

¹M.Kh. Narmuratova , ²Céline Cakir-Kiefer , ^{1*}Zh.B. Narmuratova 

¹Al-Farabi Kazakh National University, Almaty, Kazakhstan

²Université de Lorraine, INRA, URAFPA, Nancy, France

*e-mail: janarka.90b@gmail.com

Isolation and purification of lactoferrin from Kazakhstan mare milk

Abstract: The single chemical composition of mare milk, rich in whey proteins is similar to human milk. Lactoferrin is one of the important compounds contained in whey protein fraction, and has multiple biological functions such as antimicrobial and activation of human and animal immune system. Due to its strong antimicrobial activity, lactoferrin has potential pharmaceutical applications. The present work is mainly focused on the isolation and purification of the lactoferrin from mare's milk. First, the lactoferrin from Kazakhstan mare milk has been purified by gel filtration Sephadex G-100 chromatography in two steps. The column of Sephadex G-100 was eluted with 0.01 M sodium phosphate buffer (pH 6.8). Lactoferrin enriched fractions were detected using UV absorbance at 280 nm and were identified in the first peak. Second, the purity of lactoferrin was checked by 12% SDS-PAGE and the molecular weight of lactoferrin (in the range of 80-82 kDa) was estimated using protein standard, which is recombinant human lactoferrin (expressed in rice, iron saturated, molecular weight 82.4 kDa).

Key words: Kazakhstan mare milk, isolation, whey protein, lactoferrin, purification, analysis.

Introduction

Traditionally, mare milk has been an integral part of the daily diet for centuries in many countries of Central Asia and Eastern Europe. In terms of protein composition, mare milk is close to human milk. It is then a promising alternative to cow's milk for human infant feeding, especially due to its low-fat content, large amount of valuable nutrients with several health benefits [1], high abundance of whey proteins, including lactoferrin, lysozyme and immunoglobulins [2; 3], which have positive effects on the human immune system and infectious diseases [4]. The percentage of whey proteins in mare's milk (39%) [5] is higher than in cow milk (20%), but is lower than in human milk (50%). Cow milk contains the largest amount of caseins. That is why cow milk is defined as casein type milk, while human and mare milk is called albumin type milk [3-5]. The composition of mare milk makes it a beneficial source of nutrients for people.

Lactoferrin is an iron-binding protein of the transferrins family, which plays multifunctional roles in the formation of the innate immune system [6]. Lactoferrin also has ability to bind free iron in biological fluids of mammals [7]. It has been suggested that the

antimicrobial activity of this protein is due to its iron-binding properties, but the exact role of lactoferrin in iron binding in milk is unknown [8]. In the last thirty years, research has discovered several milk proteins and related peptides with interesting antimicrobial properties, particularly lactoferrin, which protects against microbial pathogens, and its antibacterial activity has already been well-described [9]. With the steady increase in the number of multidrug-resistant pathogens, many researchers are looking to alternative medicine instead of classical antibiotics. Indeed, it has become necessary to explore natural resources for new, alternative and/or complementary medicines. In this search for novel antimicrobial agents for the future, lactoferrin, a multifunctional protein that participates in a range of essential physiological processes, offers a new source with potential pharmaceutical applications. In addition, lactoferrin containing antimicrobial fragments are still being explored, through both chemical synthesis and enzymatic digestion, and the peptides have potential applications as pharmaceutical products [10; 11] as recently been described [12].

Kee-Sung et al. (2009) purified lactoferrin from Mongolian mare milk and compared its 20 amino acids N-terminal sequence with mare diferric lacto-

ferrin and bovine lactoferrin. The molecular weight of Mongolian mare milk lactoferrin is 81 kDa [9]. Mare lactoferrin contains 689 amino acid residues, which is similar to bovine lactoferrin [7]. Many studies were performed on isolated milk proteins, which are well described [13-15]. However, in Kazakhstan, the research on isolation and purification of lactoferrin from mare milk has not been studied adequately. The purpose of the current study was to isolate and purify the lactoferrin of whey mare milk by using gel filtration, Sephadex G-100. SDS-PAGE was used to check the level of purity of the lactoferrin enriched fractions.

Materials and methods

Fresh mare milk sample was procured from the local mare dairy farm in Irgelyi village in Almaty region. Temperate grasslands were used to feed horses, because feeding is one of the factors that determine the chemical composition of mare milk. Sephadex G-100 and woman milk lactoferrin marker for determination of protein molecular weight were from Sigma. The molecular weight of human recombinant lactoferrin, expressed in rice, iron saturated, $\geq 90\%$ (SDS-PAGE), is 82.4 kDa.

Sample preparation. Mare milk was stored frozen at 4°C for 30 min and the sample was prepared as follows: defatting (100 mL) was done by centrifugation at $10.000 \times g$ for 30 min at 4°C and filtration. Casein was eliminated by acid precipitation at pH 4.2 with a 1 M HCl solution followed by a centrifugation step ($10.000 \times g$ for 30 min at 20°C).

The pH of the obtained supernatant (containing whey proteins) was adjusted to pH 6.8 with 1 M NaOH and dialyzed (cut-off of dialysis membranes = 6-8 000 Da, SpectraPor; Spectrum Labs Inc., Rancho Dominguez, CA, USA) against ultrapure water for 72 h to remove salts. After dialysis, the whey samples were then kept at -20°C before being used for lactoferrin purification [9; 16].

Isolation and purification of mare lactoferrin.

In order to isolate lactoferrin from the whey proteins, a column of Sephadex G-100 was used. A column was a glass tube (50×2.0 cm) with a dropping funnel on the bottom. That was set vertically in a ring stand. Glass wool was put to the bottom of the column to prevent Sephadex gel from coming out of the column during washing with 0.01 M sodium phosphate buffer. The height of poured gel in the column was 70% of the height of a glass tube. Then the loaded whey proteins (2 mL sample each time) were eluted with 0.01 M sodium phosphate buffer (pH 6.8)

and the flow rate was 0.4-0.5 mL/min. The presence of proteins was measured at 280 nm by a spectrophotometer PD-303 UV (Apel, Japan).

Estimation of lactoferrin concentration. A common and practical expression of the Beer-Lambert law relates the optical attenuation of a material containing a concentration of attenuating species to the optical path length through the sample and absorptivity of the species. This expression is:

$$A = \epsilon lc,$$

where:

A – amount of light absorbed by the sample for a particular wavelength;

ϵ – coefficient of molar extinction;

l – distance that the light travels through the solution;

c – molar concentration of the absorbing species per unit volume.

Estimation of molecular weight (SDS-PAGE). Electrophoresis was performed in Consort EV 265 apparatus with 12% SDS-PAGE with stacking gel (Tris-HCl, pH 6.8) and separation gel (Tris-HCl, pH 8.8). Tris-glycine buffer 5x concentrate (300 mL of 5x Tris-glycine buffer mixed with 1200 mL of distilled water) was used as a running buffer. To provide protein migration in the gel, obtained protein fraction was denatured for with buffer (deionized H₂O, 0.5 M Tris-HCl, pH 6.8, glycerol 50 %, 10 % SDS, 1% bromophenol blue, 2-mercaptoethanol) in the presence of sodium dodecylsulfate in water bath at 100°C 2 min. 10 μ L of eluted fraction was loaded into the each well. The fixation of protein samples in the gel was carried out in 10% trichloroacetic acid solution during 30 min. In order to detect protein a band after electrophoresis, the gel was stained with 0.1% Coomassie Brilliant Blue R-250 for 1 h. The gel was washed with 7% acetic acid for further visualization analyses.

Results and discussion

Chromatography is designed to purify molecules based their size, chemical composition, chemical and physical properties. Size-exclusion chromatography is particularly designed to isolate pure molecules based their molecular weight. Standard markers allow to determine molecular weight of isolated proteins. Gel filtration chromatography has been widely used in the purification of whey proteins. Sephadex G-100 gel filtration chromatography was effective in isolating the major whey proteins [17]. That is why in the present study, lactoferrin was separated by gel filtration

chromatography through Sephadex G-100 column. Whey protein dissolved in 0.01 M sodium phosphate buffer (pH 6.8) was loaded into the column. The protein elution done with the same buffer and the flow rate of 0.4-0.5 mL/min, was monitored by UV absorbance measurement at 280 nm. The elution of the protein was evaluated by the measure of absorbance at 280 nm in a spectrophotometer. After loading samples into a column of gel chromatography large mo-

lecules move through porous beads faster than small molecules. Consequently, large molecules elute first, then small molecules are washed out from the gel. Prepared Sephadex G-100 gel may be used several times only if it is protected from degradation due to contamination with microorganisms. The results of gel filtration are shown on the Figure 1. The lactoferrin enriched fractions were loaded second time in order to get pure lactoferrin.

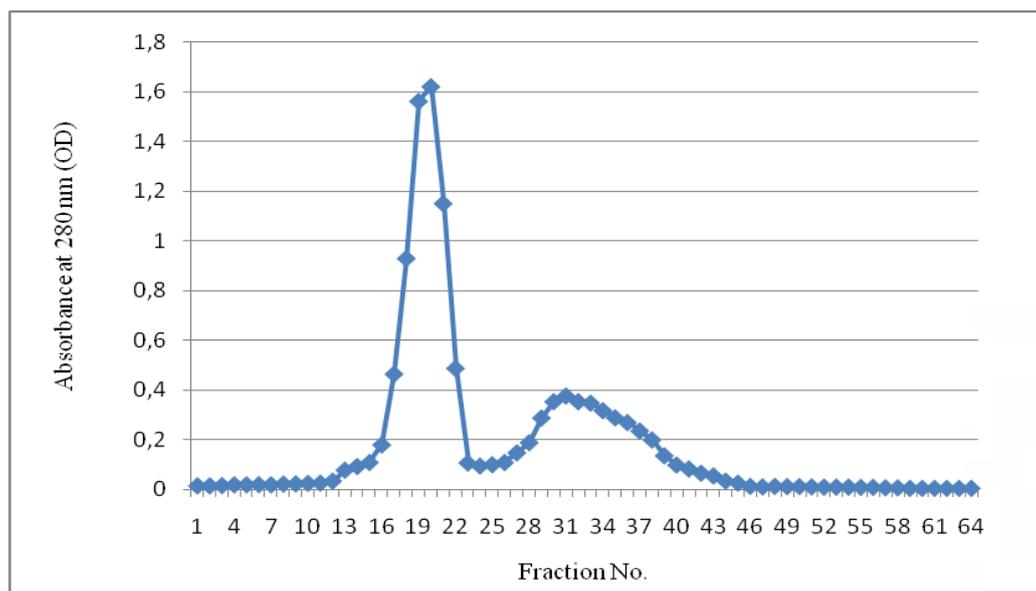


Figure 1 – Purification of lactoferrin from mare's whey protein on Sephadex G-100

Figure 1 shows that two protein peaks appeared in the eluted fractions 15-25 and 28-42, however a strong peak was observed between 15-25 fractions. In order to check purity, samples which showed the highest level of lactoferrin (fractions 17-22 and fractions 33-35) were selected.

Gel filtration has also been useful in the isolation of whey proteins, to examine the purity of commercial preparations of whey proteins [16], to separate glycopeptides of immunoglobulins, and to study the composition of whey proteins from other species [15-19].

Further amino acid composition of lactoferrin is identified. According to the information received from the ProtParam software (<https://web.expasy.org/cgi-bin/protparam/protparam>), The amino acid sequence of the lactoferrin from mare's milk consists of 708 amino acids; the theoretical molecular weight is 77.361 Da, the theoretical pI is 8.36 and the molar extinction coefficient at 280 nm is $81425 \text{ M}^{-1}\text{cm}^{-1}$. Amino acid composition of lactoferrin is presented

in Table 1.

The total number of negatively charged residues (Asp + Glu): 77. The total number of positively charged residues (Arg + Lys): 86.

Estimation of lactoferrin purity and molecular weight by SDS-PAGE. In order to determine required protein samples from fractions SDS-PAGE is used. Before loading samples into the gel of SDS-PAGE, the samples were denatured with buffer. Due to differences in charge, size and shape, proteins have different movement ability in the gel of SDS-PAGE. In order to detect protein bands after electrophoresis, the gel was stained with 0.1% Coomassie Brilliant Blue R-250. The single band of isolated lactoferrin from 17-22 fractions obtained on performing SDS-PAGE. Molecular weight was determined by applying the purified lactoferrin on 12% SDS-PAGE. According to Figure 2, lactoferrin enriched fractions gave different results, while the quantity of loaded samples was the same (10 μL). The protein concentration was determined for selected fractions by measuring the

absorbance A at 280 nm and by applying the Beer – Lambert formula.

Table 1 – Amino acid composition of mare’s milk lactoferrin

Amino acid composition		
Ala (A)	74	10.5%
Arg (R)	37	5.2%
Asn (N)	35	4.9%
Asp (D)	34	4.8%
Cys (C)	35	4.9%
Gln (Q)	29	4.1%
Glu (E)	43	6.1%
Gly (G)	52	7.3%
His (H)	10	1.4%
Ile (I)	13	1.8%
Leu (L)	69	9.7%
Lys (K)	49	6.9%
Met (M)	4	0.6%
Phe (F)	30	4.2%
Pro (P)	36	5.1%
Ser (S)	50	7.1%
Thr (T)	31	4.4%
Trp (W)	9	1.3%
Tyr (Y)	20	2.8%
Val (V)	48	6.8%

The protein concentrations are approximately:
 for 17th fraction: 5.7 μ M;
 for 18th fraction: 11.5 μ M;
 for 19th fraction: 19 μ M;
 for 20th fraction: 20 μ M;
 for 21th fraction: 14 μ M;
 for 22nd fraction: 6 μ M

Figure 2 shows the molecular weight of mare’s milk lactoferrin approximately in the range of 80-82 kDa.

According to the type of animal and carbohydrate content, molecular weight of lactoferrin differs slightly. Jolles *et al.* purified lactoferrin from E. Wettstein mare’s milk by Sephadex G-100 filtration and estimated its molecular mass as 81 kDa [17]. In the present study the molecular weight is 82 kDa on SDS–PAGE 12%. It was similar to the mass of Mongolian mare milk lactoferrin, which was characterized corresponding to a molecular mass of 82 kDa [9]. Konuspayeva *et al.* used gel permeation chromatography on Sephadex G-200 to purify lactoferrin from camel colostrum whey and checked its purity by polyacrylamide gel electrophoresis (12.5%) [18].

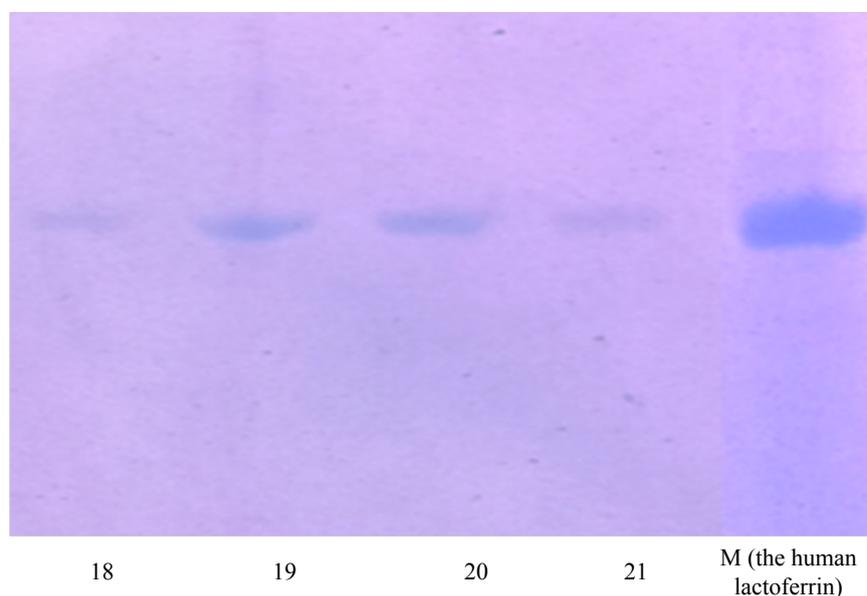


Figure 2 – SDS-PAGE 12% of lactoferrin isolated from mare’s milk whey through a column of Sephadex G-100: fractions 18-21 were loaded. Note: the molecular weight of the isolated bands of lactoferrin, is approximately 80-82 kDa; M – protein standard marker (the human lactoferrin with a molecular weight of 82.4 kDa)

Many researchers used SDS–PAGE to confirm the presence or for characterization of lactoferrin [19-24]. The purity of isolated lactoferrin was confirmed by SDS–PAGE. As a result, a single band of lactoferrin was obtained. The results of the present study were similar to other studies. Younghoon et al. checked the purity of caprine lactoferrin using SDS–PAGE [20]. The purity of isolated lactoferrin from defatted bovine colostrums was also confirmed on the SDS–PAGE gel [21]. Zainab et al. used SDS–PAGE to confirm lactoferrin purity which was isolated from goat colostrums whey [13] and cow's milk by using Sephadex G-50 [22]. Vijayan et al. employed SDS–PAGE to confirm the molecular weight of Malabari goat's colostrum lactoferrin, which was determined as 80 kDa [23]. Further, Annabelles et al. confirm the molecular weight of goat milk lactoferrin by SDS–PAGE as 78 kDa [24].

In depth research on protein properties requires purification homogeneous protein bands from samples. Isolation and purification proteins are the first and important step of protein investigation in biochemical and biomedical studies. Protein isolation methods are designed based on protein size, charge, and polarity. Moreover, these mentioned protein characteristics determine the richness and abundance of proteins. Every protein purification method allows to isolate and further purify desired proteins. In this study, size exclusion chromatography is applied to isolate pure mare's lactoferrin. Column gel filtration for isolation of lactoferrin has some advantages. First, it is convenient to isolate and classify proteins regarding their molecular weight and size. Second, the determination of molecular weight of isolated protein samples is possible by applying standard or specific markers. Sephadex G-100, which is used in this study, allows isolating proteins in a particular molecular weight. Then, in order to determine required protein samples from fractions SDS–PAGE is used. The results show that a single band of lactoferrin is isolated, and further, molecular weight of lactoferrin is determined with standard protein marker 80-82 kDa.

Conclusion

Chromatographic methods have been employed extensively in extracting many individual components of the casein and whey fractions of milk of different animals. Gel filtration, a column chromatography based on Sephadex and ion exchange chromatography are probably more frequently used than any other separation procedures. These chromatographic methods are very effective in isolating and

purifying whey lactoferrin. The results of the present study showed the effectiveness of gel filtration chromatography (Sephadex G-100) in isolation of single band lactoferrin from mare whey.

References

1. Markiewicz-Keszycka M., Wójtowski J., Kuczynska B., Puppel K., Czyzak-Runowska G., Bagnicka E., Strzalkowska N., Jozwik A., Krzyzewski J. (2013) Chemical composition and whey protein fraction of late lactation mare's milk. *Int Dairy J.*, vol. 31, pp. 62-64.
2. Karav S., Salcedo J., Frese S.A., Barile D. (2018) Thoroughbred mare's milk exhibits a unique and diverse free oligosaccharide profile. *FEBS Open Bio*, vol. 8, pp. 1219-1229.
3. Pecka E., Dobrzanski Z., Zachwieja A., Szulc T., Czyz K. (2012) Studies of composition and major protein level in milk and colostrum of mares. *Anim Sci J.*, vol. 93, pp. 162-168.
4. Jastrzebska E., Wadas E., Daszkiewicz T., Pietrzak-Fiecko R. (2017) Nutritional value and health-promoting properties of mare's milk – a review. *Czech J Anim Sci.*, vol. 62, No 12, pp. 511-518.
5. Čagalj M., Brezovečki A., Mikulec N., Antunac N. (2014) Composition and properties of mare's milk of Croatian Coldblood horse breed. *Mljekarstvo*, vol. 64 (1), pp. 3-11.
6. Ismail A.B., El Hafez S.M.A., Mahmoud M.B., Elaraby A-K. A., Hassan H.M. (2013) Development of new strategy for non-antibiotic therapy: dromedary camel lactoferrin has a potent antimicrobial and immunomodulator effects. *Adv Infect Dis.*, vol. 3, pp. 231-237.
7. Uniacke-Lowe T., Huppertz T., Fox. F. P. (2010) Equine milk proteins: Chemistry, structure and nutritional significance. *Int Dairy J.*, vol. 20, pp. 609-629.
8. Detha A., Saputra A., Ola A. (2019) Antimicrobial activity of whey mare's milk against *Salmonella enteritidis*. *Journal of Physics: Conference Series*, no. 1146, pp. 1-4.
9. Kee-Sung K., Ji-Sun K., Mi-Soon S., Hae-Won N., Sang-Dong L., Duvjir S., Alimaa J. (2009) Purification and characterization of Mongolian mare lactoferrin. *Korean J Food Sci Ani Resour.*, vol. 29, no. 2, pp. 164-167.
10. Bruni N., Capucchio M. T., Biasibetti E., Pessione E., Cirrincione S., Giraud L., Corona A., Dosio F. (2016) Antimicrobial Activity of Lactoferrin-Related Peptides and Applications in Human and Veterinary Medicine. *Molecules*, vol. 21(6), 752.

11. Théolier J., Fliss I., Jean J. (2014) Hammami, R. Antimicrobial peptides of dairy proteins: From fundamental to applications. *Food Rev Int.*, vol. 30, pp. 134–154.
12. Rastogi N., Nagpal N., Alam H., Pandey S., Gautam L., Sinha M., Shin K., Manzoor N., Viridi J.S., Kaur, P. et al. (2014) Preparation and antimicrobial action of three tryptic digested functional molecules of bovine lactoferrin. *PLoS ONE*, vol. 9, e90011.
13. Zainab H. Abbas, Kifah S. Doosh, Nahi Y. Yaseen (2015) Isolation, purification and characterization of lactoferrin from goat colostrum whey. *Pak J Nutr.*, vol. 14, pp. 517-523.
14. Niaz B., Zahoor T., Randhawa Muhammad A., Jamil A. (2017) Isolation of lactoferrin from camel milk through fast protein liquid chromatography and its antagonistic activity against: *Escherichia coli* and *Staphylococcus aureus*. *Pak J Zool.*, vol. 49, pp. 1307-1313.
15. Nicolása P., Ferreirab María L., Lassallea V. (2019) A review of magnetic separation of whey proteins and potential application to whey proteins recovery, isolation and utilization. *J Food Eng.*, vol. 246, pp. 7-15.
16. Parkar R. D., Jadhav N. R., Pimpliskar R. M. (2016) Extraction and characterization of lactoferrin from commercial milk. *Int J Pharm Res.*, vol. 6, no. 2, pp. 34-36.
17. Armstrong McD J., Hopper K.E., McKenzie H.A., Murphy W.H. (1970) On the column chromatography of bovine whey proteins. *Biochim Biophys Acta*, vol. 2014, pp. 419-426.
18. Jolles J., Donda A., Amiguet, Jolles P. (1984) Mare lactotransferrin: purification, analysis and N-terminal sequence determination. *FEBS letters*, vol. 176, no. 1, pp. 185-188.
19. Konuspayeva G., Faye B., Loiseau G., Levieux D. (2007) Lactoferrin and immunoglobulin contents in camel's milk (*Camelus bactrianus*, *Camelus dromedarius*, and Hybrids) from Kazakhstan. *J Dairy Sci.*, vol. 90, pp. 38-46.
20. Younghoon, K., J. Mae, S.H. Kyoung, Y.I. Jee, O. Sejong and H.K. SAE. (2009) Anticancer activity of lactoferrin isolated from caprin colostrums on human cancer cell lines. *Int J Dairy Techn.*, vol. 62, pp. 277-281.
21. Yafei, L., W. Xuewan, W. Mianbinand, Z. Wanping. (2011) Simultaneous isolation of lactoferrin and Lactoperoxidase from bovine colostrums by SPEC 70 SLS Cation Exchange Resin. *Int J Environ Res.*, vol. 8, pp. 3764-3776.
22. Moradian, F. (2014) Lactoferrin Isolation, purification and Antimicrobial Effects. *J Med Bio-Eng.* vol. 3, pp. 203-206.
23. Vijayan S., Radhakrishnan U., Eldho L., Jayavardhanan K. K. (2017) Isolation and purification of lactoferrin from colostrum of Malabari goats. *J Exp Biol Agric Sci.*, vol. 5, no. 4, pp. 550-555.
24. Le Parc A., Dallas D.C., Duaut S., Leonil J., Martin P., Barile D. (2014) Characterization of goat milk lactoferrin N-glycans and comparison with the N-glycomes of human and bovine milk. *Electrophor.*, vol. 35, pp. 1560-1570.