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# ISOLATION AND PURIFICATION OF PROTEIN THAT IS IMMUNOLOGICALLY SIMILAR TO HUMAN LACTOFERRIN

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**ABSTRACT** — The study aimed at isolating a substance that is immunologically similar to human lactoferrin, hereinafter — microbial-derived lactoferrin (MdLF) isolated from *K.pneumoniae* liquid culture.
Protein extraction started with isolation of ballast proteins in 2M ammonium sulphate (p.a.). Ion chromatography with cation-exchange agents was the basic method used for isolating MLF. Proteins from the isolated protein fractions were identified with AGID method. The isolated MLF is glucoprotein with the molecular mass of about 84,000, the agarose diffusion coefficient of $3.1 \times 10^{-7}$ cm$^2$ sec$^{-1}$ and the relative electrophoretic mobility of 0.42. MLF is characterized by low hydrophobicity and elutes from phenyl-Sepharose with 0.4 ammonium sulphate. Its isoelectric point equals to 9.2.

**KEYWORDS** — microbial-derived lactoferrin, chromatography, electrophoresis.

## RELEVANCE

Lactoferrin belongs to the transferrin family and is capable of active binding and transporting of $Fe^{3+}$ and other mixed-valent metals. Previously examined lacroferrins are glycoproteins with the molecular masses within the range of 75–80 kDa, containing intramolecular disulfide bonds. A polypeptide chain of lactoferrin forms two globular domains called M- and C-lobes, connected with an alpha helix. Each lobe has an iron-binding centre. The tertiary structures of apo-lactoferrin (iron-free lactoferrin) and iron-binding lactoferrin differ [4, 6, 7, 8, 9, 10, 13]. For example, it is found that $Fe^{3+}$ binding plays an essential role in implementing the antioxidant and bacteriostatic function of lactoferrin. Along with antimicrobial activity, lactoferrin also has other biological properties — it can act as an immunomodulatory agent, antioxidant and anti-inflammatory agent and transcriptional factor and definitely participates in iron metabolism [1, 5, 14, 15, 17].

Comparative studies play a significant role in examining biological properties of lactoferrin, for which reason it is important to examine the structure and properties of lactoferrin from the sources covering different species. Interestingly, literature sources still contain only sporadic data on the presence of lactoferrin in prokaryotes with no protein isolated and no molecular mass measured [2, 3, 10, 11, 12].

Anyway, microbial cells have been increasingly used as potent protein factories because of their versatility and cost-effectiveness [18].

We have made an attempt to isolate and purify a protein that is immunologically similar to human lactoferrin from Klebsiella pneumoniae. MdLF presence in the samples of the pre-centrifuged (5,000 rpm during 45 minutes) liquid culture of *K.pneumoniae* (meat-peptone broth, incubation time — 24 hours at t=37° C) was assumed on the basis of EIA results with the use of *Hycult biotech* test kits (Netherlands) [3].

## MATERIALS AND METHODS

Currently, lactoferrin is commonly isolated by the method of ion chromatography with cation-exchange agents. This method is based on a significant difference of the LF isoelectric point from other proteins at the pH values of 7–7.4.

Protein extraction started from isolation of ballast proteins in 2M ammonium sulphate (p.a.). MLF does not precipitate at this concentration, and once a precipitate was centrifugally isolated (10,000 rpm during 45 minutes), it was resuspended in a buffer (10 mM tris-HCl, pH 7.6; 1 mM EDTA; 1 mM iron(III)-ammonium) to saturate LFP with iron.

After this, the sample is thoroughly desalted and buffered with a phosphate buffer, pH=7.0. Then the obtained solution was applied on a column (4.5×60 cm) of Heparin Sepharose (Sigma, USA) which had been preliminarily equilibrated with 20 mM tris-HCl, pH 7.5. The sorbent was washed with 20 mM tris-HCl, pH 7.5, and then — with the same buffer, containing 1% triton X-100, and then again – with the buffer with no triton until the optical absorption disappeared. Elution was carried out with NaCl linear gradient with the concentrations from 0 to 1 M

in 20 mM tris-HCl, pH 7.5. The obtained fractions were dialyzed against 10 mM tris-HCl, pH 7.5 at 4° C during 16 hours.

Unlike the general method of isolating MdLF, the heparin sepharose column had been preliminary treated with 0.2–0.3% formaldehyde solution in order to oxidize minor functional groups of heparin, which leads to an increase in its negative charge and, as a result, increases it affinity in relation to lactoferrin.

The molecular mass of lactoferrin was measured with DC-Na-polyacrylamide gel electrophoresis according to Schägger and von Jagow's method with the use of 8% acrylamide gel. The molecular mass was also evaluated upon gel filtration results.

## RESULTS

The isolated MdLF is glucoprotein with the molecular mass of about 84,000, the agarose diffusion coefficient of $3.1 \times 10^{-7}$ $cm^2$ $sec^{-1}$ and the relative electrophoretic mobility of 0.42. LFP is characterized by low hydrophobicity and elutes from phenyl-Sepharose with 0.4 ammonium sulphate. Its isoelectric point equals to 9.2.

Despite its immunochemical identity with human milk lactoferrin, MdLF differs from lactoferrin by some of its physiochemical parameters. The difference is especially evident in the electrophoretic mobility of MdLF and human milk lactoferrin. According to the obtained data, the MdLF relative electrophoretic mobility is 0.41±0.006, while the one of human milk lactoferrin is 0.47±0.003. These differences may be considered another evidence of the presence of several isoforms of lactoferrin that have the same molecular mass and are immunochemically similar but differ in their affinity to different ionogenic groups.

Dissociation of the LF and $Fe^{3+}$complex was examined with two methods — at low concentrations of chelating anions (Pe^ dissociation synchronically happens from two iron-binding centres of LF) and at high concentrations of chelating anions ($Fe^{3+}$ dissociation from two iron-binding centres happens separately). Iron-saturated LF (chololactoferrin) has a typical visible absorption spectrum. The spectra appeared to be similar for the bacteria and human proteins. No difference in the bacteria and human proteins was identified in the experiments on the LF and $Fe^{3+}$ complex dissociation. Summary data on the MdLF physicochemical properties are presented in Table 1.

*Table 1. MdLF physicochemical properties*

| Properties | LFP |
|---|---|
| Molecular mass | 85,000±5,000 |
| Relative electrophoretic mobility in agar | 0.42±0.005 |
| Agarose diffusion coefficient, $cm^2$ $sec^{-1}$ | $3.1 \times 10^{-7}$ |
| Hydrophobicity | Elution from phenyl-Sepharose with 0.4 ammonium sulphate |
| Precipitation with ammonium sulphate, % saturation and precipitation range | 50–75 |
| Full precipitation | 75 |
| Precipitation with 50% ethanol | No precipitation |
| Precipitation with 60% acetone | Full (reversible) |
| Precipitation with 0.9M chloric acid | Irreversible |
| Isoelectric point | 9.2 |
| Thermal resistance | Resistant at 65° C — 30 minutes |

## CONCLUSION

Modern research allows to state that iron is a universal factor of microbial growth. Being in the environment, iron is exposed to oxidation and hydrolysis processes, leading to a decrease in the concentration of $Fe^{2+}$ and $Fe^{3+}$ free ions down to $10^{-9}$–$10^{-18}$ M, which is not sufficient for optimal vital activity of most of the microorganisms. Likewise, iron is bound to iron-binding proteins in the mammal organisms and is unavailable for microorganisms. That is why microorganisms have evolved ways of getting iron in iron-deficiency conditions, which is important for optimal development in environment and, in certain cases, may be critical for survival of the population. In this case, microbial strategies for getting iron, including MLF synthesis, can be considered as one of the factors that determine the formation and stability of microbial associations, playing an important role in the functioning of normal and pathological microbiocenoses in the host.

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