

Structure and Function of Biopolymers

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EVALUATION OF LACTOFERRIN BINDING TO PLASMINOGEN FRAGMENTS

Aim. The aim of the study was to determine affinity of lactoferrin binding to Glu-plasminogen (Glu-Pg) and its fragments: kringles 1–3, 4 and 5 (K1–3, K4 and K5), and Val442-plasminogen (mini-Pg) in order to reveal plausible lactoferrin influence on the plasminogen/plasmin system. **Methods.** Glu-Pg, K1–3, K4, K5, and mini-Pg were tested for their ability to bind lactoferrin by ELISA using polyclonal antibodies to lactoferrin. Additionally, the complex formation between lactoferrin and K4, K5 and mini-Pg was confirmed by Far-western blotting technique. **Results.** We demonstrated lactoferrin binding to Glu-Pg, K1–3, K4, K5, and mini-Pg in a concentration-dependent manner. The calculated dissociation constants range from 2.09×10^{-7} M to 7.29×10^{-8} M, indicating a strong interaction between tested Glu-Pg/Glu-Pg domains and lactoferrin. Overall, lactoferrin exhibits the strongest affinity for K5, suggesting a potential preferential binding site within the whole plasminogen molecule. **Conclusions.** The obtained data support a previously reported potential inhibitory effect of lactoferrin on plasminogen/plasmin function.

Keywords: plasminogen/plasmin system; kringles; lactoferrin; protein-protein interactions; ELISA.

Introduction

Glu-Pg (Fig. 1), the native circulating precursor of plasmin, is structurally organized into five kringle (K) domains and a serine protease domain (SPD), which together govern its activation and functional diversity [1].

The plasminogen/plasmin system of mammalian blood ensures the dissolution of fibrin clots and

the maintenance of haemostatic balance [2]. Over the past few decades, using the gene inactivation technology, transgenic mice deficient in certain proteins, and various cell lines, a large body of experimental data has been obtained proving the involvement of this system in various normal and pathological processes such as tissue remodelling, cell migration, angiogenesis, inflammation and immune regulation, wound healing, reproduction,

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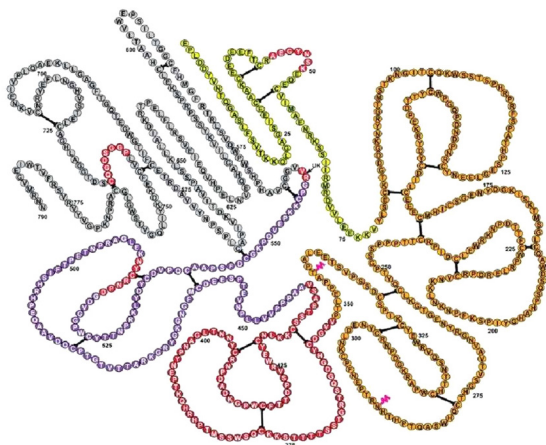


Fig. 1. Schematic illustration of Glu-Pg structural organization (K — kringle, SPD — serine-proteinase domain) [1]

neural plasticity, cancer progression and metastasis [3–10]. Notably, plasminogen is an important virulence factor for invasive pathogens, including group A *Streptococci*, *Staphylococci*, and *Borrelia*. Plasminogen binding to bacteria and its subsequent activation allow bacteria to degrade the extracellular matrix and basement membranes of the host either through the direct protease activity of plasmin or through plasmin's activation of host matrix metalloproteases (MMPs) [11, 12]. Thus, plasminogen plays a central role in fibrinolysis and various physiological processes, but its dysregulation contributes to the development of pathological conditions such as cancer cell invasion, hemostatic disorders and bacterial virulence.

Plasmin's multifunctionality arises from alterations in its substrate specificity mediated by interactions with modulatory proteins and cell-surface receptors [13]. Consequently, identifying novel plasminogen partners represents a significant biomedical challenge. Recently, Zwirzitz *et al.* showed that human lactoferrin, an iron-binding glycoprotein, blocks uPA-mediated plasminogen activation on the surface of the human monocytic THP-1 cells by directly binding to human plasminogen [14]. Lactoferrin in the human body is primarily secreted by exocrine glands and is found in milk, saliva, tears, nasal secretions, and other mucosal

fluids, as well as stored in the secondary granules of neutrophils, from which it is released during immune responses [15]. Studies show that lactoferrin can bind to platelet membranes, influencing platelet function and potentially modulating coagulation and fibrinolysis [16]. Lactoferrin primarily functions as an iron-binding glycoprotein with antimicrobial, anti-inflammatory, and immunomodulatory roles, while newly discovered activities include its ability to modulate proteolytic systems, influence tumor progression, and degrade specific bacterial virulence factors, expanding its significance beyond classical host defence [17]. Due to its ubiquitous presence in the human body, lactoferrin may exert its biological functions by modulating the activity of the plasminogen/plasmin system, although the precise mechanisms and peculiarities of these protein-protein interactions remain largely unexplored. Understanding lactoferrin-plasminogen/plasmin interaction may open therapeutic avenues for controlling fibrinolysis, limiting cancer invasion, and enhancing antimicrobial defence.

Thus, the aim of this study was to characterize the affinity of lactoferrin binding to Glu-Pg and its kringle-containing fragments, including K1–3, K4, K5, and mini-Pg by ELISA.

Materials and Methods

Glu-Pg was isolated from donor human citrated blood plasma on Lysine-Sepharose 4B (GE Healthcare BioScience, Uppsala, Sweden) in the presence of proteinase inhibitor aprotinin (Contrykal®AWD, Germany) [18]. Written informed consent was obtained from all healthy adult donors before blood collection.

Plasminogen fragments: K1–3, K4 and mini-Pg were obtained from plasminogen elastase hydrolysate followed by separation on a Sephadex G-75 (GE Healthcare BioScience, Uppsala, Sweden) and Lysine-Sepharose 4B [19]. K5 was obtained from pepsin hydrolysate of mini-Pg on AH-Sepharose 4B (Amersham Biosciences, Sweden) [20].

IgG pool of blood serum of rabbits immunized with lactoferrin (Novax®Pharma, Monaco) was

purified from globulin fraction by affinity chromatography on protein A-Sepharose (Sigma Aldrich, USA). The purity and specificity of IgG to lactoferrin in this pool were studied using gel electrophoresis and Western blot analysis as described elsewhere [21]. The working concentration of antibodies against lactoferrin was established by direct ELISA. Adsorption of lactoferrin in the wells of 96-well plates ("Nunc", Denmark) at a concentration of 10 µg/ml in 0.02 M bicarbonate buffer (pH 9.5), necessary washings and blocking of free adsorbing groups of the plastic were performed in a similar manner. After that, the wells were washed three times with 0.01 M potassium phosphate buffer (pH 7.4) with 0.14 M NaCl and 0.05% Tween-20 (PBST), and 0.1 ml of IgG to lactoferrin, titrated at concentrations of 1.56; 3.12; 6.25; 12.5; 25 and 50 µg/ml in PBST, were added to them. All subsequent steps followed the same procedure. The working concentration was defined as the value corresponding to the midpoint of the linear portion of the binding curve.

Tricine electrophoresis was performed in 15% polyacrylamide gel (PAGE) in the presence of 0.1% sodium dodecyl sulfate (SDS) [22].

The interaction of lactoferrin with Glu-Pg, K1—3, K4, K5, and mini-Pg was evaluated by ELISA. Glu-Pg and its fragments at a concentration of 10 µg/ml in 0.02 M bicarbonate buffer, pH 9.5, in the volume of 0.11 ml were added to the wells of 96-well plates ("Nunc", Denmark). The protein adsorption was allowed to proceed for 18 hours at 4 °C. The unbound protein was removed and the wells were washed several times in PBST. To prevent nonspecific sorption, 0.15 ml of 2.5% bovine serum albumin (BSA) was added to the wells and incubated for 1 h at 37 °C. After that, the wells were washed three times with PBST and 0.1 ml of lactoferrin solution titrated at concentrations of 0.78; 1.56; 3.12; 6.25; 12.5; 25 and 50 µg/ml in PBST were added to them. Incubation was carried out at 37 °C for 2 h, then plates were washed by PBST three times. After that, 0.1 ml of a solution containing 3 µg/ml anti-lactoferrin IgG dissolved in PBST was added to the wells. Incubation was

carried out at 37 °C for 1 h, then PBST was used for three-time washing. Then, 0.1 ml of a solution of goat anti-rabbit IgG-HRP Conjugate (Sigma-Aldrich, USA), diluted in PBST 1 : 2000, was added to the wells. Incubation was carried out at 37 °C for 1 h, then plates were washed by PBST three times, and 0.1 ml of 0.05 M potassium phosphate buffer (pH 6.0) with 0.03% H₂O₂ and 0.04% orthophenylenediamine (Synbias Ltd, Life Chemicals group, Kyiv, Ukraine) was added to the wells. The reaction was terminated by adding 50 µl of 2 M H₂SO₄ to each well. Optical density was measured using an automatic microreader (Titertek Multiskan MC, Finland) in dual-beam mode at 492 nm, with 620 nm as the reference wavelength.

Far-western blotting was used to examine lactoferrin interactions with K4, K5, and mini-Pg. The proteins were electrotransferred from SDS-PAGE onto a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Sweden) using a buffer system containing 48 mM Tris-HCl, 39 mM glycine, 0.037% SDS, and 20% methanol. Transfer was carried out for 2 h at a current density of 0.8 mA/cm² of membrane. The membranes were immersed in a 4% BSA solution in PBST for 15 h at 22 °C, washed three times with PBST and incubated in a solution of lactoferrin (5 µg/ml) for 2 h at 37 °C. After washing, the membranes were incubated with anti-lactoferrin IgG (3 µg/ml) in PBS for 2 h at 37 °C. The membranes were washed three times with PBS and immersed in a solution of goat anti-rabbit IgG-HRP conjugate (Sigma Aldrich, USA) in PBST, diluted 1 : 5000, for 60 min at 37 °C. After washing the membranes with PBST, immunoreactive bands were visualized by the enhanced chemiluminescence method (ECL) [23].

Statistical analysis of the experimental data was performed using Prism 5 software (Graph-Pad Software Inc., USA). The curves shown in the figures are representative of a series of repeated experiments (at least three per series). The dissociation constant ($K_{d(ops)}$) for protein-protein complex formation was determined from plots of ligand concentration versus ligand binding to the

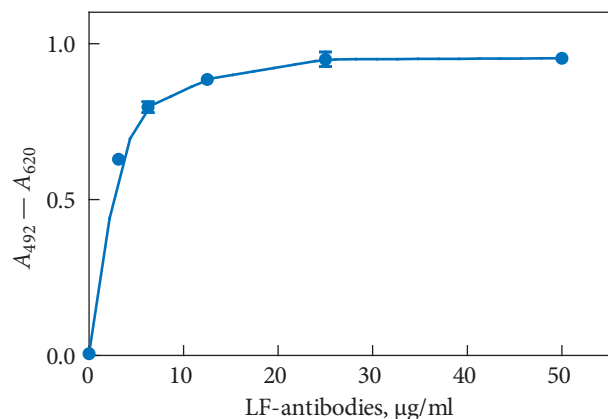


Fig. 2. Binding of polyclonal antibodies specific to lactoferrin to immobilized lactoferrin (ELISA)

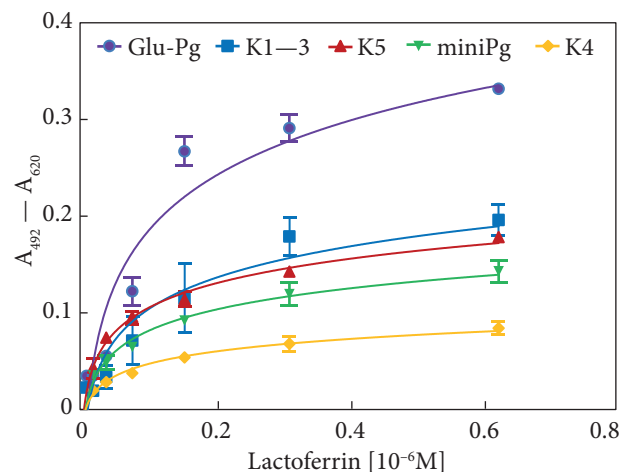


Fig. 3. Concentration-dependent binding of lactoferrin to immobilized Glu-Pg, K1—3, K4, K5, and mini-Pg, determined by ELISA using polyclonal anti-lactoferrin antibodies (3 µg/ml)

Table 1. Dissociation constants ($K_{d(obs)}$) for lactoferrin binding to Glu-Pg, K1—3, K4, K5, and mini-Pg, determined by ELISA. Values of Pearson correlation coefficient (r) calculated from the binding curves in Fig. 3 are also presented

Kringle-containing protein/fragment	$K_{d(obs)}$	r
Glu-Pg	$(1.56 \pm 0.07) \times 10^{-7}$	0.95
K1—3	$(2.09 \pm 0.10) \times 10^{-7}$	0.86
Mini-Pg	$(1.04 \pm 0.05) \times 10^{-7}$	0.92
K4	$(1.06 \pm 0.05) \times 10^{-7}$	0.92
K5	$(7.29 \pm 0.11) \times 10^{-8}$	0.96

immobilized protein, according to the following equation:

$$A = A_{\max} / (1 + K_{d(obs)} / [L]),$$

where A denotes the absorbance of ophenylenediamine, which is proportional to the amount of bound ligand (goat anti-rabbit IgG-HRP conjugate) on the linear portion of the curve; $A_{\max}$ is the absorbance at ligand saturation; $[L]$ is the molar concentration of the ligand; and $K_{d(obs)}$ represents the dissociation constant [24].

Data in the table are presented as arithmetic means (M) with standard errors of the mean (m). The electrophoregram and immunoblot shown in Fig. 3 are representative of repeated experiments (at least three replicates). For the analysis, only results with a permissible error not exceeding 5% ($P < 0.05$) were considered.

Results and Discussion

The binding of polyclonal anti-lactoferrin antibodies to immobilized lactoferrin in ELISA was examined, and the working concentration was determined to be 3 µg/ml (Fig. 2).

The binding of lactoferrin to immobilized Glu-Pg, mini-Pg, K1—3, and K4, and K5 was investigated by the ELISA. We demonstrated that lactoferrin binds to plasminogen-derived kringle-containing proteins and fragments and this binding depends on lactoferrin concentration..

From the binding curves shown in Fig. 3, dissociation constants were calculated for each fragment (Table 1). These values indicate the strongest affinity of lactoferrin for mini-Pg, K4, and particularly K5. The dissociation constant values appear unusual, as the affinity to the isolated K5 domain was found to be higher than to the intact Glu-Pg molecule. This phenomenon can be explained by the conformational properties of native plasminogen. Glu-Pg is the proenzyme precursor of plasmin and adopts a closed globular conformation stabilized by intramolecular interactions between the Pan-apple domain and K5 [1]. It is

therefore reasonable to assume that in the intact molecule, the lactoferrin-binding site located in K5 is partially shielded and less accessible, whereas in the isolated K5 fragment it is exposed and available for binding. However, upon conversion of the zymogen into the active protease plasmin, conformational rearrangements occur that lead to the exposure of K5, thereby enabling its recognition and binding by lactoferrin. This structural feature may account for the higher “observed” affinity to K5 compared with Glu-Pg.

Immunoblotting with polyclonal anti-lactoferrin antibodies confirmed the ELISA results, demonstrating specific binding of lactoferrin to Glu-Pg, K1–3, K4, K5, and mini-Pg, immobilized on nitrocellulose membranes (Fig. 4). Also, Fig. 4 shows the SDS-PAGE data (lanes 1–3) alongside Far-Western blot analysis developed by the ECL method (lanes 4–6). As an example, the Far-Western blot illustrates the interaction of lactoferrin with the plasminogen fragments exhibiting the highest affinity, namely mini-Pg, K4, and K5.

The specificity of intermolecular interactions of plasminogen/plasmin and participation in various biological processes are largely determined by the structural features of its molecule consisting of several structural domains, including NH₂-terminal PAN domain [25], five kringle domains and a serine proteinase domain, which contains a catalytic triad of amino acid residues of His603, Asp646, and Ser74. The kringle domains, each comprising 80 amino acids, are double-looped disulfide-bonded structures that contain the lysine binding sites (LBS) and are responsible for the specific recognition of α_2 -antiplasmin, fibrin, plasma proteins, the extracellular matrix molecules and cell receptors. In addition to the LBS, plasminogen also has benzamidine binding sites (BBS): one in K5, a second in the active site of the proteinase domain [26, 27]. Mini-Pg contains the essential protease domain and truncated kringle regions, making it a compact model for studying activation mechanisms.

Strong binding of lactoferrin to plasminogen fragments, especially K5, suggests promising bio-

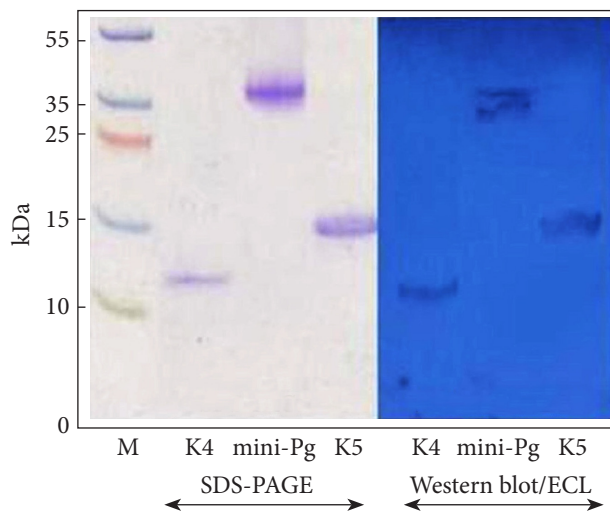


Fig. 4. Binding of lactoferrin to human plasminogen fragments (mini-Pg, K4, and K5), determined by Far-Western blot/ECL using polyclonal anti-lactoferrin antibodies. The immunoblot confirms the ELISA results

medical perspectives. It may act as a natural regulator of plasminogen activation on cell surfaces, thereby modulating key cellular function. This property could be harnessed to limit tumor cell invasion by disrupting plasminogen-driven proteolysis. At the same time, lactoferrin may help block pathogenic bacteria such as *Streptococcus* or *Borrelia* from exploiting the plasminogen system to cross tissue barriers. The interaction with kringle domains also opens the possibility of designing peptide mimetics or inhibitors that replicate the regulatory effects. Dual role of lactoferrin as an iron-binding glycoprotein and plasminogen modulator may provide added benefits in inflammatory conditions. Incorporating lactoferrin into biomaterials or medical device coatings could reduce pathogen colonization by interfering with plasminogen binding. Altogether, these perspectives highlight lactoferrin as a multifunctional molecule with therapeutic potential in hemostasis, oncology, and infectious disease regulation. Thus, lactoferrin biotechnology could be advanced significantly, with sources including bovine milk, human milk, and recombinant production systems in yeast, rice, or transgenic animals. These biotechnological approaches not only

ensure sustainable large-scale production but also provide high-purity lactoferrin for therapeutic and industrial applications, making it a valuable molecule for pharmaceutical development and functional food supplement. Importantly, there is currently no internal industrial production of lactoferrin for the Ukrainian biotechnological market, despite the availability of relatively inexpensive raw materials. This highlights the necessity of establishing domestic production capacities to compensate for this deficit and ensure sustainable access to high-purity lactoferrin for scientific and applied purposes.

Conclusions

Overall, the findings establish that lactoferrin binds specifically and with high affinity with the native form of plasminogen — Glu-Pg and its kringle-containing fragments: K1—3, K4, mini-Pg, and K5. We found that lactoferrin demonstrates different affinity toward Glu-Pg and its fragment, as follows: K5 > mini-Pg > K4 > Glu-Pg > K1—3. The results confirm its role as a natural modulator of the plasminogen/plasmin system and highlight K5 as a preferential binding site for lactoferrin. These findings suggest potential applications of lactoferrin in regulating plasminogen activity on cell surfaces, influen-

cing platelet-mediated fibrinolysis, and preventing bacterial exploitation of the plasminogen system. Taken together, lactoferrin emerges as a multifunctional molecule with promising therapeutic and biotechnological relevance in haemostasis, oncology, and infectious disease control, where modulation of plasminogen activation plays a key role.

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Conflict of Interest. The authors declare no conflict of interest.

Author Contribution. Conceptualization: Artem O. Tykhomyrov, Methodology and Investigation: Lada G. Kapustianenko, Olena I. Yusova, Viktoriia V. Korsa. Data Curation: Lada G. Kapustianenko. Writing — Original Draft: Lada G. Kapustianenko, Olena I. Yusova; Review and Editing: Artem O. Tykhomyrov. The final manuscript was approved and read by all authors.

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ВИЗНАЧЕННЯ ЗВ'ЯЗУВАННЯ ЛАКТОФЕРИНУ З ФРАГМЕНТАМИ ПЛАЗМІНОГЕНУ

Мета. Метою дослідження було визначити афінність зв'язування лактоферину з Glu-плазміногеном (Glu-Pg) та його фрагментами: кринглами 1—3, 4 та 5 (K1—3, K4 та K5), а також Val442-плазміногеном (міні-Pg) для вивчення впливу лактоферину на систему плазміноген/плазмін. **Методи.** Glu-Pg, K1—3, K4, K5 та міні-Pg було протестовано на їх здатність зв'язувати лактоферин методом імуноензимного аналізу з використанням поліклональних антитіл до лактоферину. Крім того, утворення комплексу між лактоферином та K4, K5 та міні-Pg було підтверджено методом Far-western блоттингу. **Результати.** Продемонстровано концентраційно-залежне зв'язування лактоферину з Glu-Pg, K1—3, K4, K5 та міні-Pg. Розраховані константи дисоціації знаходяться у межах від 2.09×10^{-7} М до 7.29×10^{-8} М, що вказує на високу афінність між Glu-Pg/крингловими доменами Glu-Pg та лактоферином. Загалом, лактоферин демонструє найсильнішу спорідненість до K5, що свідчить про потенційний переважний сайт зв'язування в межах усієї молекули плазміногену. **Висновки.** Отримані результати свідчать про можливий модуляторний ефект лактоферину на функцію плазміноген/плазміну та визначають K5 як основний сайт взаємодії.

Ключові слова: система плазміногену/плазміну; крингли; лактоферин; протеїн-протеїнові взаємодії; імуноензимний аналіз.