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Chapter 4

Human Milk Lactoferrin and Antibodies: Catalytic Activities, Complexes, and Other Features

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Additional information is available at the end of the chapter

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Abstract

Human milk is a source of biologically active proteins, including lactoferrin (LF) and antibodies (Abs). These proteins are considered as the most polyfunctional proteins of human milk. Apparently, human milk is not a simple mixture of proteins and peptides: recently it was shown that human milk contains stable supramolecular protein complex, composed of LF, α-lactalbumin, milk albumin, β-casein, IgG, and sIgA molecules. We believe that the whole set of different biological functions of the individual milk proteins is significantly supplemented by features of their complexes.

Keywords: antibodies, catalytic antibodies, Fab arm exchange, lactoferrin, protein complexes

1. Introduction

Human milk not only has nutritional value but also is a source of biologically active proteins, including lactoferrin (LF) and antibodies (Abs). LF and Abs are often considered as the most polyfunctional proteins of human milk. LF is an iron-binding glycoprotein and is assumed to play a key role in iron uptake by the intestinal mucosa; it also keeps iron from iron-requiring bacteria acting as a bacteriostatic agent. Major immunoglobulin of human milk is sIgA; besides that milk contains significant amounts of IgG, along with conventional Abs, human milk contains anti-idiotypic Abs capable of enhancing infant antibody response. Maternal milk Abs coat infant mucosal surfaces, play protective role, and also catalyze various chemical reactions (so-called abzymes). Recently it was shown that human milk contains stable supramolecular protein

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complex, which is composed of LF, α-lactalbumin, milk albumin, β-casein, IgG, and sIgA molecules. Here we describe the biological roles of LF and Abs as the major multifunctional proteins of human milk.

2. Human milk lactoferrin and its known functions

LF is a single polypeptide chain of 76–80 kDa containing two lobes [1] either of which binds one Fe^{3+} ion and contains one glycan chain [2]. LF was first isolated and purified from bovine and human milk [3–5]; then LF was described in human epithelial secretions and barrier body fluids [6–9]. The concentration of LF in human milk (~1 mg/ml) is much higher comparing to cow's milk (up to 0.01 mg/ml) [10]. Considering that LF binds iron [4], it was hypothesized that LF transports iron in the infant gut. In 1991 the LF receptor specifically binding and transporting LF was identified on the gut mucosa cells [11, 12]. Since there is no evidence that iron then enters the bloodstream, it can be concluded that LF does not facilitate iron absorption in the infant gut [13].

Many different functions have been attributed to LF: immunomodulation and cell growth regulation [9, 14], protection from iron-induced lipid peroxidation, DNA binding [9], RNA hydrolysis [15, 16], and transcriptional activation of specific DNA sequences [17, 18]. LF also activates natural killer cells [19] and may be effective in antitumor defense, which is not dependent on iron [14, 20]. It was shown that LF induces granulopoiesis [21], cytotoxicity mediated by Abs [22], production of cytokines [23], and growth of some cells in vitro [24]. The role of LF in physiology of these activities is not yet clear, but there are suggestions that LF is effective for primary defense against viral and microbial infections [6, 8]. The highest concentration of LF is usually detected in the inflammatory sites since LF is known as a protein of the acute phase. The blood of newborn babies contains LF during several hours after feeding and it was shown that LF easily penetrates plasmatic and nuclear membranes of the cells [25]. Protective activity of LF against viral and microbial diseases may increase the newborns' passive immune system. Since the removal of iron from bacteria eliminates this important microelement which is ultimately needed for the proliferation of microflora, it was initially suggested that the antimicrobial properties might be attributed to LF iron-binding capacity [26]. Surface receptors for LF are expressed on many microorganisms that may explain different iron-independent antimicrobial and antiviral properties of LF [27, 28] in which mechanisms are still unknown.

It has been proposed that the existence of several oligomeric forms of LF may provide polyfunctional properties of this relatively small protein. The oligomerization and dissociation of LF may be under control of specific ligands such as ATP and others [29, 30]. There were shown some peculiarities of LF molecule ATP-binding site: interaction of the protein with ATP leads to changes of LF interaction with DNA, proteins, and polysaccharides [30]. Further it was demonstrated that LF possesses two DNA-binding sites which interact with specific and nonspecific DNAs in an anti-cooperative manner and may coincide or overlap with the known polyanion-binding and antimicrobial domains of the protein [31].

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3. Oligomerization of human milk lactoferrin

It has been reported that at physiological concentrations of NaCl and KCl LF in solution predominantly exist in two forms—monomeric and tetrameric [19, 32]. However, LF oligomerization has been so far studied by gel filtration using polysaccharide sorbents [19, 32], but it is known that LF can efficiently interact with polysaccharides [33]. Therefore, LF can bind to such polysaccharide sorbents and as a result may be eluted only with buffers with high ionic strength which promote dissociation of LF oligomers [34]. Thus, gel filtration is inadequate for studying LF and may lead to improper conclusions about the state of LF in solution in the presence or absence of salts.

The analysis of the LF oligomerization by gel filtration [35] has shown that the LF monomer and oligomer interactions with resins are remarkably weaker in case of Sepharose 4B. Prior to gel filtration, the solutions of lyophilized LF (3 mg/ml) were incubated in the absence or in the presence of different ligands and 0.1–0.15 M KCl for 10 days at 20°C. Gel filtration of LF preincubated in 50 mM Tris-HCl pH 7.5 on the Sepharose 4B column resolves the LF sample into several fractions (**Figure 1A**, curve 1) demonstrating small reproducible peak at ~800 kDa presumably containing LF decamers, major wide peak at ~310–275 kDa (a tetramer), and small peaks at ~150 kDa (a dimer) and at ~70 kDa (a monomer).

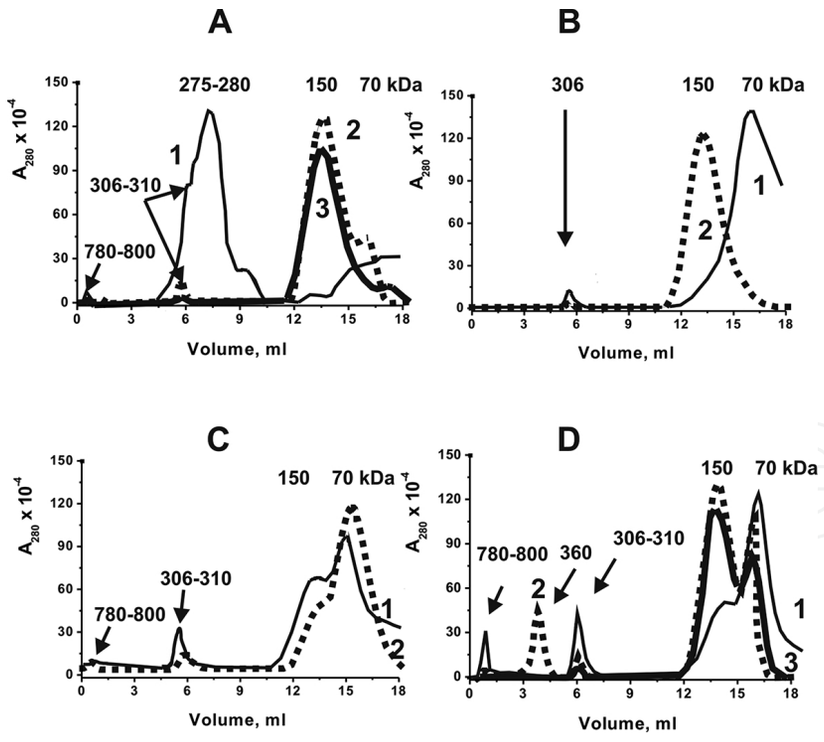

Figure 1. Gel filtration analysis of LF oligomeric forms on Sepharose 4B. Prior to gel filtration, LF was incubated for 20 days at 22°C in 50 mM Tris-HCl (pH 7.5) in the absence of other ligands (A) or in the presence of 5 mM oligosaccharide (B), 50 mM ATP (C), or 50 mM AMP (D). The reaction mixtures containing no salt (curve 1), containing 0.1 M (curve 2), or 0.15 M KCl (curve 3) were used. The column was equilibrated and LF was eluted with the buffer used for the LF preincubation.

The addition of 0.1–0.15 M KCl to the buffers leads to the significant decrease of the LF oligomer forms (**Figure 1A**, curves 2 and 3). One can suppose that absence of KCl leads to nonspecific absorbing of dimer and monomer LF forms on the Sepharose 4B resin [35]. Preincubation of LF in the presence of 0.1 M KCl prior to gel filtration leads to complete dissociation of the tetramer form (**Figure 1B**, curve 2) with the most of LF fraction being a dimer. The gel filtration profile of LF sample incubated with 50 mM ATP in the presence and in the absence of KCl indicates that such conditions are favorable either for decamer (~800 kDa) and for tetramer (~306 kDa) forms and especially favorable for the dimer and monomer forms of LF (**Figure 1C**). As it can be seen from **Figure 1D**, 50 mM AMP allows formation of even more stable complexes: in this condition the most expressed peaks of decamer and tetramer forms are observed, and again, addition of 0.1 M KCl leads to dissociation of decamer form, but increase of KCl to 0.15 M stabilizes dimer LF forms. Hereby, oligosaccharide, ATP, and AMP stimulate the formation of LF oligomers [35].

Denaturing PAGE with 0.1% SDS of the LF preparations preheated with 1% SDS at 100°C reveals on the gel only one band corresponding to the 80 kDa LF monomer [29–31, 35]. Mild treatment with 0.1% SDS without heating leads to the detection of oligomeric forms containing up to six monomers [36]. This leads us to suggestion that LF forms oligomers with a higher monomer number in solution and that these oligomers are quite stable under mild concentrations of SDS. The native electrophoresis allowed us to estimate the initial degree of LF oligomerization and changes of the oligomerization degree due to the presence of various ligands. The gel after the native electrophoresis revealed several weak bands with different electrophoretic mobility, including two major bands that could be attributed to the monomer and the tetramer. After the preincubation with ATP or AMP, most of the proteins remain at the start of the gel, which leads to suggestion that generation of LF oligomer forms with high molecular weight is taking place [36]. The results demonstrated that gel filtration and electrophoresis are not the optimal methods for the detection of the LF oligomers existing before and after binding with different ligands. Therefore, the methods allowing direct detection of protein oligomer forms in solutions were used: small-angle X-ray scattering (SAXS) and light scattering (LS) [35].

According to the data of 2.8 Å X-ray analysis and calculations of LF hydration [1], the volume LF globule is ~132 $Å^3$. The shape of molecule is close to the ellipsoid and the average radius of gyration (Rg) and is ~26.7 Å. The SAXS method allows detecting only small particles, which are comparable with LF oligomers containing one to four monomers. SAXS spectra from a standard LF solution (5 mg/ml) were obtained and the average Rg was calculated as 43.7 Å [35]. LF oligomers dissociate in the presence of 1 M NaCl; therefore the changes in the SAXS spectra after adding NaCl to LF solution to 1 M final concentration were studied. Values of Rg after adding NaCl were 38.6 Å after 1 h, 32.9 Å after 20 h, and 27.4 Å after 42 h; during the further incubation SAXS spectra were stable and the profile matched expected Rg 26.7 Å for LF monomer. Then the LF solutions were analyzed by the LS method: Rg values estimated for the solutions of various LF preparations ranged from 450 to 575 Å and depended on the time of storage and the initial LF concentration. Rg values obtained using LS were one order higher than obtained by SAXS (43.7 Å). LF oligomers analyzed by LS demonstrated approximately

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the same slow rate of monomer dissociation, and the final values of Rg after long incubation were similar to SAXS data. Interestingly, lyophilized LF preparations that were solubilized in a neutral buffer contained substantially lower quantities of oligomer LF: the Rg values for such solutions were 50–100 Å, which is 5–10 times lower than for the nonlyophilized LF solutions (450–575 Å). Thereby LF exists in neutral solutions in different oligomer forms, the increase of LF concentration promotes oligomer formation, but all the oligomers dissociate in the presence of 1 M NaCl [35].

There may be different possible modes of monomer association into oligomers: a sphere (model 1), a cylinder (model 2), and a plate of the height *h* and the radius *R* (model 3) [35]; the estimation of LF oligomerization type was done taking into account these three different models. The LF oligomers with Rg 30–44 Å (SAXS) or Rg 50–100 Å (LS) can consist of 8–70, 5–25, or 2–5 monomers depending on the proposed shape of molecule. According to the gel filtration data (**Figure 1**), the LF solutions contain at least ~10-mer complexes. These results comply with the model assuming formation of cylindrical structures composed with 10–25 monomers. LS method has shown that the presence of 0.1 M KCl slows the initial rate of LF oligomerization in 1.5- to twofolds compared to one without salts. The presence of the nucleotides, oligonucleotides (ODNs), and oligosaccharides increases the Rg values of LF complexes up to 300–700 Å which corresponds to 5–25 monomers in cylindrical oligomer model. The relatively low correlation level of Rg values can be explained by conformational changes of LF molecules in the presence of 1 M KCl due to high conformational lability of LF. According to the SAXS and LS data, the increase of LF concentration promotes oligomer formation, but all the oligomers dissociate in the presence of 1 M NaCl.

The effect of $d(pT)_{10}$ and maltoheptaose on the oligomerization of 3.5 μM LF was analyzed in detail. In the presence of the oligosaccharide and $d(pT)_{10}$, Rg was increased from the initial Rg = 75–100 Å to 304 ± 30 Å and 260 ± 20 Å, respectively [35]. Interestingly, AMP exerted a greater effect on the efficiency of oligomerization in several LF preparations (Rg = 75–100 Å) to oligomers demonstrating Rg. = 450–480 Å. Addition of 1 M NaCl to the oligomer complexes formed in the presence of $d(pT)_{10}$ and maltoheptaose led to the dissociation into free LF monomers with Rg 27.3 Å. Remarkably, according to the LS data, the LF decamer can be formed in all analyzed reaction mixtures and the best ligand-stimulating decameric complex formation is AMP and then ATP and the last maltoheptaose. It should be mentioned that each of the three LF ligands demonstrated two K_d values characterizing the interactions with LF (see below). Taken together, the results show that interaction of LF with mono-oligonucleotides, ODNs, and oligosaccharides can lead to the formation of complexes with various degrees of oligomerization.

4. Oligonucleotide recognition and catalytic activity of human milk lactoferrin

It was shown that LF interacts with RNA [15, 16, 37, 38]. Later it was shown that LF can be considered as sequence-specific protein, since it interacts with DNA molecules containing

three specific sequences including specific d(TAGAAGATCAAA) ODN [17, 29, 30, 39]. Later, it was shown that human milk LF interacts with ATP, NTP, and oligosaccharides [34] and contains two DNA-binding sites, interacting with specific and nonspecific ODNs [31].

Detailed analyses of LF interaction with a number of nucleotides, NTPs, NDPs, NMPs, dNTPs, dNDPs, and dNMPs and their derivatives (e.g., cAMP, cGMP, NAD, ppppA) by fluorescence analysis have shown that LF contains three nucleotide-binding sites, which are characterized by three dissociation constants (K_d). In the case of the first site, the K_d values for various nucleotides and their derivatives varied from 1.4×10^{-5} M for ADP to 3.6×10^{-7} M for dGDP [40]. Similar results were observed for the second site: the K_d values varied from 3.9×10^{-4} M for NAD to 7.3×10^{-6} M for cAMP, while the K_d for the third site were in the range from 1.1×10^{-3} M for NAD to 9.2×10^{-5} M for dCMP [40]. The results obtained by using fluorescence analysis were confirmed with the measurements of the circular dichroism spectra for LF in the absence and in the presence of ATP: the K_d for LF-ATP complex (1.8×10^{-6} M) was comparable with the fluorescence data (8.3×10^{-6} M). It has been shown that LF binding to the different nucleotides leads to a change of the intrinsic LF fluorescence indicating that conformation changes in the protein are caused by LF-nucleotide complex formation [40].

Using affinity modification of LF by chemically active derivatives of ATP, it was shown that ATP-binding site is localized in the C-terminal domain of LF in contrast to the antibacterial and polyanion-binding sites which are located in the N-terminal domain [30]. In addition, it was shown that LF has two anti-cooperative DNA-binding centers which are localized on the N-domain [31]. Interaction of LF with three ATP molecules consists with binding one of the ATP molecules (high affinity) with a specific ATP-binding center on C-terminal domain and binding of two other ATP molecules (with a lower affinity) with two sites on N-terminal domain of LF [41].

It was shown that human milk LF possesses two DNA-binding sites with different affinity for specific ODNs: $K_{d1} \approx 8$ nM (high-affinity site), while $K_{d2} \approx 0.1$ mM (low-affinity site) [31]. Considering the wide range of LF biological functions, it was highly interesting to analyze how the high-affinity site of LF recognizes DNA and what are the differences of LF interactions with DNA comparing to other canonical DNA metabolism enzymes. To evaluate the relative contributions of individual DNA elements to the enzyme affinity for long DNA, a new approach, stepwise increase in ligand complexity (or SILC), was developed (reviewed in [42–44]). Using the SILC approach, many DNA-dependent enzymes were studied, including those of not specific for DNA structure or sequence such as *E. coli* RecA; specific for DNA structure but not for sequence, such as DNA polymerases of prokaryotes, eukaryotes, viruses, and human DNA ligase I; specific for DNA damage such as human uracil DNA glycosylase (UDG), *E. coli* Fpg and human 8-oxoguanine DNA glycosylases (OGG1), and human apurinic/apyrimidinic endonuclease (AP endonuclease); and specific for DNA sequence such as *Eco*RI restriction endonuclease, human topoisomerase I (Topo I), and HIV integrase. It was shown that complex formation, including formation of contacts between all these enzymes and specific sequences, cannot provide high enzyme affinity for DNA or substrate specificity. All mentioned enzymes recognize DNA by forming multiple additive contacts with all DNA units covered by the protein globule (7–20 units depending on the enzyme), and the total interaction

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is a combination of weak electrostatic, hydrophobic, and/or van der Waals interactions of the enzyme with the individual structural elements of DNA. Thus total interaction can be described by the following geometric progression:

$$K_d\left[d\left(pN\right)_n\right] = K_d\left[P_i\right] \times e^{-n} \times h_A^{-a} \times h_C^{-c} \times h_G^{-g} \times h_T^{-t}$$

where $K_d[P_i]$ is the constant of dissociation (K_d) for the *ortho*phosphate (P_i); *e* is the electrostatic factor reflecting the interaction with one inter-nucleoside phosphate group and increase in enzyme affinity; and *h* is hydrophobic factor for A, C, G, and T nucleotide bases, corresponding numbers of which are, respectively, *a*, *c*, *g*, and *t*. It might be proposed that the interaction of noncognate single-stranded (ss) and double-stranded (ds) DNA with any enzyme can be described by this equation. The *h*, K_d, and *e* values for *ortho*phosphate were reviewed in [42–44]. The affinity of some DNA-dependent enzymes to nonspecific $d(pN)_n$ does not always depend on the relative hydrophobicity of the bases. In the case of repair enzymes (8-oxoguanine DNA glycosylases FPG and OGG1, apurinic/apyrimidinic endonuclease APE) and *Eco*RI restriction endonuclease, all hydrophobic factors (*a*, *c*, *g*, and *t*) were equal to one. These enzymes interact mainly with internucleotide phosphate groups, and these weak electrostatic interactions provide high affinity of the $d(pN)_n$ for these enzymes (reviewed in [42–44]).

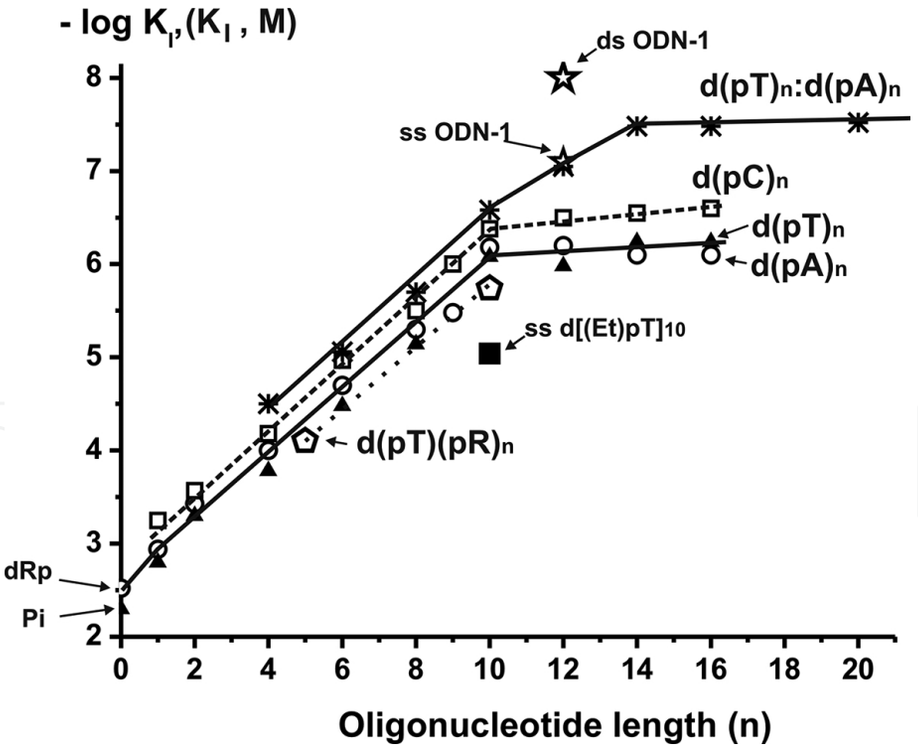

Figure 2. Logarithmic dependencies of LF affinity ($K_i = K_d$ values) for ss and ds; homo- and hetero-ODNs of different lengths (*n*) were calculated by LF complexation analysis with ss [^{32}P]sODN1 and its inhibition. Positions of -log(K_i) values for ethylated $d[(pEt)T)_{10}$, $d[(pT)(pR)_9]$ (pR is a tetrahydrofuran analog of deoxyribose), specific ss and ds sODN1, are shown.

Using the SILC method, the formation of the LF-DNA complexes with $d(pA)_{1–16}$, $d(pT)_{1–16}$, $d(pC)_{1–16}$, $r(pA)_{10}$, $r(pU)_{10}$, $r(pC)_{10}$, specific ODN, and several oligodeoxyribonucleotides was analyzed by electrophoretic mobility shift assay (EMSA) and the Scatchard plot [45]. It was shown that the interaction of the first high-affinity LF DNA-binding site with single-stranded (ss) specific and nonspecific ODNs as well as double-stranded (ds) ODNs is competitive, and K_i ($K_d = K_i$) values were estimated [45]. Affinity of LF (K_i) for homo- and hetero-ss and hetero-ds ODNs of different lengths (n) is determined by using inhibition of LF complexation with ss specific for LF [^{32}P]sODN1. It was shown that like all abovementioned DNA-dependent enzymes [42–44], the minimal ligands of two LF DNA-binding sites are mononucleotides and nucleotide derivatives [45]. The K_d values for minimal ligands of the first LF DNA-binding site were calculated: orthophosphate (5×10^{-6} M), deoxyribose phosphate (3×10^{-6} M), and different dNMPs ($(0.56–1.6) \times 10^{-6}$ M). It means that similarly to other analyzed enzymes [42–44], LF recognizes free dNMPs through interaction with all structural elements (base, sugar, phosphate), and the phosphate group makes the major contribution to the affinity for dNMPs [45]. Logarithmic analysis of dependencies the K_d for $d(pN)_n$ versus the number of mononucleotide units ($n = 1–20$) has shown the additivity of free ΔG^0 values for the interaction of 10–11 individual nucleotide units of ODNs with LF and the absence of strong cooperative interactions (**Figure 2**).

Values of factor f (2.28 ± 0.02), the increase in affinity of LF for various $d(pT)_n$ and $d(pA)_n$, were evaluated from the slopes of the linear parts of these curves (**Figure 2**). The factor f (2.36 ± 0.03) for $d(pC)_n$ was slightly higher. The values of $K_d = 1/f = 0.44 \pm 0.004$ M for $d(pT)_n$ and $d(pA)_n$ and 0.42 ± 0.005 M for $d(pC)_n$ correspond to the increase of enzyme K_d values. These are reflecting the interaction between one of ss DNA units and enzyme active center and in turn are equal to the reciprocals of these factors. The affinity of the first LF-binding site for dNMPs (0.56–1.6 mM) is approximately 81–770-fold higher than for other 9–10 nucleotides ($K_d = 0.42–0.44$ M) of an extended ODN [45]. In contrast to DNA polymerases, UDG, AP endonuclease, and Topo I but similarly to *E. coli* Fpg, human 8-oxoG OGG1 glycosylase, and *Eco*RI endonuclease [42–44], the affinity of LF for $d(pC)_n$, $d(pT)_n$, and $d(pA)_n$ did not depend on the relative hydrophobicity of their bases (**Figure 2**). Moreover, LF does not contact 9 of the 10–11 DNA bases but mainly interacts with the sugar-phosphate backbone of the ligands. The internucleoside moieties of ethylated phosphates neutralize their charges and increase the affinity of $d(pT)_{10}$ (0.83 μM) up to 12-fold comparing to ethylated $d[p(Et)T]_{10}$ ($K_i = 10.0$ μM) (**Figure 2**), indicating that negative charges play an important role in DNA internucleoside phosphate group interaction with LF. It might be supposed that the lack of all bases except one nucleotide unit of $d(pN)_n$ interacting with LF active center does not influence significantly the LF interactions with the sugar-phosphate backbone of ss DNA. Similar to other enzymes [42–44], the interactions between LF and sugar-phosphate backbone of DNA may be considered as weak interactions of oppositely charged surfaces by ion-dipole and dipole-dipole interactions. Most probably, DNA- and RNA-binding sites of LF may be the same or at least significantly overlap. It was shown that LF binding to polyanions such as heparin and tRNA inhibits the binding to DNA [31]. Moreover, LF demonstrates higher affinity for $r(pA)_{10}$ (2.9-fold), $r(pC)_{10}$ (2.8-fold), and $r(pU)_{10}$ (7.5-fold) than that corresponding for $d(pN)_{10}$. This can be attributed to extreme biological polyfunctionality of LF, including its ability to activate

transcription; also it cannot be excluded that $r(pN)_n$ can be easier adopted to the first LF nucleic acids–binding site [45].

It was shown previously that LF interacts specifically with three sequences of ds DNAs and activates transcription: TAGAAGATCAAA (ODN1), ACTACAGTCTACA (ODN2), and GGCACTTAC (ODN3) [17]. The affinity of these three ODNs for LF was estimated. It was shown that ss and ds ODN3 demonstrate the K_d values of 8.0×10^{-7} M and 2.5×10^{-7} M, respectively. Interestingly, the ss $d(pN)_9$ ($(1–3) \times 10^{-6}$ M)) and ds $d(pT)_{10}$:$d(pA)_{10}$ (2.7×10^{-7} M) values are comparable for those different substrated. It has been proposed that the interaction of LF DNA-binding site's central cytosine bases of GGCACTTAC and in the case of different hetero-ODNs results in equality of f factor for d(pG)-mononucleotide units, d(pT)- and d(pA)-links ($f = 2.28$; see above). Thereby K_d value for ODN3 was calculated from the equation:

$$K_d[ODN3] = K_d\left[d(CMP)\right] \cdot \left[1/f_{T,A,G}\right]^6 \cdot \left[1/f_C\right]^2 = 5.6 \cdot 10^{-4} \cdot \left(1/2.28\right)^6 \cdot \left(1/2.36\right)^2 = 7.1 \cdot 10^{-7} M$$

The calculated K_d value $(7.1 \pm 0.7) \times 10^{-7}$ M was comparable with experimental one $(8.0 \pm 0.7) \times 10^{-7}$ M. In a similar way the K_d values for five different nonspecific hetero-ODNs of different lengths and sequences were estimated. The experimental K_d values for all five ODNs were also comparable with the calculated values. The affinity of LF for specific ss ODN2 ($K_d = 2.2 \times 10^{-7}$ M) was ~3.9-fold higher than that for ss $d(pT)_{12–14}$ or ss $d(pA)_{12–14}$ (average $K_d = (8.6 \pm 2.0) \times 10^{-7}$ M). Interestingly, these values for ss $d(pC)_{12–14}$ ($(3.0 \pm 0.2) \times 10^{-7}$ M) are 1.4-fold lower, but ds ODN2 ($K_d = 1.8 \times 10^{-7}$ M) affinity was approximately twofold lower than that for ds $d(pT)_{12}$:$d(pA)_{12}$ ($K_d = 9.0 \times 10^{-8}$ M), which are completely nonspecific. Specific ss ODN1 ($K_d = 8.0 \times 10^{-8}$ M; increase in affinity 4.0–7.9-fold) and ds ODN1 ($K_d = 1.0 \times 10^{-8}$ M; increase in affinity ninefold) but not others, compared to nonspecific ss ($K_d = (3.2–6.3) \times 10^{-7}$ M) and ds ($K_d = 9.0 \times 10^{-8}$ M) homo-$d(pN)_{12}$ of the same length, demonstrated significantly higher affinity for LF. Of all hetero-ODNs only specific ss and ds ODN1 demonstrates significant increase in the affinity compared to nonspecific ODNs and only this ODN contains TCA trinucleotide. Therefore, one cannot exclude that this particular trinucleotide sequence may be important for specific interaction of all nucleotide units of ODN1 with the DNA-binding site of LF [45]. Similar to all analyzed enzymes, the second strand contributes to the LF affinity for nonspecific and specific ds DNA (14–24-fold; $(4–7) \times 10^{-2}$ M) in a less degree than the first strand ($(3–8) \times 10^{-7}$ M) (**Figure 2**) [45].

Interestingly, the relative contributions of specific interactions with specific modified nucleotide units in the case of UDG, AP endonuclease, and Fpg are comparable on the level of minimal ligands (dNMP), ss $d(pN)_n$, and ds ODNs [42–44]. This may explain why total affinity for these repair enzymes is close to additive in case of the specific and nonspecific interactions of specific DNA with different nucleotides. The cooperative effect on the DNA-binding groove of the active site of human OGG1 enzyme may be due to the formation of specific contacts with the oxoG unit [46]. Some nonspecific, previously weak interactions with noncognate ODNs may also greatly strengthen when cognate DNA is bound with LF. Since the ratio in the affinity

(K_d values) for specific and nonspecific ligands significantly increases from mononucleotides (1.8–2.9 ratio) to ss (4.0–7.9 ratio) and ds ODNs (9.0 ratio), it seems likely that the TCA motif of ODN1 may be important for possible cooperative interaction of LF DNA-binding groove with the specific ds ODN1 [45].

Many data indicate that all enzymes acting on extended DNA molecules first bind to any sequence of DNA and then slide to the site containing a specific sequence or a lesion (reviewed in [42–44]). The increase in the affinity (estimated 5 to 790-fold for different enzymes) for specific sequence of DNA can change the DNA backbone conformation in multiple ways. The promotion of the proper fit of specific bases into recognition pockets of the enzymes may be a result of these bonds formation. Similar mechanism was proposed for specific sequence searching and DNA recognition by LF. Moreover it was shown that the interaction of LF with nucleic acids demonstrates some specific features of DNA and RNA recognition compared to other enzymes studied [45].

5. Five catalytic activities of human milk LF

It was previously shown that human LF possesses protease [47] and RNase [15, 16] activities. Later the evidence that homogeneous human milk LF possesses five different enzymatic activities was presented: DNase, RNase, ATPase, phosphatase, and amylase [34]. Several rigid criteria were applied to show that all hydrolyzing activities are intrinsic properties of LF, rather than a result of contamination with canonical enzymes. The most important of these criteria are given below: (a) electrophoretic homogeneity of LF after SDS-PAGE with silver staining, (b) adsorption of LF hydrolyzing activities to the anti-LF Sepharose leading to the disappearance of catalytic activities from solution and the elution by acidic pH of peak possessing five catalytic activities, and (c) FPLC gel filtration of LF under conditions of "acidic shock" (pH 2.6) that does not lead to the disappearance of the activity and to the elution of five in one peak corresponding to ~80 kDa LF.

To exclude possible artifacts of contaminating enzymes, modified with affinity reagents, LF preparations were separated by SDS-PAGE (**Figure 3**). It was shown that only LF contains ^{32}P-label after its affinity modification with [α-^{32}P]oxATP (2′,3′-dialdehyde derivative of ATP), 2′, 3′-dialdehyde derivatives of [5′-^{32}P]-d(pT)$_9$r(pU), and [5′-^{32}P](pU)$_{10}$ (**Figure 3**, lanes 3–5). Two LF catalytic activities were detected after extraction of proteins from the gel slices (**Figure 3A**, lanes 9 and 10) or were detected in situ using gels containing DNA and RNA (lanes 6 and 7; ethidium bromide staining after 16 h at 37°C revealed dark bands corresponding to LF molecular mass on a fluorescing background of copolymerized DNA or RNA).

The same results were obtained using in gel ATPase assay in which enzyme-generated P_i was precipitated by Pb^{2+} resulting in $Pb_2(PO_4)_3$ formation in gel regions containing ATP-hydrolyzing molecules (**Figure 3**, lane 8). Since SDS dissociates any protein complexes, the detection of five activities in the gel regions corresponding to intact LF molecular mass and the absence of any other bands of the activity (**Figure 3**) provide direct evidence that LF possesses five catalytic activities. It was shown that trypsin at pH 8.2 cleaves the LF molecule between Lys283

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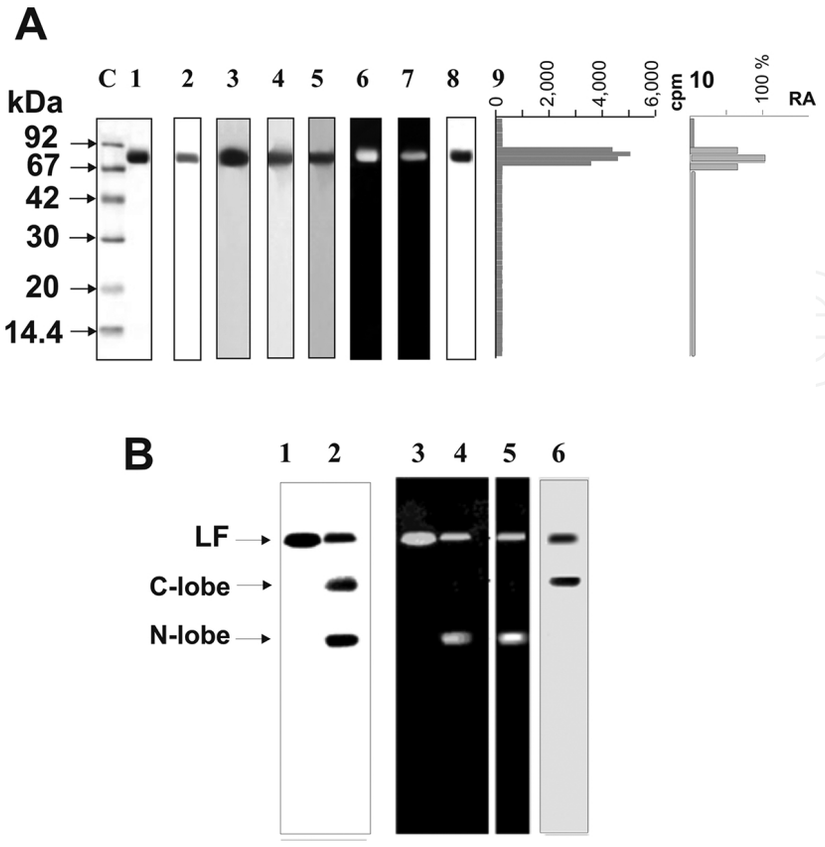

Figure 3. In-gel detection of enzymatic activities of intact LF and tryptic fragments in 12% SDS-PAGE gels. (A) Lane 1, silver stained; lane 2, immunoblotting (alkaline phosphatase-conjugated anti-LF antibodies); lanes 3–5, affinity labeling of LF with periodate-oxidized [α-^{32}P]ATP (3), 5′-[^{32}P](pU)$_{10}$ (4), or 5′-[^{32}P]d(pT)$_9$r(pU) (5) (autoradiographs); lanes 6–7 (negatives), DNase and RNase in gels containing calf thymus DNA (6) or yeast RNA (7); lane 8, ATPase activity; lane 9, phosphatase activity, lane 10, amylolytic activity (RA, relative activity): extracts from 2 to 3 mm gel slices were incubated with 5′-[^{32}P](pT)$_8$ (line 9) or with or with oligosaccharide. (B) Lanes 1 and 2, Coomassie R250-stained LF (1) and LF tryptic fragments (2); lanes 3–5, the negatives of the films corresponding to DNase (3, 4), RNase (5), and ATPase (6; [^{32}P]Pb$_3$(PO$_4$)$_2$ activity of LF (3) and its tryptic fragments (4–6).

and Ser284 residues resulting in N-lobe (30 kDa) and C-lobe (50 kDa) formation [48]. There were compared DNase and ATPase activities of LF before and after trypsinolysis (**Figure 3B**). Interestingly, the high-affinity DNA (lane 4)- and RNA (lane 5)-binding sites are located on the N-fragment (**Figure 3B**), while ATP-binding site is located on the C-fragment (**Figure 3B**, lane 6). Overall, our results demonstrate that all five catalytic activities are intrinsic properties of LF [34].

The above data were obtained using total preparations of LF from different donors. At the same time, it was shown that LF can be separated by chromatography on Cibacron Blue Sepharose into many fractions with different affinities for the sorbent [16]. It was analyzed whether LF fractions exhibiting different affinities for Cibacron Blue Sepharose possess different catalytic activities [34]. The main subfraction of LF (peak 4, LF-5, **Figure 4**) had the highest affinity for this sorbent, as for three additional subfractions (peaks 1–3, **Figure 4**) they represented ~10–20% of the total LF depending on the milk donor. The first protein peak had not demonstrated enzymatic activity; at the same time LF fractions corresponding to three

other peaks possessed ODN 5′-phosphatase, DNase, RNase, ATPase, and maltooligosaccharide-hydrolyzing activities, and each activity was eluted in several peaks (**Figure 4A–C**). LF subfraction corresponding to the peak 2 possessed four different activities: phosphatase, DNase, RNase, and ATPase. Eluate corresponding to the protein peak 3 demonstrated three prominent peaks of ODN 5′-phosphatase activity (**Figure 4B**) and two peaks of RNase activity (**Figure 4C**). Thus, LF subfractions LF-1-LF-5 (**Figure 4**) possess different combinations of various catalytic activities.

The nature of the structural variations that gives rise to these profound functional differences is not defined. The LF molecule contains two potential glycosylation sites [2], and the degree of different molecules' glycosylation varies. LF can contain hexose, mannose, hexoseamines, or other saccharides [49] and may also differ in level of phosphorylation. Therefore, one cannot exclude that different affinities of LF fractions to Cibacron Blue Sepharose and specific distribution of various catalytic activities among these fractions may be due to different types of LF glycosylation and/or phosphorylation.

DNase activity of human LF differs significantly from other known canonical DNases [34]. The pH optimum of LF is 7.0–7.5—significantly higher than that (5.0–5.5) of human blood DNase II. The LF activates significantly (100–150%) by 100 mM NaCl, whereas DNase I is 70% inhibited by 50 mM NaCl [50, 51]. LF-mediated cleavage of [^{32}P]-ODNs and DNA is stimulated three- to fivefold by Ca^{2+}, Cu^{2+}, and Zn^{2+} and eight to ninefold by Mn^{2+} or Mg^{2+} ions. In contrast to known human DNases, LF DNase activity is activated by ATP, dATP, and NAD (150 mM) by 1.5–2.5-fold [34]. Human LF hydrolyzes supercoiled plasmid DNA ~30–200 times faster (k_{cat} = 2–9 min^{-1}) than ODNs; the rate is comparable to some DNA restriction endonucleases. It was shown that in case of RNA hydrolysis LF substrate specificity stands out from RNase A and other human milk RNases [34]. LF preparations from different donors hydrolyze ATP with K_m ~3.5×10^{-5} M, and the k_{cat} value ~2×10^{-3} min^{-1}. As it was shown, maltooligosaccharide is the best substrate for different LF preparations demonstrating $K_m \sim 10^{-6}$ M [34].

Taking into account the relative k_{cat} values for LF in the hydrolysis of DNA, RNA, and ATP and the relative content of LF in human milk comparing to canonical DNases, RNases, and ATPases, it was shown that the LF is the major DNase, RNase, and ATPase of human milk [34, 41, 52]. Unlike the main LF fraction with the highest affinity for Blue Sepharose, all LF fractions with DNase activity are cytotoxic and suppress growth of mouse and human tumor cells [52]. The discovery of LF catalytic activities may contribute to understanding of multiple physiological functions of this extremely polyfunctional protein and explain its protective role against microbial and viral infections.

6. Catalytic antibodies of human milk

Abs are produced by the plasmatic cells. In traditional view they have a unique function binding antigens and elicit the immune response. However, it was shown that Abs raised against chemically stable analogs of the transition states of chemical reaction can possess different enzymatic activities. These artificial catalytic Abs were termed abzymes (*a*nti*b*ody

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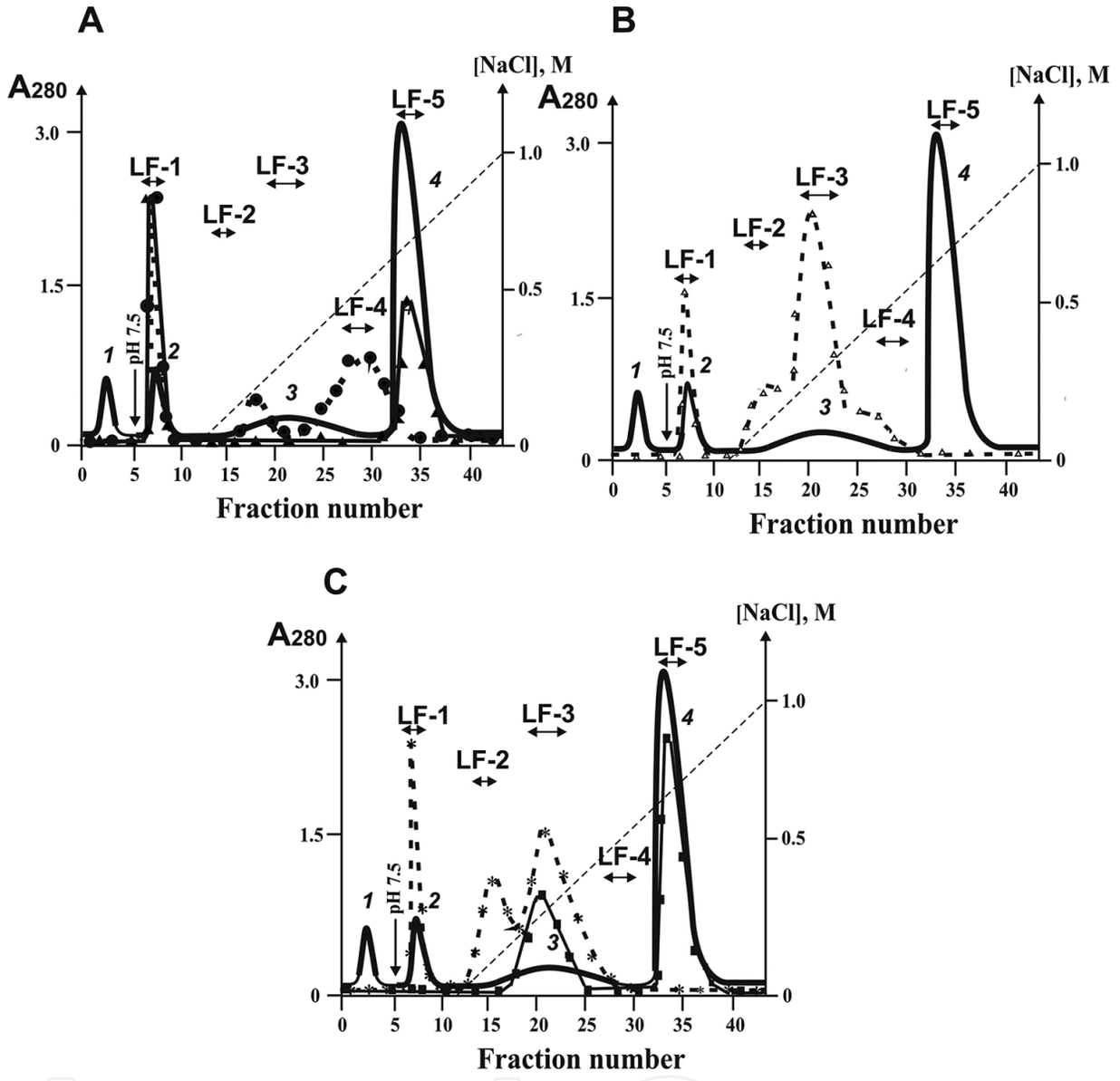

Figure 4. Chromatography of LF on Blue Sepharose and enzymatic activities in five fractions of LF (LF-1-LF-5): (A) solid line (-), A_{280}; DNase (•) and ATPase (▲); (B) 5′- (Δ); (C) RNase (*) and amylase (■). Aliquots (1–3 μl) of chromatography fractions were used for the determination of DNase (supercoiled DNA), RNase (5′-[^{32}P](pU)$_{10}$), phosphatase (5′-[^{32}P](pT)$_8$), ATPase (γ-[^{32}P]ATP), and amylase (4-nitrophenyl-4,6-O-ethylidene-α-D-maltoheptaose) activities.

enz*ymes*). Abzymes catalyzing more than 100 distinct chemical reactions are reviewed in Refs. [53–60].

During past two decades, it was shown that the sera of patients with autoimmune diseases contain auto-Abs that possess catalytic activities. Now it is obvious that the occurrence of catalytic Abs is a distinctive feature of AIDs (reviewed in [61, 62]). Naturally occurring abzymes may be Abs raised directly against enzymes acting as haptens, and mimicking transition states of catalytic reactions, this process is similar to artificial catalytic Abs raised against stable analogs of chemical reactions transition states [53–60]. Moreover, the induction

of anti-idiotypic Abs in AIDs with a primary antigen may result in some antibody catalytic activities. IgGs and/or IgAs, IgM abzymes hydrolyzing different oligopeptides, proteins, DNA, RNA, nucleotides, and polysaccharides were detected in the sera of patients with several AIDs. Healthy humans usually do not develop catalytic Abs or their activities are very low. The detection of abzymes has been recognized as the earliest indicator of autoimmune process [61, 62].

During pregnancy and immediately after delivery, women are often undergoing immune system changes similar to that of AIDs (autoimmune diseases) [61–63]. The published data suggest that pregnant women may be directly immunized through certain compounds of viral, bacterial, or food origin, which can efficiently stimulate production of different Abs. Immunization of animals by direct injection of antigens (mainly proteins) into the bloodstream or by oral administration 1–3 months before delivery leads to the production of Abs against corresponding proteins which later may be detected in the milk [64]. The generation of human milk Abs several months after the immunization indicates on the existence of a specific "immune memory" in lactating and in pregnant women. During the pregnancy, the autoimmunization of mothers may be similar to the processes occurring in blood of autoimmune patients. It has been reported that the level of DNA in the serum of normal women increases during the first 3 months of pregnancy and this resembles the situation in autoimmune diseases [65]. In addition, an increased level of apoptosis during the last 3 months of pregnancy has been demonstrated [66] together with the presence of low numbers of embryonic cells in the blood of pregnant women [67, 68]. This means that, in contrast to healthy humans, pregnant women may be efficiently immunized by compounds of various viruses and bacteria.

It was shown that IgGs from the sera of normal 2–7-month-old (CBAxC57BL)F1 and BALB/c mice and from the sera of 2–3-month-old autoimmune-prone MRL-lpr/lpr mice (conditionally healthy mice) are catalytically inactive [69–71]. During spontaneous development of deep system lupus erythematosus like pathology, the specific reorganization of immune system in these mice leads to the production of IgGs hydrolyzing DNA, ATP, and polysaccharides with low level of catalytic activity (conditionally prediseased mice). A significant increase in DNase, ATPase, and amylase activity of IgG is associated with the transition from predisease to deep disease condition and correlates with additional changes in differentiation and proliferation of bone marrow hematopoietic stem cells (HSC) and lymphocyte proliferation in different organs. Abzyme activities in the serum of pregnant females were comparable with those of predisease mice, but the profile of HSC differentiation and cell apoptosis levels in pregnant and predisease mice were quite different. Right after the beginning of lactation (4 days after delivery) and in a late time of lactation (14 days after delivery), the increase in cell apoptosis and significant changes in two different stages of the HSC differentiation profiles were observed; the first stage was accompanied with a significant increase and the second with a remarkable decrease in abzyme activity [69–71]. These data indicate that women blood and milk can contain various Abs against foreign and self-antigens (including abzymes) in high concentrations.

sIgAs and IgGs possessing DNase, RNase, amylase, and ATPase activities were found in blood serum and milk of pregnant and lactating females [63, 72–77]. It was shown that milk of

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clinically healthy human mothers contains quite unusual sIgA and IgGs possessing protein [78–80], lipid [81–83], and polysaccharide kinase activities [84–86]. The most impressive catalytic activities of human milk Abs are synthesizing ones (kinase activities), which are not detected for any serum antibody under any autoimmune disease. It was shown that IgGs and sIgAs phosphorylate not only casein but also approximately 15 other milk proteins [78–80]. It was shown that milk sIgA and IgG contain tightly bound lipids and oligosaccharides of unusual structure and these Abs effectively phosphorylate these ligands [81–85]. In contrast to canonical kinases, milk abzymes can transfer phosphate group to proteins, lipids, and oligosaccharides not only from ATP but also from all NTPs and dNTPs; the most surprising is that inorganic *ortho*phosphate is also very good substrate [78–85]. Canonical enzymes using *ortho*phosphate as a donor of phosphate groups are not yet found.

Many autoimmune pathologies can be "activated" or "triggered" in clinically healthy women during pregnancy and soon after childbirth [87, 88]. Independent of detectable autoimmune reactions during pregnancy woman may sometimes develop postnatal autoimmune pathologies such as system lupus erythematosus, Hashimoto's thyroiditis, phospholipid syndrome, polymyositis, and autoimmune myocarditis. One of the most frequently found postnatal autoimmune pathologies is Hashimoto's thyroiditis (1.9–16.7%) [88–90]. Various manifestations of these AIDs can be detected during the first 3–6 postnatal months. Interestingly, the relative activity of human blood Abs significantly increases after delivery and in the beginning of lactation. Moreover, catalytic activity of human milk Abs is 5–600-fold higher comparing the sera of the same women [63, 77]. Interestingly, the antibody DNase activity in blood of healthy pregnant women was four- to fivefold lower than that of pregnant women with pronounced autoimmune thyroiditis [63]. Thus, one cannot exclude that molecular mechanisms of immune system activation which leads to production of autoreactive or autoantibodies with and without catalytic activity are, to some extent, similar or overlapping in both autoimmune patients and human mothers.

Overall, pregnancy and especially the beginning of lactation may be considered as important periods associated with the production not only of different Abs against foreign antigens, auto-Abs, but also of Abs hydrolyzing foreign and self-antigens. Many abzymes from sera of autoimmune patients and sera and milk of human mothers possess the same activities including hydrolysis of DNA, RNA, nucleotides, and proteins. At the same time, they can have different biological functions. For example, DNase abzymes from sera of system lupus erythematosus [91], multiple sclerosis [92], and DNA-hydrolyzing Bence-Jones proteins from multiple myeloma patients [93] are cytotoxic, cause nuclear DNA fragmentation, and induce cell death by apoptosis, at the same time, DNase abzymes from human milk are not cytotoxic. Antiviral and antibacterial defenses of gut mucosal membranes are among the most important functions of human milk Abs. Immune exclusion may be achieved by entrapping viruses in immune complexes and its transcytosis and excretion into the lumen by inhibiting pathogens adherence to mucosal surface and by agglutination of microorganisms or interference with bacterial flagella [94]. According to our data, in contract to destructive abzymes of autoimmune patients, abzymes of human milk have a clear protective role against different harmful antigens and different infections [95, 96].

7. Fab arm exchange of human milk antibodies

Abs usually considered as the products of clonal B cell populations each producing Abs which recognize a single antigen. There is a common belief that mammalian biological fluids contain monovalent IgG molecules with two identical antigen-binding sites and stable structures. The Fab arm exchange of Abs was first described for isotype 4 of immunoglobulin G (IgG4) [97–100]. As the result of posttranslational modification, IgG4 molecule exchange with half molecules (HL fragments) becomes bispecific and provides anti-inflammatory activity. Mutagenesis studies revealed that for this activity CH3-domain is critical, and the in vitro experiments have shown that adding 0.1–10 mM of reduced glutathione (GSH) is sufficient to induce Fab arm exchange of IgG4 [98]. Later it was shown that natalizumab and other monoclonal Abs also exchange by Fab arms in vivo with endogenous human IgG4 in treated individuals that makes IgG4 unsuitable for human immunotherapy [101].

Polyspecificity may be defined as binding of Ab molecule to a large panel of diverse antigens. It was shown that the large number of monoclonal Abs can bind to the variety of totally unrelated foreign and self-antigens. Therefore, it was proposed that the best explanation of Abs polyreactivity is the flexibility of the antigen-binding "pocket" which can change conformation and accommodate different unrelated antigens [102]. There is no doubt that due to change of the conformation, some monoclonal Abs can bind not only specific antigen with high affinity but also some cognate and even foreign compounds with lower affinity. It is known that canonical enzymes can sometimes interact nonspecifically with foreign ligands demonstrating lower affinity comparing to specific substrates, but they usually cannot catalyze conversion of molecules of noncognate compounds. For example, it was shown that affinity of many sequence-specific enzymes for specific and nonspecific DNAs differs more often ~tenfold and rarely up to 100-fold. However, these enzymes catalyze the conversion of only specific DNAs [43].

Human milk IgGs and sIgAs were separated by affinity chromatography on DNA cellulose [103, 104]. The fractions of Abs with different affinity for DNA were analyzed in the hydrolysis of DNA, ATP, and oligosaccharides as well as in phosphorylation of proteins, lipids, and oligosaccharides. It was surprising that all IgG and sIgA fractions including those eluted under the conditions destroying strong complexes of antigen-binding sites with specific antigens (3 M NaCl, 2 M $MgCl_2$) have not only efficiently hydrolyzed DNA but also ATP and oligosaccharides as well as phosphorylated casein, oligosaccharides, and lipids. The same preparations of IgGs and sIgAs were separated on ATP-Sepharose, casein-Sepharose, and lipid-saturated silica gel; in all cases all antibody catalytic activities were distributed all over the profiles of all chromatograms (**Figure 5**).

These results cannot be explained with the current concept of one type of two (IgGs) or more (sIgAs) identical antigen-binding site of Abs. Using this concept, it was impossible even to explain the high affinity of several fractions of Abs for several different affinity sorbents and moreover the catalytic polyreactivity of different fractions. But the presence of two different antigen-binding sites in one IgG or up to four sites in one sIgA molecule can easily explain the nature of binding polyspecificity and catalytic polyreactivity. Our data indicated that human

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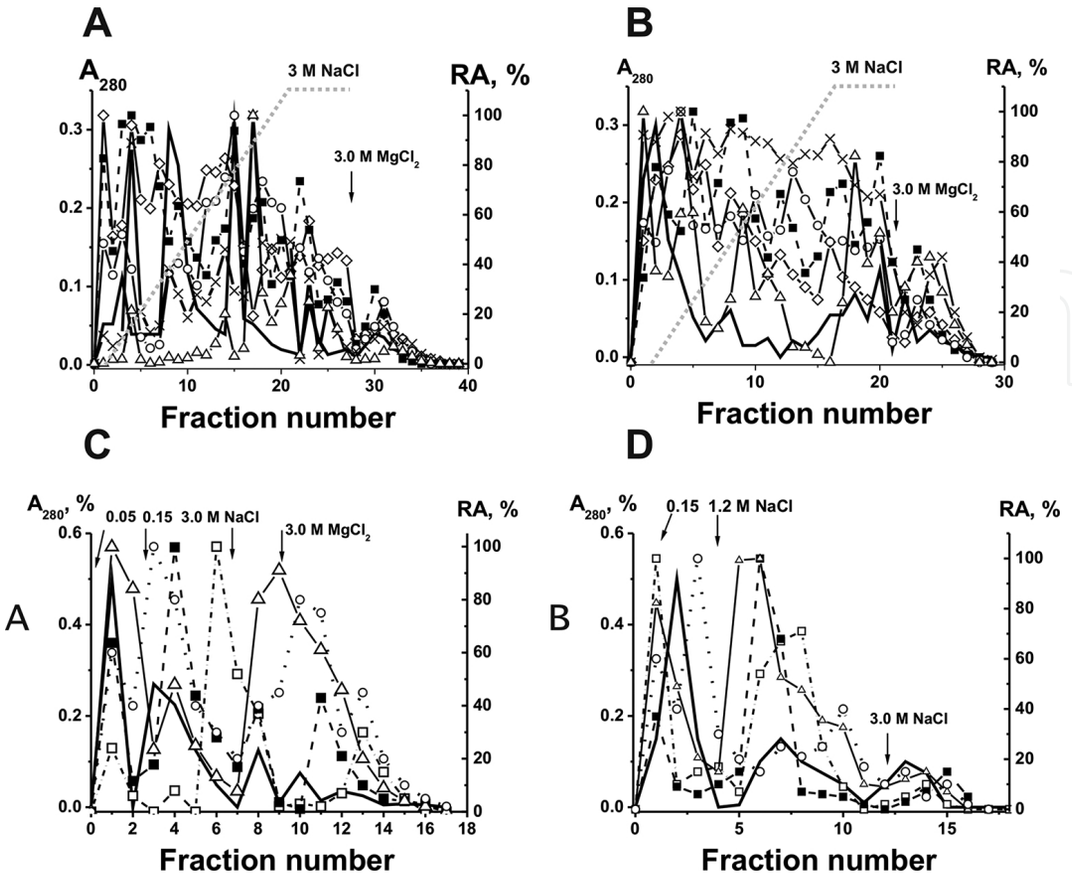

Figure 5. Affinity chromatography of human milk polyclonal IgGs on DNA cellulose (A), ATP-Sepharose (B), casein-Sepharose (C), and lipid-resin (D): (—), absorbance at 280 nm; symbols correspond to the relative catalytic activities (RA) in the hydrolysis of DNA (Δ), ATP (◎), and oligosaccharides (×); phosphorylation of lipids (■) and polysaccharides (◎) tightly bound to IgGs (A and B), hydrolysis of DNA (Δ), ATP (◎), and phosphorylation of casein (◎) and lipids tightly bound with Abs (■) (C and D). Depending on the RA and reaction analyzed, the reaction mixtures were incubated for 0.5–2 h and then the RAs were normalized to the standard conditions and the RA of the fraction with the highest activity was taken for 100%. The average error in the initial rate determination from two experiments in each case did not exceed 7–10%.

milk contains not only monofunctional catalytic Abs but also hybrid bifunctional IgGs and polyfunctional sIgAs with different combinations of HL fragments possessing distinct affinities and catalytic activities [103, 104].

To establish in vivo Abs Fab arm exchange in human milk, IgGs and sIgAs were separated by affinity chromatography on anti-κ-L-Sepharose and anti-λ-L-Sepharose bearing immobilized monoclonal Abs to human κ- and λ-light chains. The IgG fraction having affinity for anti-κ-L-Sepharose was re-chromatographed on anti-λ-L-Sepharose and vice versa. The relative percentage of Abs having affinity only for anti-κ-L-Sepharose, only to anti-λ-L-Sepharose, and to both of these sorbents was evaluated. In addition, using ELISA it was confirmed that IgGs having affinity only for anti-λ-L-Sepharose do not contain κ-IgGs, while IgGs with affinity for anti-κ-L-Sepharose are free of λ-IgGs. But IgGs interacting with both affinity sorbents demonstrated positive response to mouse Abs against human λ- and κ-Abs [103]. Similar results were obtained in the case of milk sIgAs [104]. It was shown that milk IgG preparations contain ~33%

of Abs only with κ-light chains, ~13% only with λ-chains, and ~54% of IgGs with both κ- and λ-light chains simultaneously proving the bispecific nature of milk IgG molecules. Nearly the same results were obtained in case of sIgA: ~48% of Abs contained only κ-light chains, ~35% contained only λ-chains, and ~17% were presented by chimeric molecules simultaneously containing both types of the light chains [104]. Significantly lower quantity of bispecific sIgAs comparing to IgGs can appear due to secretory component and/or J-chain which may hinder the exchange of sIgA by HL fragments. It is well known that B-lymphocytes or plasmatic cells producing Abs express only one gene coding light chain—of κ- or λ-type—as the other copies of light chain genes are removed during cell maturation. The exceptions to this rule are described in transformed hybridoma, leukemia, and myeloma cells and appear as the result of abnormalities; they are not observed in human milk donors [105, 106].

It was shown that chimeric bispecific human milk IgGs are presented mostly by IgG1 (74%) and lower amounts of IgG2–IgG4 (5–16%). Our data provided the evidence that in milk not only IgG4 but all IgG subclasses and also sIgA molecules can exchange with HL fragments, but not with individual light or heavy chains [103, 104].

The experiments on monoclonal IgG4 have shown that exchange with Fab arms in vitro occurs in the presence of reduced glutathione [99, 101]. The exchange of HL fragments between IgG and between sIgA molecules in vitro was studied using FITC and ^{32}P-labeled Abs. Nonlabeled and labeled IgG and sIgA fractions with different affinities for DNA were eluted from DNA cellulose with gradient of NaCl (0–3 M) and 8 M urea (initial chromatography). First, in control experiments the fractions of ^{32}P/FITC-labeled and ^{32}P/FITC-unlabeled Abs exhibiting lower and higher affinities for DNA were mixed in the presence of only reduced glutathione (GSH) or only milk plasma, incubated for 24 h, and subjected to re-chromatography on DNA cellulose (e.g., **Figure 6**). The radioactive (fluorescent) label was eluted with the same concentrations of salt (or urea) as in the case of the initial chromatography indicating the impossibility of the HL fragment exchange in these conditions (**Figure 6B** and **C**).

But in experiments when mixtures of 0.6 M [^{32}P]IgGs and 0.15 M IgGs were incubated in the presence of both GSH and milk plasma after re-chromatography on DNA cellulose, the radioactive label was mainly distributed between three peaks. The main part of the total ^{32}P-label moved to two IgG peaks with low affinity, and one corresponded to the initially nonradioactive IgGs 0.15 M IgGs (e.g., **Figure 6A**). Reverse situation was observed in the case of 0.15 M [^{32}P]IgGs and 0.6 M IgGs: as the result of Fab arm exchange, the most of ^{32}P label was detected in the IgG peaks and eluted with high NaCl concentration (≥0.6 M). Similar results were obtained in the case of IgGs labeled with FITC. Overall, after the exchange, 25–60% of labeled IgGs changed the affinity for DNA cellulose [103]. In the case of sIgA HL fragments, exchange led to the transition of only 11–20% of Ab from one fraction to another [104]. This is consistent with a lower content of chimeric κλ-sIgAs in human milk, and it may be due to hampering of the exchange with HL fragments by secretory and J-components of sIgA.

Thus, it has been shown that human milk contains unknown factor(s) stimulating the IgG and sIgA Fab arms exchange in the presence of reduced glutathione. Since the intact human milk contains GSH and other reducing compounds in significant concentrations, HL-fragment exchange can occur in vivo directly in human milk [103, 104]. In addition, it cannot be excluded

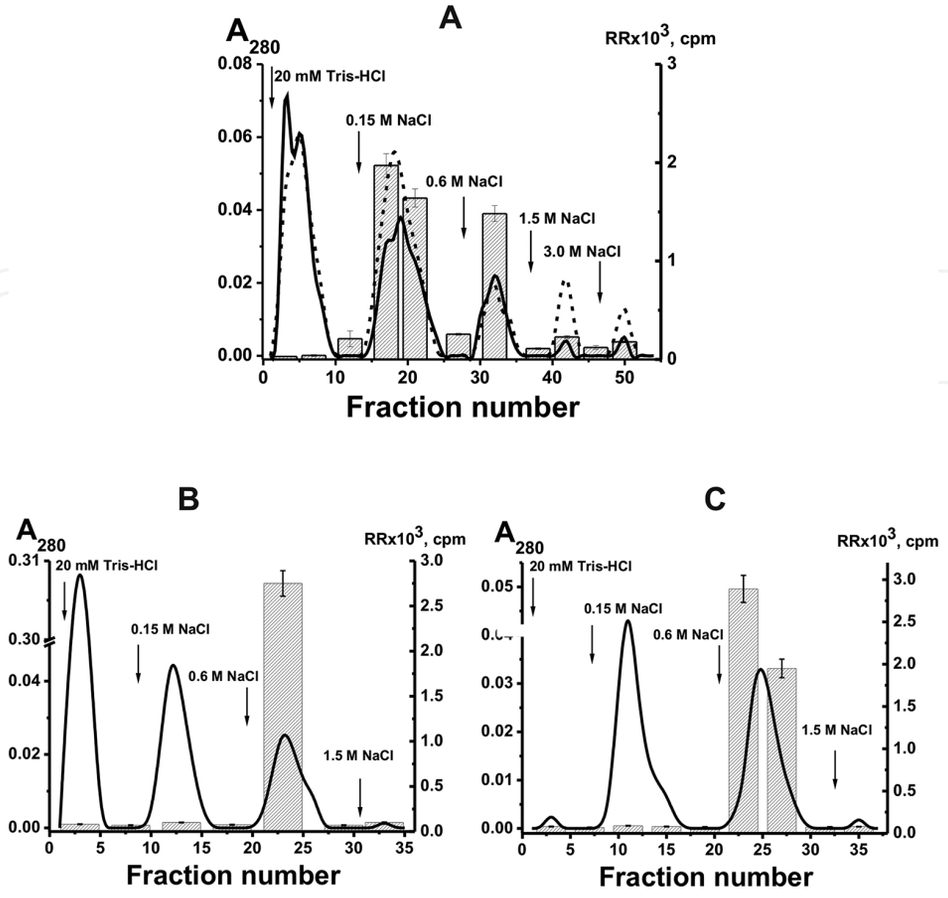

Figure 6. Affinity chromatography of nonmodified and ^{32}P-labeled polyclonal IgGs on DNA cellulose: (---) and (—), absorbance of IgGs at 280 nm before and after phosphorylation using γ-[^{32}P]ATP, respectively; the bars correspond to the relative radioactivity (RR) of [^{32}P]IgG fractions (A–C). Analysis of a relative efficiency of half-molecule exchange under different conditions between nonmodified IgGs and [^{32}P]IgGs having different affinity for DNA cellulose (A–C). Before chromatography the IgG preparations eluted from DNA cellulose by 0.15 M NaCl (0.15 M IgGs) were incubated with [^{32}P]IgGs eluted by 0.6 M NaCl (0.6 M [^{32}P]IgG) in the presence of GSH and milk plasma containing no Abs (A) or in the presence of only GSH (B) or only milk plasma containing no Abs (C).

that during the penetration of IgAs through the specific milk barrier, the secretory component (S) and the joint chain (J) can combine molecules of dimeric H_2L_2 λλ-IgAs, κκ-IgAs, and chimeric κλ-IgAs against different antigens forming many different variants of $(HL)_4$SJ sIgA molecules. Therefore, some chimeric molecules of sIgAs can in principle contain from two up to four HL fragments to various antigens interacting with high affinity for different sorbents and catalyzing various chemical reactions.

It was shown recently that similarly to human milk Abs placenta, Abs undergo to extensive half-molecule exchange and IgG pools in average consist of ~43.5% κκ-IgGs and 41.6% of λλ-IgGs, while 15.0% of the IgGs contained both κ- and λ-light chains [107]. κκ-IgGs and λλ-IgGs contained, respectively, IgG1 (47.7% and 34.4%), IgG2 (36.3% and 44.5%), IgG3 (7.4% and 11.8%), and IgG4 (7.5% and 9.1%), while chimeric κλ-IgGs consisted of 43.5% IgG1, 41.0% IgG2, 5.6% IgG3, and 7.9% IgG4. The relative content of chimeric IgGs (~15.0%) in placenta is significantly lower than that of the milk (up to 54%). It can be supposed that the observed

phenomenon may appear due to a lower content of factor(s) stimulating the exchange in placenta comparing with milk.

8. Complexes of human milk, containing antibodies and lactoferrin: structure, stability, and potential functions

Since the most biological processes are performed by protein complexes and many components of human milk are multifunctional and cooperate with other factors to produce specific effects modulating growth and development of neonates [108], identification and characterization of human milk protein complexes are important for understanding sophisticated functions of human milk. The complexes of LF with other proteins may be the most widely represented in human milk due to LF polyfunctionality and ability to form oligomers, as it was described above.

It was shown that LF interacts with calmodulin, a ubiquitous 17-kDa regulatory calcium-binding protein localized in the cytoplasm and nucleus of activated cells [109], with casein micelles [110], with β-lactoglobulin and albumin [111], and with ceruloplasmin [112]. All these complexes are relatively unstable.

Different methods to analyze whether in human milk LF can form any very stable protein complexes with other milk proteins were used [113]. Using gel filtration of milk proteins on Sepharose 4B column, there was a purified stable high molecular mass (~1000 kDa) multiprotein complex (SPC) from 15 preparations of human milk (**Figure 7A** and **7B**). By the LS and gel filtration, the stability of SPC was shown in the presence of high concentrations of NaCl and $MgCl_2$; however, the SPC efficiently dissociated in 2 M $MgCl_2$, 0.5 M NaCl, and 10 mM dithiothreitol—the conditions of immune complex dissociation (**Figure 7C**).

Obviously, it is unlikely that such stable complex is an occasional protein association. The amount of SPCs in total milk protein content varied from 6 to 25%. With electrophoretic analysis and MALDI TOF MS data, it was shown that all 15 SPCs contained LF and α-lactalbumin as major proteins, while human milk albumin and β-casein were present in moderate or minor amounts; a different content of IgGs and sIgAs depending on milk samples was observed. In addition, this complex contains several proteins/peptides with relatively low molecular masses (3–9 kDa) which have not yet been identified. All SPCs efficiently hydrolyzed DNA and maltoheptaose. Some fresh SPC preparations contained not only intact LF but also small amounts of its fragments which appeared in all SPCs during their prolonged storage. The fragments similarly to intact LF possessed DNase and amylase activities. It may be a consequence of a presence in SPCs of any canonical proteases in very low amounts or manifestations of the proteolytic activity directly by LF.

As it was mentioned above, oligomers of electrophoretic homogeneous LF completely dissociate during the gel filtration in the presence of NaCl [35]. However, human milk SPCs contain several other proteins and are very stable, requiring high concentrations of $MgCl_2$, NaCl, and DTT for effective dissociation [113]. These data suggest that large and small proteins

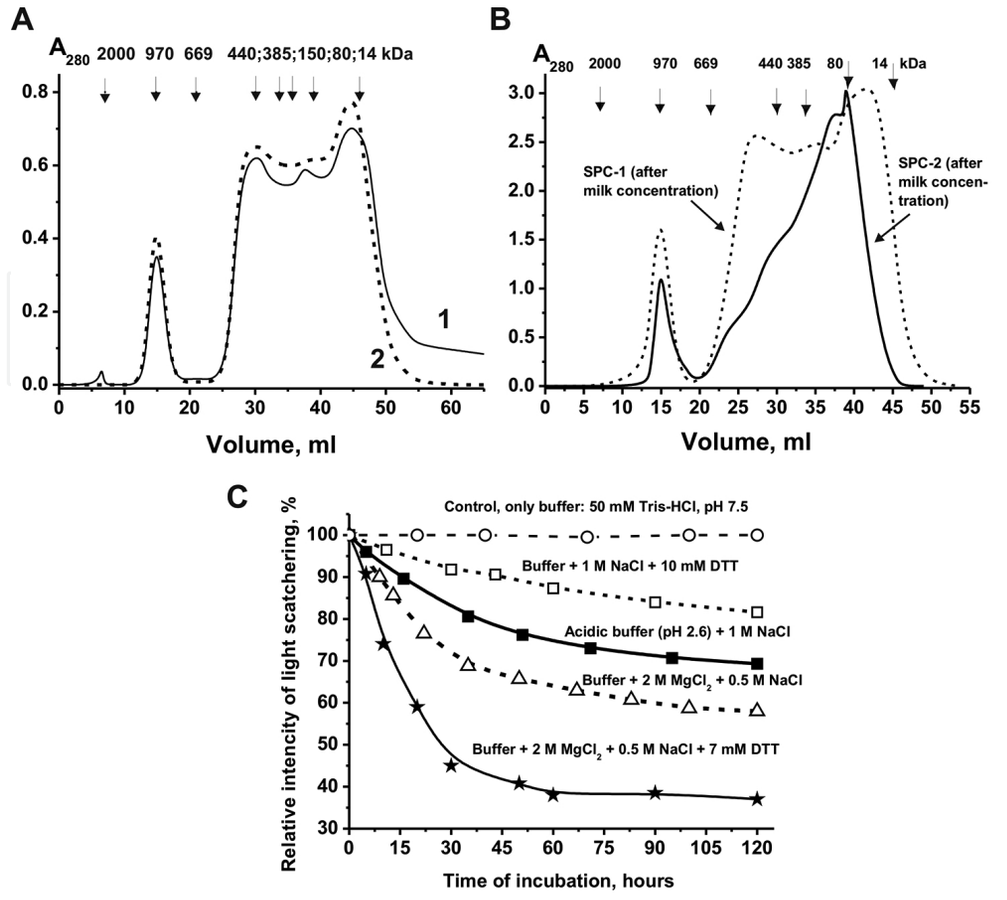

Figure 7. Isolation and analysis of milk protein complex stability. Gel filtration of untreated human milk (1 ml) of first donor (profile 1) and its plasma after removal of fat-lipid fraction (profile 2) on a Sepharose 4B column: (–) and (---), absorbance at 280 nm (A_{280}) corresponding to milk and plasma, respectively (A). Concentrated milk plasma (1 ml) of two donors was gel filtrated: (---) and (–); absorbance at 280 nm (A_{280}) corresponds to different donors (B). Changes of the relative LS intensity were measured in the equimolar mixture of SPCs from 15 milk samples (0.5 mg/ml) under different conditions (C); all conditions are shown in panel C. The highest relative LS at zero time of the incubation was taken for 100% (C).

in the SPCs are mainly interacting by electrostatic forces. At the same time, SPCs can be partially destroyed in the presence of 8 M urea which basically breaks the hydrogen bonds. Since DTTs increase the SPC dissociation, the binding of some proteins with disulfide bonds should be considered important for high stability of the SPCs. The presence of stable supramolecular complex in human milk exhibiting DNA and oligosaccharide hydrolyzing activity can be another reason for the extreme diversity of the human milk biological functions. Taking into consideration the LF functions listed above, complexes of LF with other milk proteins may be important for LF in vivo activity.

9. Conclusion

Obviously, human milk proteome is not a simple mixture of proteins and peptides. Milk proteins interact with each other, form supramolecular complexes, and catalyze various

processes—hydrolytic, synthetic, and exchange reactions. The biological roles of milk proteins and protein complexes are very diverse: milk proteins may hydrolyze foreign (viral, bacterial) DNA and oligosaccharides of antigenic epitopes, and dietary milk proteins (casein) may increase the diversity of antigen-binding sites combinations in one molecule and regulate and enhance infant immune response. Some new binding sites for different ligands and catalytic centers with new enzymatic functions may be formed at interfaces of the LF molecules with other proteins, as well as in contact zones of different proteins incorporated to the possible supramolecular protein structures. We believe that the whole set of different biological functions of the individual milk proteins and their various complexes, extremely unusual abzymes with hydrolytic and synthetic function, as well as complexes of proteins, Abs, and abzymes with nucleic acids, lipids, and oligosaccharides, are still very poorly studied.

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