Equine lactoferrin: Antioxidant properties related to divalent metal chelation

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ABSTRACT

Lactoferrin is a minor whey protein known for its multifunctionalities, particularly for its rule in regulation of immune system and for its antimicrobial activities. Although bovine lactoferrin has been extensively studied, very few information exists on lactoferrin from mare's milk. This article studies the antioxidant properties *via* metal chelation of purified lactoferrin from Kazakhstan mare's milk. Equine lactoferrin shows free radical-scavenger activity when tested with radical DPPH and ABTS and has reducing capacities testing by FRAP. Moreover, the ferrous(II) and copper(II) ion-chelating power is highlighted by chemical colorimetric tests. Finally, the newly introduced biophysics switchSENSE® technology allowed to measure in real time the molecular interactions between equine lactoferrin and divalent cations. The order of magnitude of the K_D values was tens or so μM for the four metal ions tested: Zn^{2+} ($K_D = 23.9 \pm 4.0$ μM), Ca^{2+} ($K_D = 28.2 \pm 4.4$ μM), Cu^{2+} ($K_D = 43.5 \pm 5.1$ μM) and Fe^{2+} ($K_D = 54.1 \pm 8.9$ μM). According to these results, we can conclude that equine lactoferrin shows antioxidant activity by radical-scavenging mechanism, reducing capacities and divalent pro-oxidant metals chelation ability.

1. Introduction

Equine milk and equine milk co-products are consumed routinely in Central Asia, particularly in Turkey, Bashkortostan (Russia), Kazakhstan, Kyrgyzstan, Mongolia, and Uzbekistan (Potočnik, Gantner, Kuterovac, & Cividini, 2011). According to Miraglia, Salimei, and Fantuz (2020), equine milk is used in the non-food sector (as an ingredient in cosmetic products), but also in food field especially for debilitated or immunocompromised people, and sensitive consumers like children having allergies to cow's milk protein. According to Uniacke-Lowe, Huppertz, and Fox (2010) and more recently to Miraglia et al. (2020), no data are available on the equine milk worldwide production, but mare's milk has been reported to be consumed by 30 million people (especially in Central Asia). In Europe, the dairy equine industry started in France in the context of a project on the preservation of animal diversity (Miraglia et al., 2020), and expanded then in several marginal biotopes of the world where these monogastric herbivores are absolutely adapted to different environmental issues, with forages scarce availability, frequently of poor-quality. Equine milk is considered as a "whey milk" because whey protein is considerably greater in mare's milk than other mammals. The proportion of whey proteins in equine milk is about 39%, higher than in bovine milk (18%), but less than in human milk (54%) (Pieszka et al., 2016). Among these, lactoferrin (LF) plays a crucial role in developing immune systems in the organism and displays several biological activities such as specific host defense against infection by micro-organisms, anti-inflammatory activity (Håversen, Ohlsson, Hahn-Zoric, Hanson, & Mattsby-Baltzer, 2002), anti-hypertensive properties (Ruiz-Giménez et al., 2012), and it has others various physiological and functional activities including antioxidant properties (Habib, Ibrahim, Schneider-Stock, & Hassan, 2013). The concentration of LF in equine milk is around 0.2–2 g/L (Pieszka et al., 2016), which is lower (1–7 g/L) than that of human milk (Rai et al., 2014), similar (0.02–2.1 g/L) to that of camel milk (Jahanzaib, Tahereh, Masroor, & Tanveer, 2020), but higher (0.03–0.2 g/L) than that of bovine milk (Lan, Rulan, Jianxin, & Bo, 2020). Lactoferrin is ~80 kDa single polypeptide chain glycoprotein containing two homologous globular domains (N- and C-lobes) (Baker & Baker, 2004; Bobreneva & Rokhlova, 2021), highly conserved between the species studied, suggesting that LF displays identical biological functions in all species (Wang, Timilsena,

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Abbreviations

ABTS	2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
AERC	Ascorbic acid Equivalent Reducing Capacity
bLF	bovine lactoferrin
DPPH	2,2-diphenyl-1-picrylhydrazyl
CUPRAC	Cupric Reducing Antioxidant Capacity
eLF	equine lactoferrin
FRAP	Ferric Reducing Antioxidant Power
FPLC	Fast protein liquid chromatography
LF	Lactoferrin
ROS	Reactive oxygen species
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TA40	NaCl 40 mM-containing Tris Buffer

Blanch, & Adhikari, 2019). Lactoferrin belongs to transferrin group, which has a ferric iron binding ability with high affinity ($K_D \approx 10^{-20}$ M) (Baker, 1994). The N- and the C-lobes each contain about 345 amino acids and are made up of two domains N1, N2 and C1, C2, respectively, with the iron-binding site situated in the interdomain cleft (Sharma, Paramasivam, Srinivasan, Yadav, & Singh, 1999). The C-lobe of LF shows higher iron binding ability compared to its N-lobe (Hu et al., 2008). Moreover, the LF apo-form can bind different ions, usually with high positively charged cations, including Cu^{2+}, Co^{3+}, Mn^{3+} (Ainscough, Brodie, & Plowman, 1979), Cr^{3+}, Al^{3+}, Ga^{3+}, Zn^{2+}, Cd^{2+}, Ni^{2+} (Baker & Baker, 2005; Bobreneva & Rokhlova, 2021), VO^{2+} (Harris, 1984), lanthanide cations (Bobreneva & Rokhlova, 2021), and Th^{4+} (Harris, Carrano, Pecoraro, & Raymond, 1981). Since the formation of free radicals, which can be generated due to the catalytic role of metals in an unbounded state, generates many other harmful biochemical processes, fixation of metal on LF can potentially protect cells from oxidative stress damage (Honarparvar, Kanchi, & Bisetty, 2019). Furthermore, different studies reported that LF presents antioxidant activities, due its role in activation of some antioxidative enzymes, which participate in redox-based reactions (Wang, Xu, An, Liu, & Feng, 2008), or by displaying radical scavenging properties (Belizy et al., 2001).

Contrary to bovine LF (bLF) which has been widely the subject of a lot of works in the last decades, there are very few articles on mare's LF properties (Cieslak et al., 2017; Miraglia et al., 2020; Uniacke-Lowe et al., 2010). Some differences among species are reported and the equine protein is revealed to possess similar iron-binding behavior to that of human LF but not to bLF (Shimazaki, Oota, Nitta, & Ke, 1994), conferring to the equine protein a great interest for human health (*e.g.* for curing anemia, a major issue in pregnant women and newborn babies; Niaz et al., 2019). Moreover, considering that horse's milk is widely consumed in Central Asia and that LF is present in higher concentration than in bovine milk (bovine milk is hardly consumed in countries such as Kazakhstan), it is relevant to study the LF in equine milk. The aim of this work was to study the antioxidant properties of equine LF (eLF) related to its chelation capacities of divalent pro-oxidant metals. After isolation and purification of eLF from milk, we determined its antioxidant activity (i) by determining its radical-scavenging power and metal reducing capacity and (ii) by investigating its divalent pro-oxidant metal chelation activity. This last point was completed by using of a new biophysics switchSENSE® technology, allowing to measure in real time the molecular interactions between two partners. Thus, we were able to determine the association k_{on} and dissociation k_{off} kinetics constants and the affinity constant K_D of the complex formation between eLF and calcium (Ca^{2+}), zinc (Zn^{2+}), ferrous iron (Fe^{2+}), or copper (Cu^{2+}).

2. Materials and methods

2.1. Preparation of equine milk whey proteins

The equine milk sample was stored at 4 °C for 30 min and was prepared as follows: defatting (5.0 L) was done by centrifugation at 10,000×*g* for 30 min at 4 °C. Casein was eliminated by acid precipitation at pH 4.2 with 1 M hydrochloric acid (HCl) solution followed by a centrifugation step (10,000×*g* for 30 min at 20 °C). The pH of the obtained supernatant (containing whey proteins) was adjusted to pH 6.8 with 1 M sodium hydroxide (NaOH) and dialyzed against ultrapure water at 4 °C for 72 h to remove salts and whey proteins were lyophilized. All chemicals were from Sigma-Aldrich and of analytical grade.

2.2. Purification of equine LF

Equine whey proteins (1.0 g) were suspended in 10 mL Tris-HCl buffer at 20 mM, pH 8.0 (100 mg/mL) and then fractionated by cation-exchange fast protein liquid chromatography (FPLC) on a HiTrap SP Fast Flow column connected to an ÄKTA-pure device (GE Healthcare, Uppsala, Sweden) according to El Hatmi, Jrad, Khorchani, Dary, and Girardet (2014). Briefly, sample volume of 2 mL was loaded onto the column equilibrated in 30 mM Tris-HCl buffer, pH 8.0. A 0–0.5 M NaCl linear gradient in the same buffer was applied for the elution. The flow-rate was 2 mL/min and detection monitored at 280 nm. Several runs were carried out and the fractions collected were pooled, dialyzed against distilled water at 4 °C for 3 days, and freezed-dried. Protein composition of each fraction was determined by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli and Favre (1973). Bands were identified according to Girardet et al. (2004).

2.3. Determination of antioxidative properties of LF

2.3.1. DPPH radical-scavenging activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging capacity of LF was evaluated by the method of Kirby and Schmidt (1997). Briefly, 20 μL of samples in Milli-Q water at different concentration of 0–3.0 mg/mL was mixed with ethanol (99%, 90 μL) and a solution of DPPH in ethanol (0.04% (w/v), 90 μL). After homogenization, the mixture was incubated for 45 min at ambient temperature in the dark. Ascorbic acid was used as positive standard. The absorbance was read at λ = 517 nm (A_{517}) using a 96-well microplate reader (Multiskan Spectrum, ThermoLab Systems, MA, USA). The experiments were run in triplicate and the DPPH scavenging activity was estimated following Equation (1).

$$\text{DPPH radical} - \text{scavenging activity } (\%) = \left[\frac{A_{control} - (A_{sample} - A_{blank})}{A_{control}}\right] \times 100 \quad (1)$$

Where $A_{control}$, A_{blank} and A_{sample} were respectively the absorbances of the control reaction (absence of sample), the protein (LF) sample (except the DPPH solution) and the protein in the DPPH solution.

2.3.2. ABTS radical-scavenging activity

The radical-scavenging assay was carried out according to Sadat et al. (2011), a method adapted from that of Re et al. (1999). The $ABTS^+$ (2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) radical was generated by dissolving 7 mM $ABTS^+$ in 2.45 mM potassium persulfate (di-potassium peroxydisulfate) for 15 h at 22 °C in the dark. The $ABTS^+$ radical reagent was then prepared by dilution in 5 mM sodium phosphate buffer, pH 7.4, just before use. The dilution was adjusted spectrophotometrically in order to reach an absorbance of 0.60 at 740 nm. The cation radical was stable for at least 1 h at 22 °C. Given sample volumes corresponding to known concentrations were lyophilized and

then, dissolved in 300 μL of the ABTS $^{+}$ radical reagent. The decrease of the absorbance was monitored at 740 nm for 10 min at 30 °C with the microplate reader. All assays were carried out in triplicate. The antioxidant activity was expressed in percent and was calculated as following Equation (2).

$$\text{ABTS radical}-\text{scavenging activity } (\%) = [\frac{(1 - A_r - A_b)}{(A_i - A_b)}] \times 100 \quad (2)$$

Where A_i was the absorbance of the initial ABTS $^{+}$ radical, A_r, the absorbance of the remaining radical, and A_b, the absorbance of the blank (phosphate buffer: $A_b = 0.09$).

The ABTS radical-scavenging activity of eLf was determined at 1 mg/mL (n = 4) and was expressed as μM eq. Trolox/mg Lf.

2.3.3. Ferric reducing activity (FRAP)

The ferric reducing abilities (FRAP) of eLF and bLF tested from 0 to 40 μM (= 3.2 mg/mL), as well as ascorbic acid used as reference from 0 to 200 μM (= 0.035 mg/mL) were performed according to the method of Yen and Chen (1995). A volume of 70 μL of sample prepared in 200 mM phosphate buffer, pH 6.6 was mixed with 35 μL of potassium ferricyanide 1% (w/v) and then incubated at 50 °C for 20 min. After incubation, 135 μL of ultra-pure water, 33 μL of trichloroacetic acid 10% (w/v) (Sigma-Aldrich), and 27 μL of ferric chloride 0.1% (w/v) (Sigma-Aldrich) were added to the reaction medium and incubated at ambient temperature for 10 min. Finally, the absorbances were measured at 700 nm (A_{700}) using a 96-well microplate reader and the FRAP capacities were expressed in percentage (%) and calculated using the following Equation (3):

$$\text{Ferric reducing ability } (\%) = 100 - (\frac{A_0 - A_{sample}}{A_0} \times 100) \quad (3)$$

Where A_0 (= 0.8) is the absorbance of a 66 μM Prussian blue solution measured in the same reaction medium free of reducing component and the A_{sample} is the absorbance of protein samples.

The slope of the curve obtained for each protein sample was compared to that of ascorbic acid and the Ascorbic acid Equivalent Reducing Capacity (AERC) index was determined as following:

$$\text{AERC} = [\frac{(a_s)}{(a_0)}] \quad (4)$$

Where (a_S) is the slope of the curve of the ferric reducing activity (%) vs. sample molar concentration and (a_0) the slope for ascorbic acid (Canabady-Rochelle et al., 2015).

2.3.4. Ferrous (II) ion-chelating power

The chelating capacities of ferrous ions by LF were determined following the method of Carter (1971) with slight modifications. Iron chloride $FeCl_2$ solution (20 μL, 2 mM) was added to 500 μL of different aqueous protein solutions at concentrations of 0–3.0 mg/mL. After agitation and incubation during 5 min at room temperature, the reactions were triggered by adding 500 μL of ferrozine solution (1 mM) and the preparations were vigorously shaken and then incubated 5 min. The positive control test was performed using EDTA (ethylenediaminetetraacetic acid) and these tests were run in triplicate. The inhibition ratio of ferrozine-Fe^{2+} complex formation was determined after measuring the absorbances at 562 nm (A_{562}) using a 96-well microplate reader. Ferrous ion chelating activity was estimated following Equation (5).

$$\text{Ferrous ion chelating activity } (\%) = [\frac{A_{control} - (A_{sample} - A_{blank})}{A_{control}}] \times 100 \quad (5)$$

Where the absorbance of the control (without sample), the absorbance of the blank (without ferrozine) and finally the absorbance of the protein fraction represented respectively $A_{control}$, A_{blank} and A_{sample}.

2.3.5. Copper (II) ion-chelating power

The copper ion-chelating power of LF was examined using the method described by Peng, Kong, Xia, and Liu (2010) with slight modifications. A volume of 200 μL of Copper (II) sulfate ($CuSO_4$, 2 mM) were thoroughly mixed with 200 μL of pyridine (10%, v/v) and 20 μL of pyrocatechol violet (0.1%, w/v). After homogenization 2 min, aqueous LF solutions at different concentrations (0–3.0 mg/mL) were added and the mixture was incubated for 2 min at ambient temperature in the dark. Note that EDTA was used as standards and the absorbances were measured at 632 nm (A_{632}) using a 96-well microplate reader. All the experiments were done in triplicate. Copper (II) ion chelating activity was determined following Equation (6).

$$\text{Copper ion chelating activity } (\%) = [\frac{A_{control} - (A_{sample} - A_{blank})}{A_{control}}] \times 100 \quad (6)$$

Where $A_{control}$, A_{blank} and A_{sample} were respectively the absorbances of the control reaction (absence of sample), the blank (except the copper sulfate) and the proteins in solutions.

2.4. Time-resolved molecular dynamics measurements

The experiments of metal binding (Ca^{2+}, Zn^{2+}, Fe^{2+} and Cu^{2+}) onto eLF were performed using an MPC-48-2-R1-S biochip put in a biosensor analyzer switchSENSE® DRX (Dynamic Biosensors GmbH, Planegg, Germany). The principle of the switchSENSE® technology is explained in details by Langer et al. (2013). The chip is composed of four independent channels, each containing six electrodes. The ligand (eLF) is covalently bound onto a 48-bases ssDNA (called cNL for complementary nanolever) which will then be hybridized with another ssDNA already embedded into the gold surface of the electrodes and carrying a fluorescent probe (Cy5 dye with maximal emission at 670 nm) at its free extremity. Fluorescence Proximity Sensing (FPS) is a size independent, real-time measurement of kinetics responding to changes to the molecular environment upon analyte binding (*e.g.*, a metal ion) that yields the kinetics constants of the interaction.

Prior to biosensor measurements, the ligand eLF was covalently bound to cNL with the amine coupling kit for 48-mer provided by Dynamic Biosensors, according to the manufacturer's instructions. Briefly, an amount of 100 μg of eLF (extinction coefficient at 280 nm of 81.425 M^{-1} cm^{-1}; UniProtKB/Swiss-Prot number: F6XLB1) solubilized in ultra-pure water was mixed to the cNL previously activated with a crosslinker and incubated for at least 1 h at 22 °C. The protein-cNL conjugate was then purified by anion-exchange chromatography using the automated analyzer proFIRE® (Dynamic Biosensors) equilibrated in phosphate-buffered saline buffer, pH 7.2. A 0.2–1 M NaCl linear gradient was applied at a flow-rate of 1 mL/min and detection was recorded at 280 nm. The fraction corresponding to the cNL-eLF conjugate was concentrated by centrifugal filtration and was treated by EDTA 10 mM for 10 min at room temperature to remove trace amounts of iron and, finally, concentrated by filtration/centrifugation again for buffer exchange with 10 mM Tris-HCl buffer, pH 7.4, containing 40 mM NaCl and 0.05% Tween 20 and concentrated to a volume of 50 μL. The concentration, amount, and purity degree of the conjugate were determined with proFIRE® software. Prior to biosensor analyses, the conjugate was diluted in the Tris-HCl buffer to a final concentration of 200 nM and a volume of 25 μL was injected into a chosen channel of the chip. For FPS measurements, serial dilutions of each kind of metal ion dissolved in Tris-HCl buffer at 100 μM were injected into the channel at flow rate of 50 μL/min for 2 min (association kinetics), then Tris-HCl buffer was injected at the same flow-rate (dissociation kinetics) and the real-time measurements were recorded at 25 °C. A blank was performed with buffer free of metal ions and subtracted to normalize the signal. For each

analyte (metal ion) investigated, a control with nano levers free of ligand (eLF) was performed and the fluorescence quenching of the probe by the analyte (expressed as normalized fluorescence), if any, was subtracted (Fig. 1). The association and dissociation kinetics constants (k_{on} and k_{off}), the dissociation constant K_D (k_{off}/k_{on}), and the respective error values were monitored in real-time using the FPS mode. All curves were analyzed by nonlinear fitting of single-exponential functions with the switchANALYSIS® software from Dynamic Biosensors. The error presented with the results corresponds to the global fit error of all measurements. The experiments were also repeated twice.

3. Results

3.1. Obtention of pure equine LF

Equine milk whey proteins were fractionated by cation-exchange FPLC (Fig. 2). The main proteins with acidic isoelectric points, α-lactalbumin, β-lactoglobulin and serum albumin, were not retained onto the column and were recovered in the flow-through, whereas lysozyme and eLF (isoelectric point: 8.32) were eluted at retention times 14–15 min and 20–25 min with high purity, respectively.

3.2. Antioxidant activity

Due to the diversity of oxidation processes, the use of a single method to evaluate the antioxidant ability of bioactive molecules cannot provide a clear idea about their real antioxidant potential (Canabady-Rochelle et al., 2015). Therefore, bLF used as reference, and eLF were assayed for antioxidative activity using various antioxidant complementary chemical assays: DPPH, ABTS radical-scavenging and reducing (FRAP) activities and pro-oxidant metal chelation abilities.

3.2.1. DPPH radical-scavenging activity

The free radical scavenging activity of eLF and bLF was evaluated by the DPPH oxidation inhibition percentage assay (Fig. 3A) and compared to the conventional natural antioxidants (ascorbic acid). As shown in Fig. 3A, eLF exhibited a concentration-dependent antiradical activity with IC_{50} value close to 1.80 mg/mL (22.5 μM), which were lower than those obtained for ascorbic acid (10 μg/mL, 56.8 μM). Ascorbic acid showed higher degree of free radical scavenging activity than did LFs at the same concentrations tested (0–3.0 mg/mL). The DPPH radical scavenging capacity of eLF was 33.5 ± 1.1% at a concentration of 0.50 mg/mL and increased to 56.2 ± 2.3% at 3.0 mg/mL (Fig. 3A). The DPPH scavenging capacity of ascorbic acid was 98.5 ± 1.0% at 0.08 mg/mL, then leveled off until 3.0 mg/mL (Fig. 3A), indicating that a saturation state could be reached as low as 0.08 mg/mL. When the activities for the same concentration (3.0 mg/mL) were compared, the DPPH scavenging capacity of eLF was 18% higher than that of bLF.

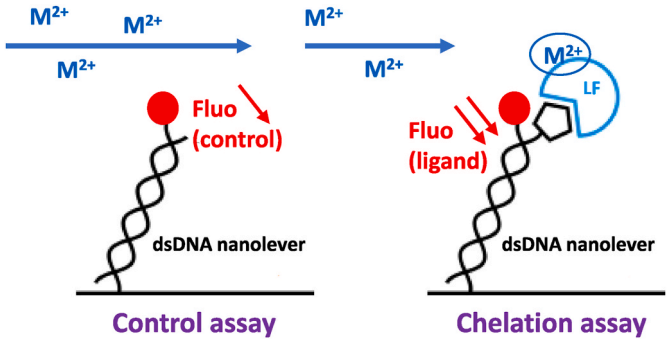

Fig. 1. Real-time switchSENSE® analysis of molecular interaction between equine lactoferrin (LF) immobilized on DNA nanolever (ligand) and various metal ions (analytes M^{2+}). A control was performed without the ligand. When some metal ions generated a quenching of fluorescence in the case of the control, the data (expressed as normalized fluorescence) were subtracted of the raw data obtained in the presence of the ligand.

3.2.2. ABTS radical-scavenging activity

In the present study, the antioxidant activity expressed as Trolox equivalent antioxidant capacity of eLF was determined (*e.g.* Sadat et al., 2011). A linear relationship was found from the concentration response curve in the range of 0–250 μM of Trolox (data not shown). Under the experimental conditions, the IC_{50} of Trolox was 157.9 μM. The results indicated that eLF at a concentration of 1 mg/mL was able to stabilize the radical formed by oxidation of ABTS with a potency of 46.3 ± 5.1 μM eq Trolox /mg of eLF (n = 4).

3.2.3. Ferric reducing ability (FRAP)

The ability to reduce Fe^{3+} (in %) and the AERC index of LF was determined for the two bovine and equine lactoferrin and compared with that of ascorbic acid, used as positive control (Fig. 3B). A linear relationship was found between the ferric reducing activity and the concentration in the range of 0–200 μM (0–0.035 mg/mL) ascorbic acid and of 0–40 μM (0–3.2 mg/mL) LFs. The IC_{50} values deduced from these curves were > to 40 μM (>3.2 mg/mL) for bLF and 35 μM (2.8 mg/mL) for eLF *vs.* 120 μM (0.021 mg/mL) for ascorbic acid. In a concentration range from 0 to 40 μM, eLF and bLF presented great reducing capacities with AERC values of 3.7 and 2.6 μM AERC, respectively.

3.2.4. Copper (II) ion-chelating activity

The copper ion chelating ability of eLF and bLF increased with the concentrations (0–3.0 mg/mL) to obtain respectively A_{632} values of 47.9 ± 0.9% and 35.7 ± 1.1% at 1.0 mg/mL. In this assay, the copper iron chelating ability of eLF was 21.8 ± 1.2% at 0.50 mg/mL, increased to 50.5% at 0.25 mg/mL, and leveled off until 3.0 mg/mL (Fig. 3C), indicating that a saturation state was almost attained with 1.0–1.50 mg/mL of eLF. The chelating ability of EDTA was approximately 75.6 ± 1.5% at 0.30 mg/mL, then leveled off until 3.0 mg/mL (Fig. 3C), indicating that a saturation state could be reached at 0.30 mg/mL of EDTA.

3.2.5. Ferrous ion-chelating activity

Lactoferrin capacities to complex Fe^{2+} were evaluated by measuring the disturbance of Ferrozine-Fe^{2+} complex at A_{562} as reported in Fig. 3D. The chelating powers of ferrous ions exerted by eLF and bLF increased similarly in a dose-dependent manner, but with values lower (64.7 ± 0.6% and 60.1 ± 0.9 for eLF and bLF, respectively) at 0.75 mg/mL than those recorded for the synthetic metal chelator EDTA (99.7 ± 0.3%) at the same concentration. The ferrous iron chelating ability of eLF was 45.9 ± 0.8% at 0.50 mg/mL, increased to 72.8 ± 1.2% at 1.0 mg/mL, and leveled off until 3.0 mg/mL (Fig. 3D), indicating that a saturation state was almost attained with 0.75–0.80 mg/mL of eLF. The chelating ability of EDTA was approximately 96.4 ± 0.9% at 0.06 mg/mL, then leveled off until 3.0 mg/mL (Fig. 3D), indicating that a saturation state could be reached at 0.06 mg/mL of EDTA.

3.3. Divalent metal ion chelation by lactoferrin using switchSENSE® technology

The ability of eLF to chelate some divalent metal ions (calcium, copper, ferrous iron, and zinc) was investigated by real-time molecular interaction analysis using the switchSENSE® technology. The latter is a sensitive approach to detect binding events of small molecules (Perrot et al., 2018; Schiedel, Daub, Itzen, & Jung, 2020). Prior to the kinetics analyses, pure cNL-eLF conjugate (100% purity; Fig. 4) was prepared with the proFIRE® device and used to functionalize the chip by hybridization. Using nano levers free of eLF, it was observed that zinc, iron, and copper were able to quench the fluorophore, whereas calcium couldn't (data not shown). Thus, controls with protein-free nano levers were performed in the presence of each of the three metal ions (zinc, iron, and copper) and the normalized fluorescence values were

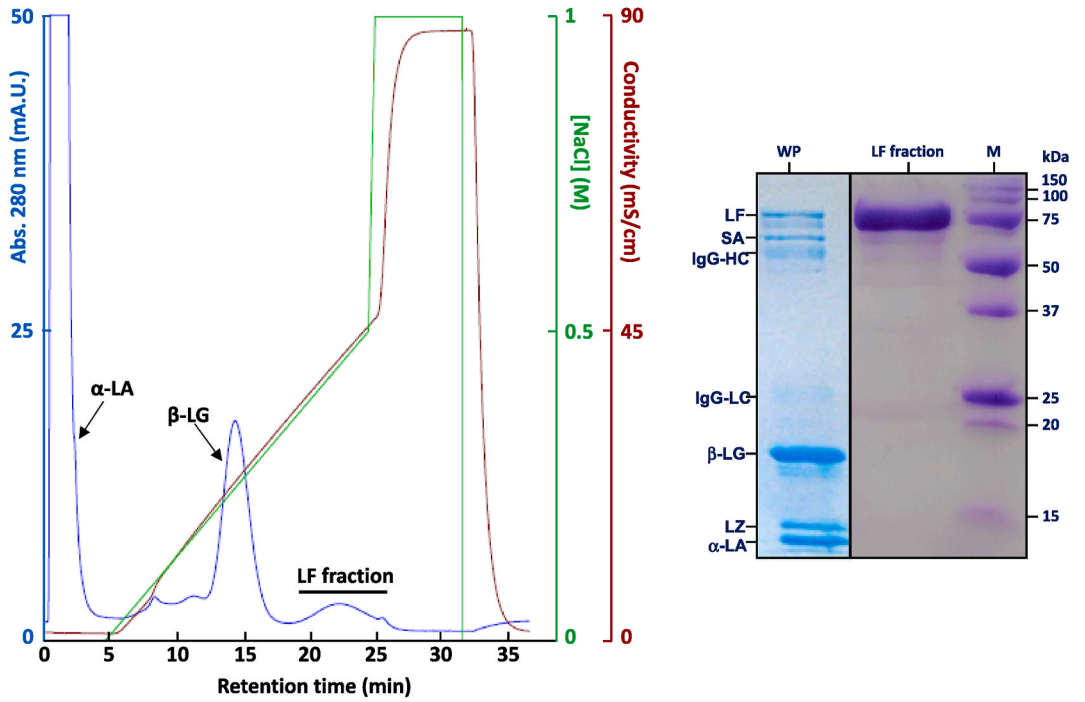

Fig. 2. Cation-exchange Fast Protein Liquid Chromatography of equine whey proteins (WP) on a HiTrap SP column and SDS-PAGE analysis of the lactoferrin (LF) fraction collected. Band identification was according to Girardet et al. (2004) Legend: A.U., absorbance unit; M, molecular weight standard; α-LA, α-lactalbumin; β-LG, β-lactoglobulin; LZ, lysozyme; IgG, immunoglobulins G; HC, heavy chain; LC, light chain; SA, serum albumin.

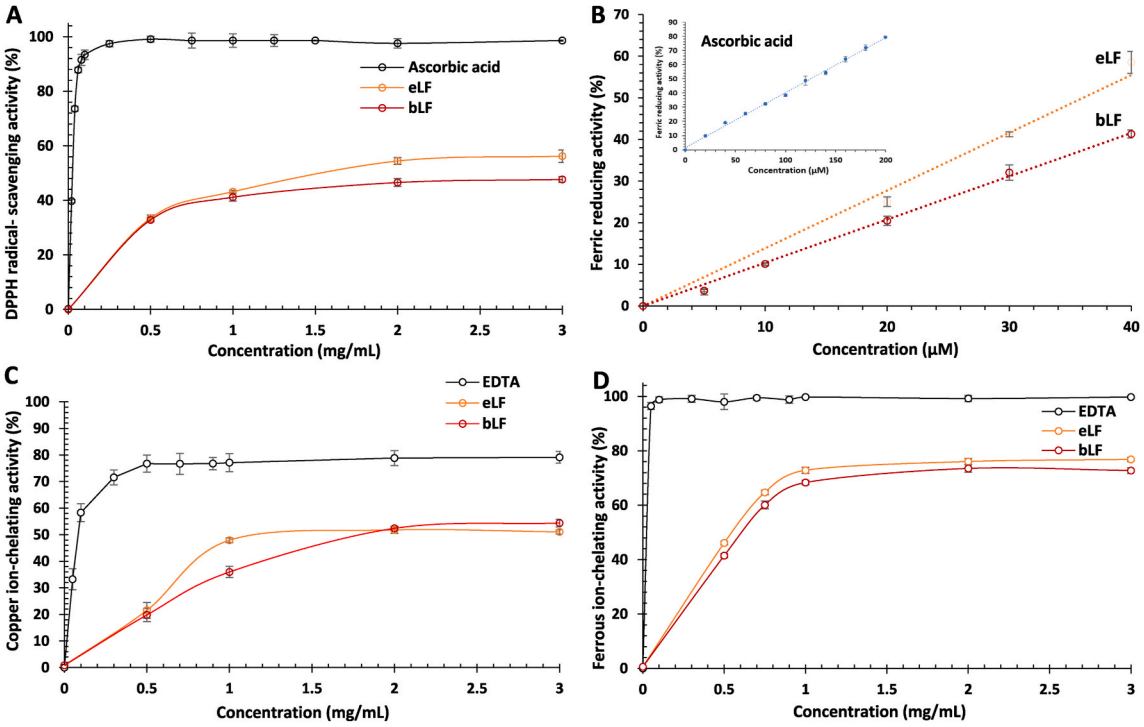

Fig. 3. **(A)** DPPH radical-scavenging activity, **(B)** FRAP reducing activity, **(C)** copper ion-chelating activity **(D)** ferrous ion-chelating activity of equine (eLF) and bovine (bLF) lactoferrin at different concentrations. The values are means ± S.D. (n = 3).

subtracted from the curves determined with the ligand. In the case of calcium, a blank was performed with a buffer free of calcium chloride and subtracted from the raw data to normalize the signal (Fig. 5). The affinity of eLF for the metal ions was investigated by determining the dissociation constant $K_D = k_{off}/k_{on}$. In the case of the association (chelation), the k_{on} value was dependent of the concentration C of the analyte and was deduced from the association constant observed, k_{obs}, with $k_{obs} = C.\ k_{on} + k_{off}$

The order of magnitude of the K_D values was tens or so μM for all the metal ions tested (Table 1). The best affinities were observed for Zn^{2+} ($K_D = 23.9 \pm 4.0$ μM) and Ca^{2+} ($K_D = 28.2 \pm 4.4$ μM) followed by Cu^{2+} ($K_D = 43.5 \pm 5.1$ μM) and Fe^{2+} ($K_D = 54.1 \pm 8.9$ μM).

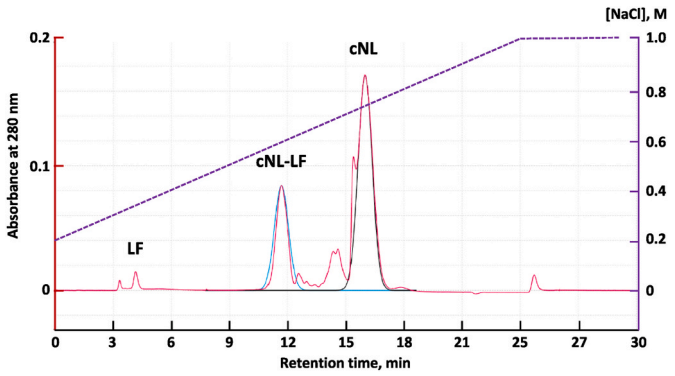

Fig. 4. Automated purification of the complementary nano lever-lactoferrin (cNL-LF) conjugate with the proFIRE® device. The protein fraction that did not react was eluted at the beginning of the linear gradient, whereas the excess of ssDNA (cNL) was eluted at the end of the 0.2–1.0 M NaCl linear gradient. The purity degree, concentration and quantity of the conjugate were determined with the software of the proFIRE® from the fit of the curve (blue line). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

Habib et al. (2013) and other authors (*e.g.*, Habib, Ibrahim, Zaim, & Ibrahim, 2021), demonstrated that most of the oxidative damage in biological systems is caused by the OH radical, which is generated by the reaction between O_2 and H_2O_2 in the presence of metal ions. Damages observed in DNA treated with UV, H_2O_2 and $FeSO_4$ were reduced in the presence of LF, which may involve in an antioxidant defense mechanism by binding catalytic iron, and inhibiting the Haber–Weiss reaction (Habib et al., 2013).

In this present paper, we first studied the radical scavenger capacity of eLF purified from mare's milk by using DPPH, ABTS chemical methods. The experimental data obtained from DPPH analyses (Fig. 3A) showed that the free radical scavenging powers of bLF and eLF were similar, concentration-dependent, and reached a maximum activity plateau at *ca.* 2 mg/mL (= 25 μM), much lower that of ascorbic acid, used as reference (100% activity reached at *ca.* 0.25 mg/mL or 1.42 mM). At 1.0 mg/mL, eLF revealed important antioxidant capacity against DPPH radicals (42.3 ± 0.5%) which is in the same order of magnitude as the one measured for a recombinant porcine LF (46.0%) at

Table 1
Kinetics and affinity constants of metal-lactoferrin interactions determined with the switchSENSE technology.

	k_{on} (10^3 M^{-1} s^{-1})	k_{off} (10^{-3} s^{-1})	K_D (μM)
$CaCl_2$	7.39 ± 1.10	208 ± 9	28.2 ± 4.4
$ZnSO_4$	2.02 ± 0.32	48.3 ± 2.4	23.9 ± 4.0
$FeSO_4$	2.64 ± 0.41	143 ± 7	54.1 ± 8.9
$CuCl_2$	2.54 ± 0.27	111 ± 6	43.5 ± 5.1

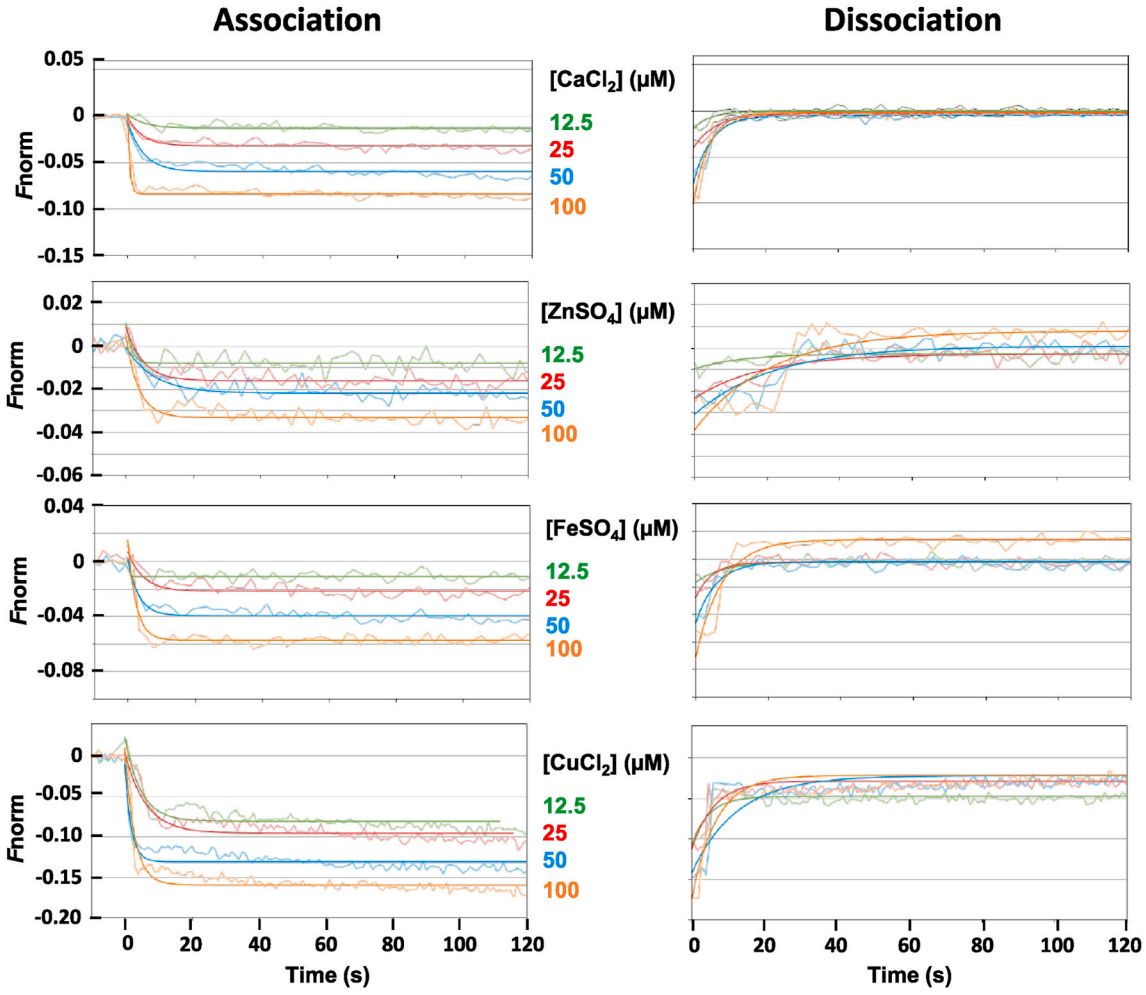

Fig. 5. Kinetics analysis of equine lactoferrin interacting with metal ions by using the switchSENSE® technology. The raw data are superimposed by global exponential fits for various concentrations of each analyte. The k_{on}, k_{off} and K_D were determined for each kinetics measurement. F_{norm}: normalized fluorescence.

the same concentration (Lee et al., 2020), and the same measured for bLF (41.1 ± 1.7%). It is appropriate to note that the scavenging radical capacities of eLF and bLF were respectively related to their protein rate, amino acid composition, molecular weight (Elzoghby et al., 2020), and to the degree of purity. This radical scavenging activity was confirmed for eLF using ABTS test.

Besides the DPPH and ABTS radicals-scavenging abilities, the metal reducing activity of eLF and bLF were investigated using the FRAP test. The equine LF was slightly more efficient to reduce ferric ion than the bovine one under the present experimental conditions. For a given concentration of 40 μM, eLF revealed important ferric reducing capacity (*ca* 61.5 ± 2.6%), which was greater than that of bLF (*ca.* 41.3 ± 1.0%) and that reported for recombinant porcine LF (*ca.* 40.0%) at the same molar concentration (Lee et al., 2020).

Furthermore, metal (iron and copper) chelating assays were performed using chemical methods. Ion (iron and copper) chelating results (Fig. 3C and 3D) revealed a similar trend for eLF and bLF to that observed using DPPH assay (Fig. 3A). At 1.0 mg/mL, eLF revealed important ferrous iron chelating capacity (72.84 ± 1.24%) but remained lower than the one measured for the recombinant porcine LF (87.0%) at a concentration of 0.25 mg/mL (Lee et al., 2020). The iron saturation degree of native LFs as well as the presence of specific chelating amino acids like tyrosine, histidine and aspartic acid can also modulate the ferrous ion-chelating activity (antioxidant properties) of proteins. Analysis of LF hydrolysates showed that the radical-scavenging ability of the protein is unveiled by small size peptides released by *in vitro* gastro-intestinal conditions and possessing residues such as Tyr, Trp, Met, Cys or His (Kontoghiorghes & Kontoghiorghe, 2020). These amino acids play a key role in binding positively charged metal ions through their atoms such as O, N and S (Kontoghiorghes & Kontoghiorghe, 2020).

To complete this study, the switchSENSE® technology was used to measure real-time molecular interaction between eLF and divalent metal ions (calcium, copper, ferrous, and zinc). Lactoferrin can bind Ca^{2+} through the carboxylate groups of the sialic acid residues, present on two glycan chains (Rossi et al., 2002). Moreover, Rodzik, Pomastowski, Sagandykova, and Buszewski (2020) suggested that Zn^{2+} could bind to the thiol group of oxidized Cys. Thanks to its mineral chelation properties, LF could play a key role in transport of mineral ions in blood (Honarparvar et al., 2019).

Several studies have determined the interactions LF-divalent or/and LF-trivalent ions using different chemical and physical methods (Harrington, Stuart, & Jones, 1987). Harrington et al. (1987) demonstrated that human LF and transferrin are capable of binding two iron(III) or copper(II) ions into specific binding sites in the presence of bicarbonate. Greater stabilization and increased resistance to protein unfolding is observed for all Fe(III) complexes compared to Cu(II) complexes of LF and transferrin as determined by isothermal unfolding and thermal denaturation. Each lobe of LF (C-lobe and N-lobe) binds one Fe^{3+} ion. For each Fe^{3+} ion, four amino acids (1 Asp, 2 Tyr and 1 His) and a carbonate ion (CO_3^{2-}) are involved in coordination bonds (Berlutti et al., 2011).

Overall and even if extensive *in vitro* antioxidant studies (for LF) are sorting out (Lee et al., 2020), the metal ion chelation mechanisms (iron, copper and others) are not comprehensively elucidated and/or confirmed (numerous conflicting results in the literature). Authors agree on at least one point, *i.e.* the antioxidant properties of LFs are not determined by a single factor but a combination of several related factors, from the purity to the structural features and the physicochemical properties of these proteins (Habib et al., 2013; Habib et al., 2021; Lee et al., 2020).

According to the present results, the antioxidant activity of eLF was due to its radical-scavenging power, its ferric iron ion reducing power, and its divalent pro-oxidant metals chelation ability. Given the important functional properties of LF and the growing interest, it is appropriate to continue to study new sources of LF, including in particular mare's milk.

CRediT authorship contribution statement

Zhanar Narmuratova: Methodology, Validation, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization. **Faiez Hentati:** Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Jean-Michel Girardet:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization. **Meyramkul Narmuratova:** Resources, Writing – original draft, Writing – review & editing, Supervision. **Céline Cakir-Kiefer:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors report no declarations of interest.

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