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Identification and Isolation from Breast Milk of Ceruloplasmin–Lactoferrin Complex

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Abstract—The presence of a complex of the copper-containing protein ceruloplasmin (Cp) with lactoferrin (Lf) in breast milk (BM) is shown for the first time. In SDS-free polyacrylamide gel electrophoresis (PAGE), electrophoretic mobility of Cp in BM is lower than that of plasma Cp, coinciding with the mobility of the complex obtained upon mixing purified Cp and Lf. Affinity chromatography of delipidated BM on Cp-Sepharose resulted in retention of Lf. SDS-PAGE of the 0.3 M NaCl eluate revealed a single band with M_r ~ 78,000 that has the N-terminal amino acid sequence of Lf and reacts with antibodies to that protein. Synthetic peptides R-R-R-R (the N-terminal amino acid stretch 2-5 in Lf) and K-R-Y-K-Q-R-V-K-N-K (the C-terminal stretch 29-38 in PACAP 38) caused efficient elution of Lf from Cp-Sepharose. Cp–Lf complex from delipidated BM is not retained on the resins used for isolation of Cp (AE-agarose) and of Lf (CM-Sephadex). Anionic peptides from Cp—(586-597), (721-734), and (905-914)—provide an efficient elution of Cp from AE-agarose, but do not cause dissociation of Cp–Lf complex. When anti-Lf is added to BM flowed through CM-Sephadex, Cp co-precipitates with Lf. Cp–Lf complex can be isolated from BM by chromatography on CM-Sephadex, ethanol precipitation, and affinity chromatography on AE-agarose, yielding 98% pure complex. The resulting complex Cp–Lf (1 : 1) was separated into components by chromatography on heparin-Sepharose. Limited tryptic hydrolysis of Cp obtained from BM and from blood plasma revealed identical proteolytic fragments.

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The complex of ceruloplasmin (Cp, ferro-O_2-oxidoreductase, EC 1.16.3.1) and lactoferrin (Lf) *in vitro* was first characterized by us [1, 2]. Formation of the Cp–Lf complex *in vivo* was also evidenced after injection of human Lf into the rat bloodstream [1]. On account of the selectivity of the interaction between the exogenous Lf and rat Cp that competed with other plasma proteins, we suggested that the Cp–Lf complex might exist in those biological fluids of an organism where both proteins are present.

Both Cp and Lf are present in breast milk (BM), which provide nutrition and host defense of a developing organism [3]. A probable function of the Cp–Lf complex in BM may be its participation in iron metabolism. Our latest studies showed that only Cp and the Cp–Lf complex in BM possess ferroxidase activity [4]. Therefore, formation of the complex between ferroxidase (Cp) and a protein from the transferrin family (Lf) is probably nonrandom because Lf can amplify the ferroxidase activity of Cp. The Cp–Lf complex is very likely to accomplish its functions in the period of early breast feeding, when the gastric pH of a newborn is close to 5-6 that does not prevent the complex formation.

Lf is a glycoprotein with M_r ~ 78,000, a member of the transferrin family. It is capable of efficient binding of

Abbreviations: BM) breast milk; Cp) ceruloplasmin; Lf) lactoferrin; PACAP 38) pituitary adenylate cyclase activating peptide.

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iron, copper, and other metal ions. The average concentration of Lf in BM is 3-6 mg/ml, which is about 25-43% of the total protein in BM depending on lactation period [3, 5]. Apart from milk, Lf has been found in secretory granules of neutrophilic leukocytes and in such secretions as saliva, tears, and seminal fluid. In the period of rearing Lf suppresses the development of pathogenic microorganisms (*Helicobacter pylori*); moreover, in the gastrointestinal tract of newborns Lf is hydrolyzed by pepsin resulting in production of lactoferricin, which has a stronger bactericidal effect than that of intact Lf. It has been also proved that Lf is a growth factor for the gastrointestinal tract cells of newborns [5].

Cp is a multifunctional copper protein of blood plasma where its concentration is 0.35-0.50 mg/ml. Its content in milk is substantially lower, being 0.005-0.05 mg/ml in the early lactation period [6]. Cp is referred to as an acute phase protein; it exhibits ferroxidase activity and acts as an antioxidant. Human Cp is extremely susceptible to proteolysis. For instance, subjected to tryptic hydrolysis, intact Cp with M_r ~ 132,000 is split into fragments with molecular weights 110, 92, 67, 52, 49, 28, 25, 19, and 18 kD [7].

Studies of Cp in BM are complicated because its low content makes it difficult to obtain sufficient amounts of the protein. The results obtained in studies of Cp from the milk of other mammalian species cannot always be extrapolated to the properties of the human protein due to significant differences in milk composition. It is known that Cp of BM is synthesized by mammary gland cells, but it is not taken up from a woman's bloodstream [8]. Milk Cp does not differ from blood Cp as judged by Western-blotting and mRNA analysis [9]. Some data show that milk and blood Cp are dissimilar in their interactions with lectins and in sensitivity to EDTA [10].

The aim of the present study was to identify Cp–Lf complex in BM, to elaborate a method of its purification, and to determine some its characteristics.

MATERIALS AND METHODS

In this study we used pre-stained molecular weight markers (BioRad, USA); cyanogen bromide (BrCN) (Fluka, Switzerland); chloroethylamine, EDTA, epichlorohydrine, triethylamine ($(C_2H_5)_3N$) (Merck, Germany); CM-Sephadex C-50, CM-Sepharose, Sephadex G-75 Superfine, Sepharose 4B, Sepharose 6B (Pharmacia, Sweden); acrylamide, arginine, medinal, methylene-bis-acrylamide, tetramethylethylene diamine, veronal (Reanal, Hungary); ammonium persulfate, complete and incomplete Freund's adjuvant, glycerol, Coomassie R-250, 2-mercaptoethanol, molecular weight markers for gel filtration (2000, 450, 240, 160, and 67 kD), sodium azide (NaN_3), Tris (Serva, Germany); glycine, *o*-dianisidine, phenylmethylsulfonyl fluoride (PMSF), salmon protamine, SDS (Sigma, USA); heparin (Spofa, Poland); Toyopearl HW-55 Fine (Toyo Soda, Japan). PBS (phosphate buffer saline) contained 0.15 M NaCl, pH 7.4, 1.9 mM Na_2HPO_4/8.1 mM NaH_2PO_4. Sodium acetate buffer (0.1 M) contained 0.089 M AcONa/0.011 M AcOH for pH 5.5, 0.0948 M AcONa/0.0052 M AcOH for pH 6.0, and 0.09977 M AcONa/0.000023 M AcOH for pH 7.4.

Peptides R-R-R-R, R-K-V-R, K-R-Y-K-Q-R-V-K-N-K, H-A-G-M-E-T-T-Y-T-V, D-Q-V-D-K-E-D-E-D-F-Q-E, E-V-E-W-D-Y-S-P-Q-R-E-W-E, and D-E-N-E-S-W-Y-L-D-D synthesized by the solid phase method (Scientific Research Institute of Extra Pure Preparations, St. Petersburg) had 99.5% purity according to HPLC and amino acid assay.

To purify Cp and identify Cp–Lf complex, we used BM samples of 32 women (1-7 days of lactation) kindly granted by Maternity Hospital No. 6, St. Petersburg. For delipidation of BM samples we centrifuged those at 500*g* (4°C) for 10 min, discarded the precipitate and the upper lipid layer, centrifuged the supernatant at 15,000*g* (4°C) for 10 min, and discarded the upper lipid layer.

Lf was purified from BM using ion-exchange chromatography on CM-Sepharose and gel filtration on Sephadex G-75 Superfine [1]. Cp preparation was isolated from blood plasma by affinity chromatography on protamine-Sepharose [11]. Cp, heparin, and protamine were immobilized on BrCN-activated Sepharose 4B [11]. AE-Agarose was obtained by treatment of Sepharose 6B with epichlorohydrine and 2-cloroethylamine [12].

Concentrations of purified proteins were measured spectrophotometrically using the molar extinction coefficients a_{280} = 1.61 ml/mg per cm and a_{610} = 0.0741 ml/mg per cm for Cp [13, 14] and a_{280} = 1.46 ml/mg per cm for Lf [15].

Molecular mass of proteins and of Cp proteolytic fragments were determined using SDS-PAGE [16]. After PAGE in SDS-free gel [17], Cp was visualized by staining with *o*-dianisidine [18] or in reaction with $K_4[Fe(CN)_6]$ for ferroxidase activity [4]. Antibodies against Cp, Lf, and serum albumin were obtained after three consecutive immunizations of rabbits with the respective proteins [19]. To concentrate proteins we used an Amicon cell with a Diaflo PM 10 filter.

Cp and Lf concentrations were assessed using rocket immunoelectrophoresis [20]. Ratio error among three parallel measurements was less than 2%. Total protein content in samples was measured according to Lowry–Folin [21], and ratio error in three parallel measurements was less than 5%.

Immunoprecipitation of Cp and Lf from BM. Portions of 50, 100, 500, or 1000 μg of anti-Lf or anti-albumin in 100 μl PBS were added to 100 μl of delipidated BM flowed through a column with CM-Sephadex, after which the samples were incubated at 37°C for 2 h. Then 100 μg of donkey antibodies against rabbit IgG were

added in 50 μl PBS and the samples were incubated at 37°C for 12 h. Immune complexes were precipitated by 1 h of centrifugation at 15,000*g* (4°C). Cp and Lf content in the supernatant was assessed by rocket immunoelectrophoresis. Samples with anti-albumin served as the control of nonspecific protein precipitation in the donkey IgG–rabbit IgG complexes. The absence of nonspecific precipitation of Cp with anti-Lf and anti-albumin was checked by adding these antibodies to Cp in PBS (0.05 mg/ml) and to the blood plasma.

Affinity chromatography of BM on Cp-Sepharose. Five milliliters of delipidated BM was applied on a column containing 1 ml of Cp-Sepharose equilibrated with PBS. The column was washed with PBS until A_{280} in the eluate became less than 0.01. The column was eluted with 2 ml of 0.3 M NaCl and the sample obtained was dialyzed against PBS. To study the competitive dissociation of the Cp–Lf complex by synthetic peptides, 5 ml of skimmed BM or 1 mg of Lf in PBS were applied on columns, each containing 1 ml of Cp-Sepharose, after which those were washed with PBS to obtain A_{280} in the eluate less 0.01. Then 1 ml of 0.1 mM peptide solution in PBS (or solely PBS for the control) was flowed through each column, which was followed by washing with 1 ml of 0.3 M NaCl.

N-Terminal amino acid Edman assay was carried out at the Institute of Bioorganic Chemistry (Moscow). After SDS-PAGE, the protein was transferred to Immobilon P membrane [22].

Limited trypsin hydrolysis was carried out at 37°C with protein to protease mass ratio 100 : 1. The reaction was stopped either after 5 min or after 1 h by adding PMSF to 0.1 mM. Samples were boiled for 2 min in the sample application buffer used in SDS-PAGE.

Isolation of the Cp–Lf complex from BM. The complex was isolated from 100 ml of BM collected on the 1st-7th day of lactation. Lf excess was removed by chromatography on a column (2.5 × 10 cm) with CM-Sephadex. Judging by the rocket immunoelectrophoresis, about 1/8 of the total milk Lf was not absorbed on the resin. Thus treated, BM was subjected to ethanol precipitation: an equal volume of 96% ethanol was added to BM at 4°C and after 30 min incubation the mixture was centrifuged at 10,000*g* (4°C) for 1 h. Again an equal volume of ethanol was added to the supernatant where Cp and Lf had been detected immunologically, after which incubation and centrifugation were repeated. The precipitate obtained was dissolved in 10 ml PBS, Cp and Lf being detected in the solution. Undissolved proteins were removed by 30 min of centrifugation at 10,000*g* (4°C). The next step was subjecting the proteins of the supernatant to affinity chromatography on a column (1 × 2 cm) with AE-agarose equilibrated with PBS. Upon sample application, the column was washed with PBS to allow A_{280} of eluate arrive at less than 0.01. Then the proteins were eluted with 0.5 M NaCl (pH 7.4). Summarized data on Cp–Lf complex isolation are presented in the table.

RESULTS AND DISCUSSION

Electrophoretic mobility of Cp within BM revealed by staining with *o*-dianisidine was less than that of purified Cp or of Cp in blood serum (Fig. 1, lanes *1*, *4*, and *7*). This mobility coincides with that of Cp contained in the Cp–Lf complex formed upon mixing the two purified proteins (Fig. 1, lane *2*). The changes in Cp mobility occurred also when Cp was added to BM, LF was added to blood serum, or when the latter was mixed with BM, which evidences the interaction of Cp with Lf (Fig. 1, lanes *3*, *5*, and *6*). Staining the gel containing these samples with $K_4[Fe(CN)_6]$ to detect the ferroxidase activity revealed the mobility of Cp equal to that in the gel shown in Fig. 1. We examined 32 BM samples (on the 1st-7th day of lactation) using this method and found the Cp–Lf complex in every sample.

It was previously speculated that an altered mobility of Cp in BM results from its microheterogeneity in milk, by existence of protein isoforms, by the presence of lipids in BM, or by alterations of the protein molecule charge upon binding additional copper ions [23, 24]. In our

Stages of purification of the Cp–Lf complex from BM

Stages and fractions	Total amount in the fraction			Purification degree of Cp	Cp yield, %
	Cp, mg	Lf, mg	protein, mg		
Skimmed BM	1.05 ± 0.01	280 ± 5	1120 ± 20	1	100
PBS eluate from CM-Sephadex	0.80 ± 0.01	32 ± 4	650 ± 5	1.3	76
After ethanol precipitation	0.70 ± 0.01	0.58 ± 0.01	120 ± 5	6.2	67
0.5 M NaCl eluate from AE-agarose	0.65 ± 0.01	0.38 ± 0.01	1.05 ± 0.01	660	62

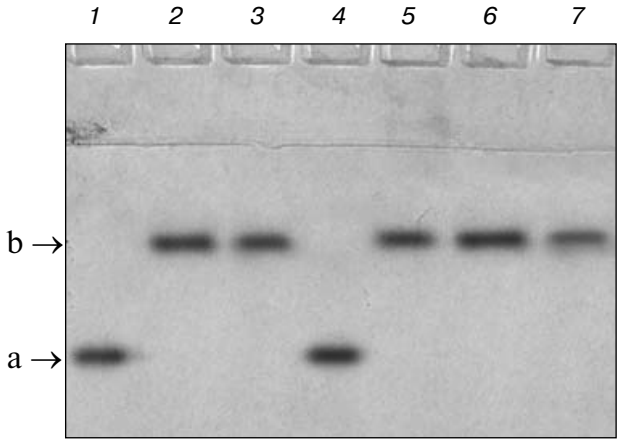

Fig. 1. Formation of the Cp–Lf complex visualized by non-denaturing PAGE with *o*-dianisidine staining: *1*) 1 μg Cp (level *a*); *2*) 1 μg Cp + 1.2 μg Lf (level *b*); *3*) 1 μg Cp + 5 μl BM; *4*) 5 μl blood serum; *5*) 5 μl blood serum + 1.2 μg Lf; *6*) 5 μl blood serum + 5 μl BM; *7*) 40 μl BM.

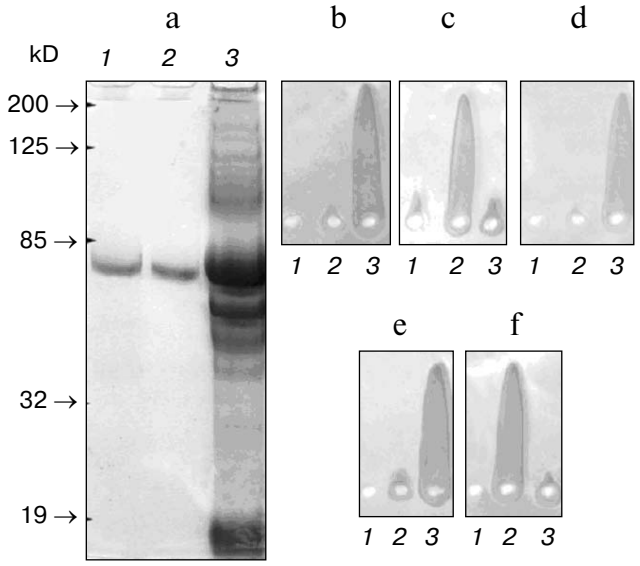

Fig. 2. Results of BM chromatography on Cp-Sepharose. a) SDS-PAGE (Coomassie R-250 staining): *1*) elution with 0.3 M NaCl in BM chromatography on Cp-Sepharose; *2*) Lf, 5 μg; *3*) BM, 5 μl. b-f) Rocket immunoelectrophoresis in antibodies against Lf (0.4 μg per ml of agarose, Coomassie R-250 staining): *1*) elution with PBS from Cp-Sepharose; *2*) elution with peptide-free PBS (b) and with 0.1 mM solutions of R-R-R-R (c), R-K-V-R (d), H-A-G-M-E-T-T-Y-T-V (e), K-R-Y-K-Q-R-V-K-N-K (f); *3*) elution with 0.3 M NaCl.

experiments, Cp interacted essentially with Lf, and other proteins of blood plasma and of BM would not prevent the complex formation. Adding Cp to BM caused solely a more intense staining of the retarded band rather than appearance of new oxidase-positive bands. The most likely explanation is that a new portion of Cp–Lf complex was formed with the mobility slower than that of the native Cp (Fig. 1). Thus, the Cp-containing band with slower mobility in PAGE is the Cp–Lf complex, but not an isoform of Cp.

Affinity chromatography of skimmed BM on Cp-Sepharose showed that Lf interacts with the resin and is eluted with 0.3 M NaCl. Its presence in the eluate was ascertained by rocket immunoelectrophoresis with anti-Lf (Fig. 2b). SDS-PAGE of the eluate revealed a single protein band with molecular weight 78 kD (Fig. 2a). The N-terminal amino acid sequence of the protein in this band (^{1}G-R-R-R-R-S-V^{7}) is identical to the amino acid stretch 1-7 of Lf.

Indeed, affinity chromatography of BM on Cp-Sepharose resulted in selective retention of Lf. A number of complexes formed by Lf with proteins of BM have been described, among which are: lysozyme [25], secretory IgA [26], casein, α-lactalbumin, and albumin [27]. The presence of these proteins did not prevent Lf from interaction with the added Cp and with Cp-Sepharose perhaps due to the fact that its concentration in milk is some 300 times higher than that of Cp.

We used synthetic peptides R-R-R-R and R-K-V-R, that match two N-terminal amino acid stretches in the Lf sequence (2-5 and 28-31), and decapeptide H-A-G-M-E-T-T-Y-T-V corresponding to the stretch 1028-1037 in the Cp sequence for competitive displacement of Lf bound to Cp-Sepharose. The column with adsorbed Lf was eluted with 0.1 mM solutions of the peptides in PBS. Efficient release of Lf occurred upon elution with R-R-R-R solution (Fig. 2c). The fragment ^{29}K-R-Y-K-Q-R-V-K-N-K^{38} of neuropeptide PACAP 38 (pituitary adenylate cyclase activating peptide) responsible for the selective interaction of PACAP 38 with Cp [22] also efficiently removed Lf from Cp-Sepharose (Fig. 2f).

We demonstrated previously that heparin causes dissociation of Cp–Lf complex *in vitro* [2]. Heparin mostly interacts with the N-terminal arginine stretch ^{2}R-R-R-R^{5} in Lf [28], though ^{28}R-K-V-R^{31} is also involved in the interaction [29]. In the present study, we demonstrate an efficient dissociation of Cp–Lf complex caused by peptide R-R-R-R. Taken separately, R-K-V-R caused no dissociation. One may suggest that dissociation of Cp–Lf complex by R-R-R-R resulted from the competition of the peptide both with ^{2}R-R-R-R^{5} and with ^{28}R-K-V-R^{31} stretches in Lf on account of their spatial proximity. Most likely R-K-V-R is not a strong competitor with heparin for the stretch ^{2}R-R-R-R^{5} in Lf.

The peptide H-A-G-M-E-T-T-Y-T-V that corresponds to the amino acid stretch 1028-1037 in Cp sequence and prevents the interaction of Cp both with protein C [30] and with ferritin [31] did not cause dissociation of the Cp–Lf complex. This suggests that the Lf-binding site in Cp is different from the contact sites for protein C and ferritin.

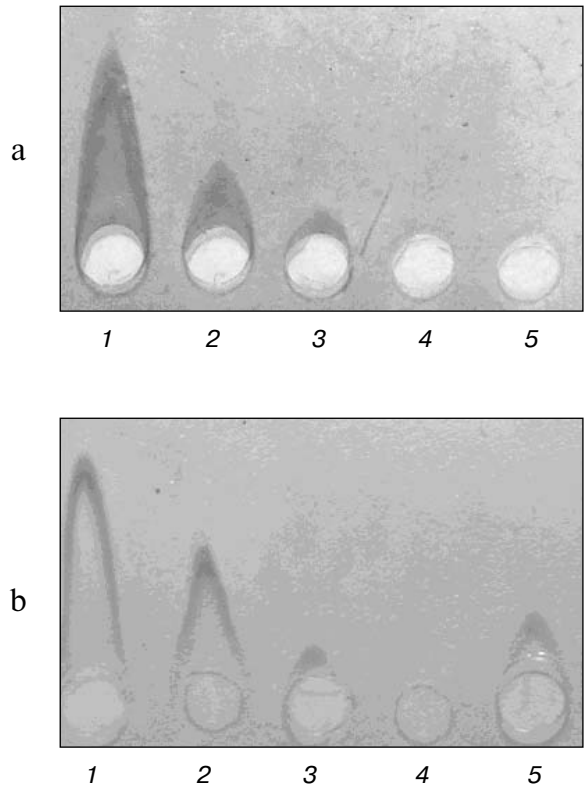

Fig. 3. Precipitation of Cp and Lf from BM flowed through CM-Sephadex. Rocket immunoelectrophoresis in antibodies against Lf (0.4 μg per ml agarose, 5 μl of sample, Coomassie R-250 staining) (a) and in antibodies against Cp (0.2 μg per ml agarose, *o*-dianisidine staining, 40 μl of sample) (b): *1*) BM (PBS added instead of antibodies); *2-5*) BM after adding of 50 μg (*2*), 100 μg (*3*), 500 μg (*4*), and 1 mg (*5*) of antibodies against Lf.

The fragment ^{29}K-R-Y-K-Q-R-V-K-N-K^{38} of neuropeptide PACAP 38 efficiently prevented interaction of Cp with Lf complex (K_d = 1.8 μM [1]), which is indicative of the likely competition between this fragment and Lf for the contact site on the Cp molecule. The capability of the PACAP 38 fragment to prevent interaction of Cp and Lf seems to result from the lower dissociation constant for the interaction of Cp with ^{29}K-R-Y-K-Q-R-V-K-N-K^{38} in PACAP 38 (K_d = 3.4 nM). It was shown previously that of all blood proteins PACAP 38 selectively interacts only with Cp [22].

Anionic peptides mimicking the amino acid stretches in Cp D-Q-V-D-K-E-D-E-D-F-Q-E (586-597), E-V-E-W-D-Y-S-P-Q-R-E-W-E (721-734), and D-E-N-E-S-W-Y-L-D-D (905-914) did not provoke dissociation of the Cp–Lf complex. Meanwhile, 0.1 mM solution of each peptide could efficiently elute Cp from AE-agarose (data not shown), an affinity resin for purification of Cp from blood plasma. It seems likely that amino acid stretches in Cp interacting with Lf and with AE-agarose do not overlap, which allows the adsorption of the complex without its dissociation.

Co-immunoprecipitation of Cp and Lf from BM. Lf concentration in BM is 300 times higher than that of Cp. The latter is not retained on AE-agarose when skimmed BM is flowed (as judged by the results of rocket immunoelectrophoresis and PAGE under non-denaturing conditions). The major part of Lf is retained on CM-Sephadex when BM is applied, while Cp virtually does not interact with the resin. Cp and Lf concentrations are closer in the fraction that does not interact with CM-Sephadex (0.015 and 0.60 mg/ml, respectively). Adding antibodies against Lf to this fraction resulted in complete precipitation of Lf and in co-immunoprecipitation of Cp. Upon complete precipitation of Lf, an increase in the amount of anti-Lf

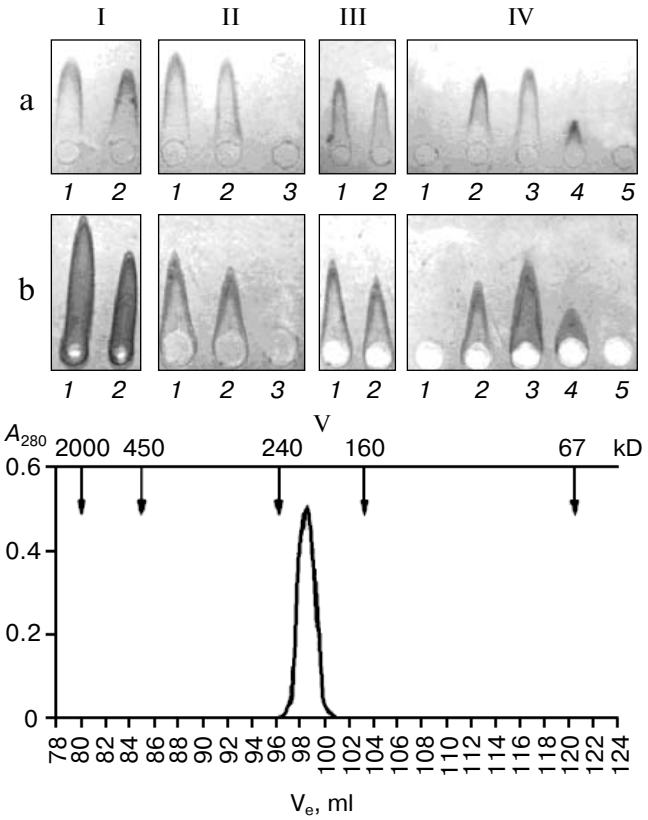

Fig. 4. Identification of Cp and Lf in samples during isolation of Cp–Lf complex from BM. Rocket immunoelectrophoresis in antibodies against Cp (0.2 μg per ml agarose, *o*-dianisidine staining) (a) and in antibodies against Lf (0.4 μg per ml agarose, Coomassie R-250 staining) (b). Panel I: *1*) 5 μl of BM before applied on CM-Sepharose ((b) dissolved 5 times); *2*) 5 μl of BM after CM-Sepharose. Panel II: *1*) 40 μl of supernatant upon ethanol precipitation 1 : 1; *2*) 5 μl of solubilized precipitate upon ethanol precipitation 1 : 2; *3*) 40 μl of supernatant upon ethanol precipitation 1 : 2. Panel III: *1*) eluate 0.5 M NaCl from AE-agarose; *2*) 0.1 μg Cp and 0.06 μg Lf (Cp/Lf, 1 : 1). Panel IV: *1-5*) 40 μl sampled from fractions corresponding to the volume of 96, 97, 98, 99, and 100 ml of eluate during gel filtration of the Cp–Lf complex on Toyopearl HW-55, respectively. Panel V: elution profile upon gel filtration of the Cp–Lf complex on Toyopearl HW-55 (1 × 150 cm), elution peak maximum is V_e = 98 ml, arrows indicate V_e of molecular mass markers.

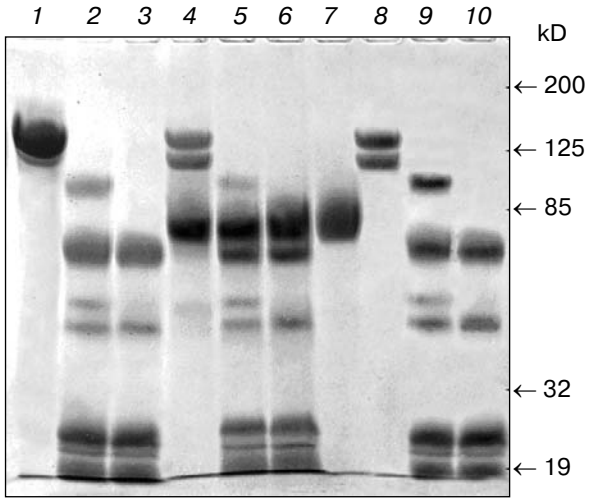

Fig. 5. SDS-PAGE of the products obtained upon limited proteolysis of Cp with trypsin (protease/protein = 1 : 100, Coomassie R-250 staining): *1*) plasma Cp, 25 μg; *2*) plasma Cp, 25 μg, after 5 min of proteolysis; *3*) plasma Cp, 25 μg, after 1 h of proteolysis; *4*) Cp–Lf from BM, 25 μg; *5*) Cp–Lf from BM, 25 μg, after 5 min of proteolysis; *6*) Cp–Lf from BM, 25 μg, after 1 h of proteolysis; *7*) Lf, 25 μg, after 1 h of proteolysis; *8*) Cp from BM (void volume from heparin-Sepharose), 25 μg; *9*) Cp from BM, 25 μg, after 5 min of proteolysis; *10*) Cp from BM, 25 μg, after 1 h of proteolysis.

in a sample did not cause the complete precipitation of Cp from BM (Fig. 3). Adding antibodies against serum albumin to the fraction under study did not result in precipitation of either Cp or Lf.

Precipitation of Cp from BM by anti-Lf clearly indicates that the Cp–Lf complex is present in BM. We observed in our previous studies that the complex formed *in vitro* dissociates when affected by anti-Cp and anti-Lf in PAGE under non-denaturing conditions and in rocket immunoelectrophoresis [2]. This can be explained by the partial coincidence of the protein epitopes with the sites of their reciprocal contact. That is a likely reason why an excess of anti-Lf did not cause the complete precipitation of Cp from BM.

Characterization of Cp–Lf complex isolated from BM. CM-Sephadex did not retain the Cp–Lf complex during its isolation (Fig. 4, I). The interaction of Cp and Lf was not altered when the proteins were precipitated by ethanol, a chaotropic agent (Fig. 4, II). The ratio Cp/Lf in the eluate collected from AE-agarose was 1 : 1 as judged by the results of rocket immunoelectrophoresis (Fig. 4, III). We obtained a 98% pure complex. The yield of Cp was 62% of its amount in crude BM (table).

Upon subsequent gel filtration on a column with Toyopearl HW-55 Fine (1 × 150 cm), the Cp–Lf complex was eluted as a single peak (Fig. 4, IV and V). It had M_r 220,000 ± 10,000 according to the calibration data. SDS-PAGE revealed 130- and 110-kD bands of Cp and 78-kD band of Lf (Fig. 5, lane *4*). In our previous studies, we observed *in vitro* formation of the Cp–Lf complexes with various stoichiometries of the components. For instance, in PAGE under non-denaturing conditions the ratios Cp/Lf were 1 : 2 and 1 : 4 [1], while gel filtration on Sephacryl S-300 produced a single complex with Cp/Lf ratio 1 : 1 [32]. During isolation of the Cp–Lf complex from BM the relation of the proteins within the complex approached 1 : 1 and remained such in the preparation of the purified complex, the molecular mass of which (220 kD) corresponds to the sum of 132 and 78 kD.

The Cp–Lf complex isolated from BM dissociated during chromatography on heparin-Sepharose (0.5 × 1 cm) as did the complex obtained upon mixing the two purified proteins. Lf was retained on the column, while Cp was eluted in the void volume. This result was expected as we had shown previously that under the influence of heparin the Cp–Lf complex dissociates *in vitro* [2].

Denaturing PAGE with SDS revealed the identity of the tryptic fragments of Cp from BM to those of Cp from blood plasma. Tryptic hydrolysis of the Cp–Lf complex obtained from BM produced proteolytic fragments of Cp, but the 78-kD band of Lf remained (Fig. 5). This favors the notion about the identical polypeptide chains in Cp isolated both from BM and from blood plasma, and does not support the concept of microheterogeneity of Cp in BM [10]. Greater resistance of Cp isolated from BM to proteolysis upon storage [24] is likely to result from the absence of admixtures of blood plasma-specific proteases that split the purified Cp preparations during storage [11].

The results are convincing proofs of the existence of a complex of Cp and Lf during early terms of lactation. The question of its function remains open. The given data clearly indicate that the Cp in the complex saves ferroxidase activity. Electrostatic interactions play a role in formation of the complex. Heparin and a fragment of PACAP specifically interact according to Lf and Cp and cause its dissociation. However, in the absence of dissociating reagents the complex is stable enough and can be isolated from BM as a highly purified preparation (with molar ratio of Cp–Lf, 1 : 1), suitable for the further research.

The results provide a number of convincing arguments favoring the existence of Cp–Lf complex in BM at early stages of lactation. The question about its function remains open and requires further investigation.

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