, N.I., [3]Korotkova, A.A. and [2]Zlobina, E.Y.

[1]Moscow State University of Food Production, 125080, 11, Volokolamskoye shosse, Moscow, Russian Federation
[2]Volga region research institute of manufacture and processing of meat-and-milk production, 400131, 6, Rokossovskogo st., Volgograd, Russian Federation
[3]Volgograd State Technical University, 400005, 28, Lenina avenue, Volgograd, Russian Federation

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Abstract

The dependence of the angiogenin content in the cow's milk on the number of the calving events, the milk producing ability, breed of cow and the lactation period has been found out. The results of the research on the relationship between the technological factors and the grade of the angiogenin concentration in milk are presented. The experiments in vivo and in vitro have revealed the antioxidant activity of the whey proteins cationic fraction enriched with angiogenin that expands the prospects for its use as a protective factor.

Introduction

As an active angiogenetic and immunomodulator, angiogenin is a subject of many scientific studies in the USA, the UK, Japan and other countries (Strydom, 1998; Harris *et al*., 2010; Oikonomou *et al*., 2011; Dungwa *et al*., 2012; Rayaprolu *et al*., 2012; Iyer *et al*., 2013; Jiang *et al*., 2014). Their research is mainly focused on the study of the properties of recombinant angiogenin and the possibilities of its application, mainly for medical purposes (Yang *et al*., 2011; Es *et al*., 2012; Eleftheriadis *et al*., 2013; Wang *et al*., 2014). This trend is provided by the spectrum of physiological properties of angiogenin (Li and Hu, 2012; Marioni *et al*., 2013; Ramani *et al*., 2013; Knight *et al*., 2014; Sheng *et al*., 2014; Yoneme *et al*., 2015).

The scientists managed to identify a number of angiogenin rich natural sources (Chang *et al*., 1997; Strydom *et al*., 1997; Riordan, 2001). First of all, it is plasma of human and bovine blood, as well as cow's milk. An average of 0.1 mg of pure angiogenin was isolated from one liter of blood plasma and 0.8-2.0 mg – from milk. So, angiogenin is a part of the whey proteins. Under that logic, the whey is assumed to have high angiogenin content. In this regard, research in the field of producing angiogenin from recycled raw milk and of its application not only for health but also food purposes are conducted (Komolova and Fedorova, 2002). For further research on obtaining angiogenin from milk raw material, it became necessary to develop methods for the quantitative determination of angiogenin in biological fluids, which are multicomponent systems, with its biological activity to be confirmed at a later stage. The methods known for determining angiogenin are mainly applied in pure solutions and are not suitable for multi-component bodily fluid such as milk.

Biologically active milk proteins, including ribonuclease, are of great physiological importance for human body, especially at an early age, when the protective system of the body is not formed. Therefore, the protein content in colostrum and milk is significant. At the same time, in the breast milk substitutes for infants, they are lacking or their concentration is significantly lower than in human milk. The data obtained showed that the preparations of pure angiogenin or of whey concentrates enriched with angiogenin are of great social and practical consequence for the baby food production.

Materials and Methods

At the initial stage of research, the angiogenin

*Corresponding author.
Email: *niimmp@mail.ru.*

content was determined in freshly drawn milk from the cows of black-and-white breed of a certain select population in the first half of lactation. The cows were kept on a cattle farm of the agro-enterprise "Kosino" in Moscow region in conditions of appropriate veterinarian requirements. The main diet included a set of feed, typical for the winter nongrazing time: haylage, silage, mixed feed, roots, mineral fertilizer, salt. The animals were divided into two groups of 10 cows each. The first group consisted of the first calving cows; the second group was made up of the 2^{nd}-7^{th} calving cows. Each group had two subgroups depending on the milk producing ability with a daily milk yield from 14 to 26 liters. The milk samples for the analysis were collected during the milling operation at the lunch hour.

For the quantitative determination of milk angiogenin, the authors developed a method of competitive enzyme immunoassay (EIA), with polyclonal antibodies conjugated with horseradish peroxidase to be applied. The development of this method was carried out in stages: selection of laboratory animals on the grounds of immunogenicity to produce antibodies to angiogenin and their immunization with angiogenin; selection of polyclonal antibodies from blood of the animals at the peak of the immune response; synthesis of conjugates of the produced antibodies to angiogeninus by horseradish peroxidase; selection of optimal concentrations of the conjugate for EIA; the EIA of the conjugates.

A method of competitive interaction of ribonuclease A (RNase) and angiogenin inhibitor for the placental RNasin was developed as a short time analysis method. The placental RNase inhibitor inhibits the RNase activity of angiogenin, forming a relatively stable complex "angiogenin - inhibitor". In the course of the analysis, a competitive reaction proceeds, with a complex "placental RNase inhibitor - pancreatic RNase – angiogenin" to be formed. As a placental RNase inhibitor, a dry commercial preparation of the firm Sigma (Germany) was used.

Due to the fact that the pancreatic RNase content in the cow's milk ranges from 12 to 32 mg/l (Shidlovskaya, 2006) that is almost an order of magnitude higher than the grade of the angiogenin concentration in it, the application of the method for the determining angiogenin in dairy raw material is possible only after its prepurification from the endogenous RNase. To reduce the mass fraction of the RNase A, the liquid sorptive chromatography with blue agarose is proposed to use, as the triazine dyes cross-linked with agarose are ligands having a high affinity for the metabolism enzymes of the nucleic acids. The RNase A concentration after the chromatographic purification must not exceed 0.4 mg/l. The modification of the method of chromatographic purification of the biological fluids from RNase allowed to adapt the new method for the angiogenin determination in raw milk.

Implementation of the scientific and practical task to obtain angiogenin from the raw milk is also associated with the development of statistically valid method of quantitative analysis. In order to obtain an objective assessment of the amount of angiogenin in milk and dairy raw material, which are a multifactorial biological system, the analytical dependence was found out experimentally and the calibration curve which allowed to determine angiogenin was constructed by the nonlinear regression analysis. Thus, to determine the angiogenin content in milk from the experimental cows, the EIA method based on polyclonal antibodies to bovine angiogenin was tested.

In our studies, the angiogenin identification was carried out by its angiogenic activity using a modification of the method of "micropocket" on the cornea in the rat eye (Komolova *et al*., 1992; Mashtakova and Komolova, 1993). The tablet of carboxymethyl cellulose or other biologically neutral polymer impregnated with the protein analyzed was introduced into the cornea at a distance from the vascular system. Three or four days later, there was the growth of blood capillaries extending from the vessel of the eye rim perpendicular to it and towards the implant. The number of the newly formed capillaries was counted and their length at different times was measured, recording the growth dynamics.

At the next stage, the studies were conducted to determine the antioxidant activity of the protein module – a whey proteins cationic fraction enriched with angiogenin. The experiments were performed in vivo on the laboratory animals and in vitro on the model systems. The *in vivo* experiments were carried out on Wistar rats. The laboratory animals (16 males and 16 females) were divided into two groups: a control group and an experimental one. Milk as a source of cationic proteins was excluded from the diet of the control group (a standard vivarium diet) and the diet of the experimental group was intervented with the whey proteins cationic fraction enriched with angiogenin at the rate of 0.6 mg/g of the body weight (in addition to the vivarium diet). This dose was calculated taking into account the physiological norms of milk consumption in the diet of baby rats. The early period of adaptation of the laboratory animals is accompanied by stress, which is known to be marked by the increased formation of reactive oxygen intermediate in their organism. At the end of

the experiment the next day, the laboratory animals were decapitated, the blood was collected, the serum was separated by centrifugation, and the products of the lipid peroxidation (LPO) were determined in the serum.

In the experiment, the method *in vitro* helped to analyze the grade of the LPO-products concentration in the model system of the lipid oxidation according to the test with thiobarbituric acid (TBA).

The techniques developed formed the basis for angiogenin determination in raw milk (depending on the zoo-technical factors) and secondary dairy raw materials (depending on the technological factors) to identify new resources to derive it. The study of the fractional composition of the cheese whey was carried out by SDS-electrophoresis in polyacrylamide gel (Laemmli U, 1970). The method uses a stepped buffer system (the buffer composition varies in separation space), which allows to reduce the diffusion and thereby to increase the resolution of the method. Due to the SD(L)S method (sodium dodecyl (lauryl) sulfate, SDS sodium dodecyl sulfate, SLS – sodium lauryl sulfate), the separation of proteins occurs only in their size. SDS is an anionic surface acting agent (SAA); its molecules in water carry a negative charge in a wide pH range. Upon heating the proteins in SDS solution, their almost complete denaturation occurs and in presence of SDS, they sorb its molecules so that a practically constant number of SDS molecules are accounted for the length unit of the polypeptide chain. So, the proteins get a negative excess constant specific charge (per length (weight) unit of the protein). In such a treatment, the self-charge of the protein is practically unobservable from the point of view of its influence on the mobility of the protein molecule. The electrophoresis was performed in the 16% polyacrylamide separating gel at the current strength of 30-40 mA and the maximum voltage of 290 V. Then the proteins were stained with the dye solution Coumassie R-250. To identify the proteins, the cheese whey was used in 10, 20, and 30-multiple dilutions. The standard high molecular marker proteins were used to identify the molecular weights of the proteins studied.

The data on different variables, obtained from the experiment, were statistically analyzed by Statistica 10 package (StatSoft Inc.). The significance of differences between the indices was determined using the criteria of nonparametric statistics for the linked populations (differences with $P < 0.05$ were considered significant). Student's t-test, Fischer test, Blackman test, χ^2 were applied for the statistical analysis. Regression and Correlation analyses were also computed to establish relationships among various parameters (Johnson and Bhattacharyya, 2010).

The mean of a set of measurements was calculated according to the formula: $\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n}$, where $\bar{x}$ –a mean value; $\sum_{i=1}^{n} x_i$ – read this as "the sum of all xi with i ranging from 1 to n"; n – number of measurements. The residual variation is expressed as a root mean square error (r.m.s.e.): $\sigma = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}}$. The standard error of mean (s.e.m.) was calculated by the formula: $s.e.m.(\bar{x}) = \frac{\sigma}{\sqrt{n}}$. The reliability of a sample difference (Student's t-distribution) was estimated by the test of the difference validity, which is the ratio between the sample difference to the non-sampling error. The test of the difference validity was determined by the formula: $t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{s.e._1^2 + s.e._2^2}} \geq t_{st}(d.f. = n_1 + n_2 - 2)$, where t – Student's t-distribution; $(\bar{x}_1 - \bar{x}_2)$ – difference of the sample mean measurements; – sample difference error; s.e.m.1, s.e.m.2 – non-sampling error of the sample statistics compared; t_{st} – standard criterion according to the t-Table for the probability threshold preset depending on degrees of freedom ("probability": $P < 0.05$); n_1, n_2 – number of measurements in the samples compared; d.f. – degrees of freedom for difference of two mean measurements. The coefficient of variation (CV) was calculated by the formula: $CV = \frac{\sigma}{\bar{x}}$.

MS Office 2010 package was employed for graphical presentation of the data.

Results and Discussion

The results of the research of the angiogenin content in milk from the cows of black-and-white breed show significant individual differences of animals on this indicator, despite the identical conditions of their keeping and feeding. The index analyzed was found to range from 2.09 to 4.85 mg/l. A statistically significant pattern was revealed: the angiogenin concentration in the milk from the first calving cows (group I) is lower than in the milk from the several calving cows (group II), with the differences between the subgroups within groups I and II to be statistically inaccurate. Thus, the study showed no significant effect of the level of milk producing ability of the cows on the angiogenin concentration in milk.

The photographs of the angiogenesis identification test clearly show the growth of the blood vessels of the experimental animals in comparison with those in the control group that confirms the angiogenic activity of angiogenin derived from the cow's milk (Figure 1). In the experiments in vivo with baby rats, their diet was intervented with the whey proteins cationic fraction enriched with angiogenin. The data obtained evidence of the change in anthropometric

Table 1. Effect of angiogenin in feed ration on indices of laboratory animals

Indices	Age, days							
	14		25		14		25	
	male (16 parents)				female (16 parents)			
	experiment	control	experiment	control	experiment	control	experiment	control
Body weight, g	from 16.9 to 17.5	from 16.5 to 17.0	from 40.1 to 58.1	from 39.0 to 57.0	from 12.4 to 19.8	from 11.8 to 18.5	from 13.1 to 21.3	from 12.9 to 20.5
Body length, mm	80±1.0	78±1.0	112±1.0	110±0.9	79±1.0	77±0.8	110±0.9	109±1.0
Tail length, mm	52±1.0	51±0.5	88±1.0	87±1.1	51±0.5	50±0.5	87±1.0	86±0.9

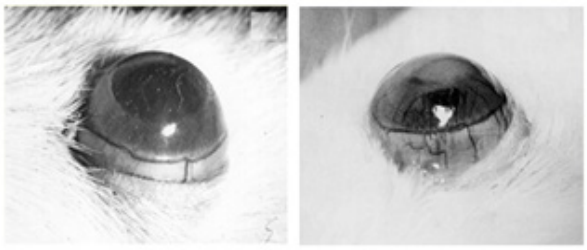

Figure 1. Angiogenesis test by "micropocket on the eye cornea of a rat" method: (A) – control (without angiogenin); (B) – experiment (350 ng of angiogenin)

indices of the laboratory animals (Table 1) and of the manifestation of antioxidant activity with sufficient reliability. Thus, the concentration of the LPO-products in blood of the laboratory animals in the experimental group was 20% ($P<0.05$) lower than that in the control group.

In the *in vitro* experiment, the whey proteins cationic fraction enriched with angiogenin was introduced into the reaction medium. The LPO-products content in its composition decreased with the increasing of the protein module dose introduced. Thus, the resulting effect is dose dependent and described by a typical S-shaped curve (Figure 2).

As follows from the analysis of the experimental data, a fitted equation (1) describing the dependence of the mass fraction of the LPO-products on the cationic fraction concentration of the whey proteins enriched with angiogenin in a model system was obtained:

$$y = a + \frac{b}{\left[1 + \exp\left(\frac{x-c}{d}\right)^{80}\right]} \quad ,(1)$$

where y is the mass fraction of LPO products, %;

x is the dose of protein module, g/cm^3;

a = 19.751395; b = 79.778943; c = 6.367171; d = 1.3950346;

the determinacy coefficient $r^2 = 0.9979178$;

the maximum percentage error of 5.670256%.

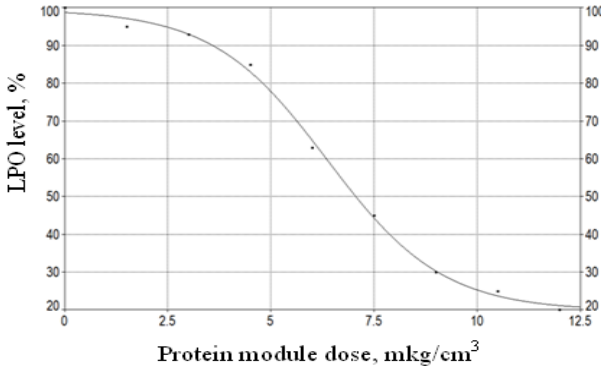

Figure 2. Dependence of the LPO-products content in a model system on the dose of the whey proteins cationic fraction enriched with angiogenin ($P < 0.80$)

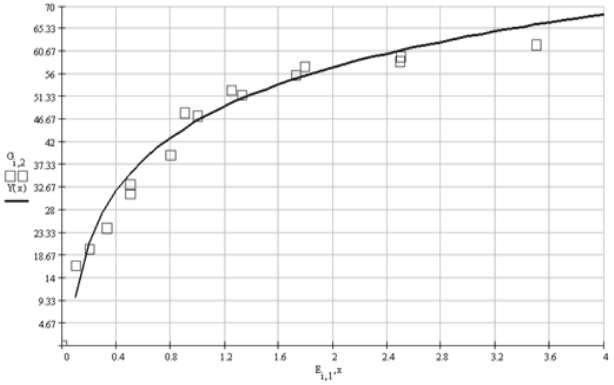

Figure 3. Calibration curve for the angiogenin determination

In order to obtain an objective assessment of the angiogenin amount in milk and dairy raw materials, which are a multifactorial biological system, research studies were carried out, with the analytical dependence to be found out and special calibration curves to be constructed. The nonlinear regression analysis helped to obtain a calibration curve that allowed determining angiogenin over the whole definition range (Figure 3).

The simplest multi-hop interpolation is a piecewise linear approximation. It helped to describe each interval of the dependence between the recovery rate of the RNase activity and the angiogenin content in the studied system by a linear function, and the

Table 2. Quantification of angiogenin in milk from cows of different breeds and angiogenin content in milk and secondary milk raw material

Breed	Colostrum, (D=1-7)	Old milk, (D=284-293)
Black-and-White	A(D) = 2.66-0.179D	A(D) = -12.35+0.047D
Kholmogorskaya	A(D) = 3.26-0.225D	A(D) = -8.87+0.036D
Holstein	A(D) = 25.71-2.07D	A(D) = -183.1+0.67D
Ayrshire	A(D) = 29.0-2.21D	A(D) = -154.8+0.58D

Kind of milk raw material	Angiogenin content, mg/l
Whole milk	(2.3-9.0)±0.07
Skim milk	(1.7-5.0)±0.09
Cheese whey	(0.9-1.2)±0.05
Curd whey	(0.5-0.8)±0.05
Butter milk:	
by creaming technology	(0.6-0.9)±0.03
by transform technology	(0.09-0.13)±0.008
Ultrafiltrate of whole and skim milk	(0.5-0.8)±0.08
Ultrafiltrate of cheese whey	(0.18-0.27)±0.005

resultant – by a system of linear equations. So, this calibration curve allows performing an accurate determination over the whole curve range as well as on each specific interval. The methods developed for the angiogenin determining formed the basis of the research of the angiogenin content according to the zoo-technical and technological factors and of determining the industrial angiogenin resources.

The results of the study of the zootechnical factors effect on the angiogenin content and of the regression analysis showed that the angiogenin content depends on the lactation period (Table 2). The highest content of angiogenin was noted for the first lactation days, with colostrum to be the richest in angiogenin that confirms the colostrum's importance for the organism of a newborn animal for the first days of life. For the last days of colostral period, the angiogenin content reduces and becomes constant at the average level, characteristic for the longest main period of lactation. The angiogenin content in old milk increases, but does not reach the values typical for the colostrum.

The study of the angiogenin content in the raw milk, with the technological factors to be taken into account, showed that the main types of the heat treatment used in the dairy industry have a significant impact on the content of angiogenin and its activity. So, the flash and short hold types of pasteurization reduce the angiogenin activity by 10±2% on the average, and the holding pasteurization – by more than 50%. All kinds of sterilization completely inactivate the enzyme. So, functional products, which are to be sterilized, should be enriched with angiogenin to the grade of the angiogenin concentration of fresh milk – about 12 mg/l.

The studies of the secondary raw milk revealed the presence of angiogenin almost in all its forms. The highest angiogenin content was noted in the skim milk and the lowest – in the buttermilk obtained when producing butter by the transform technology (Table 2). It is related to the fact that being separated, the protein fractions almost completely transform into skim milk. High-fat cream initially contains little protein that explains low angiogenin content in buttermilk. The studies have shown that the cheese whey contains more angiogenin than the curd one. The reason for the pattern is that technological methods of milk coagulation and curd processing in the cheese production provide a greater degree of transform of the whey proteins into the cheese whey.

The data show that angiogenin is found in all kinds of raw materials studied. The cheese whey, which differs little from the original milk, is the richest in angiogenin. From the point of view of the economic efficiency, the ultrafiltrate obtained from milk in production of children's cottage cheese is of particular interest. The angiogenin content is quite high there; however, the ultrafiltrate is little used for further processing unlike other types of dairy raw materials. The raw materials, which have the highest fortification with angiogenin, are obvious to be seen as a cheap source promising for large-scale production of the natural biologically active polypeptide. The possibility of industrial angiogenin production from cheese whey was investigated in production of cheese "Russian young" at Closed Joint Stock Company CJSC "Tbilisi creamery" in the Krasnodar Region of the Russian Federation.

The studies determined the fractional composition of cheese whey comprising the proteins in order of the molecular weight increasing: angiogenin, lysozyme, β-lactoglobulin, casein fraction, lactoferrin, immunoglobulin G. The analysis revealed that the fractional composition of the proteins contained unique biologically valuable proteins, namely, angiogenin and lactoferrin, in addition to the well-studied whey proteins.

It is promising to develop new medical preparations and pluripotential cosmetics, as well as biologically active dietary supplements (BADS), especially for baby and functional foods on the basis of milk angiogenin. First of all, this is particularly so with the human milk substitutes intended for infant feeding in the early postnatal period. The breast milk substitutes produced from cow milk should contain biologically active substances, including the protein factors that determine the immune status formation of

the developing organism, along with macronutrients.

In this regard, a biologically active concentrate of milk protein has been developed. This concentrate comprises angiogenin, pancreatic ribonuclease, lysozyme, and peptides having a synergistic effect. The biological activity of the preparation was tested on activation of the body defense reactions of laboratory animals (Wistar baby rats) during the transition period from breastfeeding to the standard diet.

Furthermore, the bacteriostatic effect of each biologically active protein of the concentrate (angiogenin, pancreatic RNase, lysozyme) at their minimum concentration (10 mg/l) and the bactericidal action – at their maximum concentration (50 mg/l) has been established. The data obtained on the influence of the concentrate on the pathogenic microflora by the example of coli-forms (coli group bacteria – CGB) - *E. coli* strain B-125 (origin: ATCC, USA, N 6538-p) suggests the prospect of its use for biological preservation of dairy products, including food products for infants.

The protein concentrates enriched with angiogenin are of practical interest. Their production from recycled raw milk can be added to the schemes of non-waste processing based on membrane technology.

Conclusions

The chromatographic method for purification angiogenin from the raw milk has been modified. A method for identifying angiogenin based on its angiogenic properties, a short time quantitative method for the determination of bovine angiogenin in biological fluids based on its competitive interaction with pancreatic RNase A for placental RNase inhibitor, as well as the EIA-method for the angiogenin analysis using polyclonal antibodies conjugated with the horseradish peroxidase have been developed. The content of angiogenin in cow's milk has been found to vary between 2.09-4.85 mg/l depending on the individual characteristics of animals and to increase in the several calving cows. The results of the identification test confirmed the angiogenic activity of angiogenin derived from the cow's milk. The antioxidant effect of the protein module in the test systems has been reliably established that can be a base for the whey proteins cationic fraction enriched with angiogenin to be considered as a factor being able to regulate the oxidation rate of lipids.

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