Isolation of a human lactoferrin biosimilar from frozen milk of goat producers

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Abstract. This study offers an additional source of information on isolation and purification of the human lactoferrin biosimilar from frozen milk of goat producers. Thanks to the application of an improved technique of raw material degreasing, a milk filtrate with complete absence of the fat part was obtained, which as a result had a favorable effect on the yield and properties of the finished product, whose quality was confirmed by methods of MALDI-TOF mass spectrometry and microbiological analysis. As a result 135 g of human lactoferrin biosimilar was obtained from 75 liters of frozen milk.

1 Introduction

Currently, as medical products based on human lactoferrin biosimilar, the IGB RAS produces: Goldoferrin Spray, oral hygiene product to combat xerostomia, viruses and bacteria, gum inflammation and tooth decay; Goldoferrin C, a dietary supplement to strengthen and protect immunity from vi-ruses, bacteria, fungi, prevention of colds and Goldoferrin Fam, to maintain the natural microflora of the intimate sphere.

According to Insights on the Lactoferrin Global Market, the global lactoferrin market will reach $408 million by 2027, with an increase in market growth of 8.5% over the forecast period [1].

The increase in the output of products containing lactoferrin is due to the useful properties of this glycoprotein, expressed in its ability to have a stimulating effect on the immune system, as well as to prevent cell damage caused by aging. In addition, LF maintains the balance of beneficial bacte-ria in the intestinal tract, reduces the impact of viruses, bacteria and fungi, and is a regulator of iron metabolism [2]. Lactoferrin has been shown [3, 4] to be effective in treating hepatitis C infection by inhibiting virus multiplication. LF is also potentially used in the production of personal care products, nutraceuticals, and pharmaceuticals [5, 6], thus contributing to the growth of the lactoferrin market.

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It has been proved [2, 7] that lactoferrin exhibits antiviral, antiparasitic, antibacterial, anti-allergic functions and properties. In this connection it is planned to use it widely to fight one of the most widespread dermatological skin diseases, acne. The COVID-19 pandemic has also increased the demand for lactoferrin supplements [8].

At the moment, a number of research organizations in our country are working to establish the biological properties of LF. The antibacterial properties of the protein are due to its ability to bind iron and thereby deprive bacterial cells of the microelement necessary for their growth and vital ac-tivity [9].

For further study of the properties of LF, the development of J. Lang et al. (2011) [10] and Mirabelli C. et al. The authors determined that the anti-infective and anti-inflammatory properties of the protein are also conditioned by its interaction with other biomacromolecules. Lactoferrin, being the main protein, can interact with anionic biopolymers as well as with cell surface biostructures.

It is necessary to have a clear idea of how the sequence of certain operations can influence the quality and yield of the product. Therefore, the development of new approaches to the isolation of individual proteins from dairy raw materials will make it possible to identify the factors potentially affecting the process and subsequently evaluate the most important parameters that need to be improved.

In this regard, a specific example of work with frozen milk of goat producers of LFh, from the moment of its receipt in farm conditions, followed by long-term storage at low temperatures, on the example of existing technological equipment for the isolation of milk proteins from milk of farm animals, will demonstrate the technological process of LFh extraction from raw milk.

2 Materials and methods

Frozen milk of LFh-producing goats was the object of the study. The studies were carried out at the "Biotechnological research and experimental production on animal transgenesis" and at the "Scientific and production line for obtaining recombinant lactoferrin from goat milk" at the SUE "Scientific and Production Center of the NAS of Belarus on Animal Husbandry".

The study was conducted in three stages:

1. Production and long-term storage of milk from producer goats.
2. Defrosting and preparation of raw material for the process of protein extraction.

Chromatographic extraction of lactoferrin from filtrate using single-stage ion exchange chromatography.

Production and long-term storage of milk from producer goats.

Raw materials for LFh isolation were obtained from milking goats producing 1-4 years of lactation, carrying the human lactoferrin gene in their genome.

Storage samples were stored based on control milking in August, September, and October 2020. Milk after milking was collected in one container, left to rest for 2 hours, then stirred, samples were taken to determine the LFh content in the raw milk, then milk was poured into 1 liter polyethylene (p/et) bottles and sent for storage at -24°C.

The milk was stored until January 2022.

Defrosting and preparation of raw material for the process of protein extraction.

In January 2022, three batches of frozen milk, 25 bottles each, were delivered from storage. The batches were selected taking into account the previously measured concentration of LFh in fresh milk of 2020.

75 liters of frozen milk were subjected to air thawing at +24°C for 24 hours.

After thawing the milk, a sample was taken from each packaging unit to determine the LFh con-tent. A total of 75 aliquots were taken.

In the process of defrosting of raw milk, a specially developed method of separation of milk fat was used.

As a result of defrosting 48 liters of clarified milk filtrate was obtained, which was 64% of the product yield.

LFh content was measured by enzyme immunoassay using laboratory test-system " IFA-recLF " with the use of Tecan Sunrise photometer (Tecan, Austria).

Determination of physical and chemical parameters of the obtained lactoferrin-containing filtrate was performed on an Ekomilk Ultra device (Bulgaria).

Chromatographic extraction of lactoferrin from filtrate by ion-exchange chromatography.

3 Results and discussion

The average lactoferrin content of whole goat milk was: in August 2020 - 2.4 g/L, whereas after more than two years of storage it was 2.18 g/L with a difference of 0.22 g/L or 9%; in September 2020 - 2.8 g/L, whereas after storage it was 2.46 g/L, a difference of 0.34 g/L or 12%; in October 2020 - 3.6 g/L, after storage and thawing 3.32 g/L, a difference of 0.28 g/L or 8%. Cumulatively by month, lactoferrin concentrations in frozen-thawed milk from producer goats relative to fresh milk analyzed in 2020 decreased by 10%. The average LF content in the combined batch of thawed product was 2.8 g/L.

Defrosting and preparation of raw material for the process of protein extraction.

In the process of thawing of raw milk, a specially developed method of separation of milk fat was used and maximal degreasing was achieved. As a result, 48 liters of clarified milk filtrate was obtained, which amounted to 64% of the initial amount of the product (all described in the methodology).

Thus, high quality lactoferrin-containing milk filtrate was obtained, according to physico-chemical indices, intended for further work. With the help of specially developed methodology, maximum removal of fat part of milk was carried out, preserving at the same time its basic composition.

The main indices of the obtained filtrate, which include co-fat content, pH, lactoferrin amount - were 0%, 6.3, 2.3 g/l, respectively, were kept at their "ideal" level for further work.

Chromatographic one-step extraction of a lactoferrin biosimilar from milk filtrate.

The block diagram of LFh extraction from milk filtrate is shown in Figure 1.

Fig. 1. Block diagram of LFh extraction from milk filtrate on the pilot equipment.

48 l of milk filtrate from thawed milk of goats was filtered with the Pilot Membrane System - SW40-1-MF/UF, MMS AG Membrane Systems, Switzerland (membrane type for ultrafiltration: 20 kDA, for microfiltration: 0.45-0.8 mkm Ceramic module 3 x 25 mm).

NuviaTM cation-exchange sorbent was used for isolation of recombinant lactoferrin.

The obtained retentate (46 l) was sent to a SKID 00 chromatographic system, BIO-RAD. France, prewashed with equilibration buffer (20 mM Na phosphate, 0.2M NaCl, pH 7.5) at a rate of 10 L/h, which resulted in the absorption of LF serum protein on the ion-exchange carrier.

After applying the filtrate, the column was washed with equilibration buffer until absorbance at 280 nM was reduced to baseline. Lactoferrin was eluted by a linear gradient of 0.2M to 1M NaCl in two steps. In the first step, the unbound protein fraction was washed off the column using 0.2 M NaCl at 10 l/h, and in the second step, lactoferrin was eluted using a linear gradient of 0.7-1 M NaCl, flow rate 10 l/h, time taken 60 min.

The LFh eluate was collected according to the optical density peaks (Table 1). The optical density range of the eluate collection was between 0.1 and 12.98 o.u., the concentration of LFh between 0.4 and 9.9 g/L.

Table 1. Concentration of LFh for the elution fractions, (g/L).

Optical density, o.u.	0.54	1.83	3.03	12.98	5.63	2.50	0.10
Content LF, g/l	0.5	0.8	2.9	9.9	2.9	2.6	0.4

At the end of eluate collection, the sorbent was washed with phosphate buffer until the optical density of the solution reached zero.

Diafiltration concentration of the lactoferrin solution was performed by dialysis on a PALL GmbH (Germany) unit. Chromatographically purified lactoferrin solution was diluted with phosphate buffer to a final concentration of NaCl 0.125 M, transferred into a dialyzer tank, and concentrated fivefold.

Four fractions of the concentrated eluate were collected after the step. LF content in the collected fractions (Table 2).

Table 2. LF content in concentrated eluate.

Eluate fractions	№1	№2	№3	№4
LFh, g/l	16.9	10.3	11.0	8.1

The data in the table show that the eluate after the thickening process was concentrated by a factor of 4.

Freezing and drying the lactoferrin solution.

Lactoferrin solution after dialysis and concentration was transferred into freezing tanks and placed in freezing chambers at -20ºC, where it was kept for 24 hours.

The frozen concentrate was dried using a Gamma lyophilizer, Martin Christ, Germany at -60ºC, vacuum 1.35 mbar. After 48 h, lyophilization was completed.

Protein collection and evaluation.

Ready lyophilized lactoferrin was collected in a container, weighed (the mass of the drug yield was 135 g) and placed in storage at 4ºC.

Thus, 135 g of lyophilic dried LF with the degree of purification of not less than 90% was obtained using one-step cation exchange chromatography on semi-industrial equipment, the quality of which was confirmed by MALDI-TOF mass spectrometry and QMAFAnM (CFU)/g values (content by this parameter was $1x10^3$ g/cm^3).

4 Conclusion

Long-term storage of frozen milk of LFh-producing goats has no significant effect on the biological content of the "protein of interest.

The concentration of lactoferrin in the frozen-thawed milk of goat producers that was stored at -24ºC for a long time decreased by 10% compared to fresh milk.

Storing milk in a frozen state contributes to "perfect" defatting of frozen milk, which subsequently improves the quality of the finished product. As a result, from 75 liters of frozen milk of goat producers, 48 liters of lactoferrin-containing filtrate was obtained, from which 135 grams of LFh were isolated by one-stage chromatography, whose quality was confirmed by MALDI-TOF and QMAFAnM (CFU)/g values (content in this parameter was $1x10^3$ g/cm^3).

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