

Purification and Quantification of Lactoferrin in Equine Seminal Plasma

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ABSTRACT. Lactoferrin with a molecular mass of 80 kDa was purified from equine seminal plasma by heparin-Agarose affinity chromatography and Sephacryl S-200 gel filtration. Purified lactoferrin was found to be highly homogeneous on the bases of its migration as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and of the monospecificity of rabbit antibodies to the purified protein in immunoblotting of seminal plasma proteins. A sandwich enzyme-linked immunosorbent assay was developed for quantifying lactoferrin in equine seminal plasma. Seminal plasma lactoferrin concentrations in 23 normal stallions ranged from 42 to 453 $\mu\text{g/ml}$, with a mean value of $157 \pm 118 \mu\text{g/ml}$ (S.D.).

KEY WORDS: equine, lactoferrin, seminal plasma.

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Lactoferrin is an iron-binding protein with a molecular mass of about 80 kDa [1, 3], and is found in human various secretions [13]. Lactoferrin is present in the seminal plasma of humans and boars at a level ranging from 0.1 to 1.0 mg/ml [5, 13, 15], and is identified as a sperm-binding protein [8, 10]. The physiological role of seminal lactoferrin remains unclear. No information about lactoferrin in equine seminal plasma is available to date, although lactoferrin was recently identified in stallion epididymal fluid [6]. In the present study, as lactoferrin was detected in stallion seminal plasma, it was purified to homogeneity. Furthermore, seminal plasma lactoferrin was quantified.

Equine serum transferrin, which was purified previously [17], and rabbit antiserum to bovine milk lactoferrin, which was prepared as described [18], were used. Protein was determined according to the method of Lowry *et al.* [12] using bovine serum albumin as a protein standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as described [18].

The ejaculates of 23 mature Thoroughbred stallions (aged 7 to 20 years) were each centrifuged at $1,650 \times g$ for 20 min at room temperature, and the resulting seminal plasma samples were frozen at -25°C until use.

When equine seminal plasma was subjected to immunoblotting using anti-bovine milk lactoferrin antiserum as a probe, an 80-kDa protein was specifically detected (data not shown), suggesting this protein was lactoferrin. As heparin-Sepharose affinity chromatography has been used for purifying lactoferrin from human [4], equine [11], and bovine [18] milk, this method was considered to be applicable for purifying lactoferrin from equine seminal plasma. The seminal plasma was applied to a column (1.3×4.0 cm) of heparin-Agarose (Sigma, St. Louis, MO, U.S.A.), which had been equilibrated with 20 mM Tris/HCl, 100 mM NaCl, pH

7.4, at a flow rate of 23 ml/hr. After washing the column with the same buffer, lactoferrin was eluted with a linear gradient generated by mixing 150 ml each of 100 mM and 600 mM NaCl in 20 mM Tris/HCl, pH 7.4. The pooled eluate containing lactoferrin was brought to 80% saturation by gradual addition of the calculated amount of solid ammonium sulfate. The mixture was left for 5 hr and centrifuged at $10,700 \times g$ for 15 min at 4°C . The precipitate obtained was dissolved in 3 ml of 20 mM sodium phosphate, 500 mM NaCl, pH 7.2, and the resulting solution was applied to a column (2.0×100 cm) of Sephacryl S-200 (Amersham Pharmacia Biotech AB, Uppsala, Sweden), which had been equilibrated with the same buffer, at a flow rate of 13.2 ml/hr. Lactoferrin eluted as a single symmetric peak was used in subsequent experiments.

Antisera to equine seminal plasma lactoferrin were produced in female Japanese white rabbits (Clea Japan, Tokyo, Japan) using an immunization protocol described previously [14]. Antibody specific to lactoferrin was purified from the antisera by affinity chromatography on lactoferrin-bound Sepharose 4B, which was prepared by coupling 10 mg of purified lactoferrin to 10 ml of CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech AB).

A sandwich enzyme-linked immunosorbent assay (ELISA) for measuring the concentration of lactoferrin in equine seminal plasma was performed essentially according to the procedure described for determining equine serum ferritin [14]. The concentration of affinity-purified antibody to lactoferrin for coating was 2,000 ng/ml, and that of alkaline phosphatase-labeled anti-lactoferrin antibody prepared using glutaraldehyde [2] was 300 ng/ml. Phosphate-buffered saline (PBS: 20 mM sodium phosphate, 150 mM NaCl, pH 7.2) containing 0.05% Tween 20 was used to wash the plates. ELISA buffer consisting of PBS, 0.1% gelatin, 0.1% Tween 20, and 500 mM ammonium sulfate, pH 7.2, was used to dilute equine seminal plasma, purified lactoferrin as a standard, and the enzyme-labeled antibody.

Purified equine seminal plasma lactoferrin showed a sin-

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gle band with a molecular mass of 80 kDa on SDS-PAGE (Fig. 1A). Only a single band was detected when seminal plasma proteins were subjected to immunoblotting using antibodies to equine lactoferrin (Fig. 1B). These results indicate that purified lactoferrin was highly homogeneous. Purified lactoferrin was pink-colored and showed an absorption spectrum with a peak around 465 nm, which is characteristic of iron-bound lactoferrin [1]. The iron saturation of the protein was about 80% as estimated using a value of $A_{470\text{nm}}^{1\%} = 0.510 \text{ cm}^{-1}$ of iron-saturated human lactoferrin [1].

A sandwich ELISA was developed for measuring the concentration of lactoferrin in seminal plasma. A standard curve is shown in Fig. 2, indicating that the detection limit was about 1 ng/ml. This figure also shows that anti-lactoferrin antibodies did not cross-react at all with transferrin, even at a concentration of 100 $\mu\text{g/ml}$. Recoveries of 9.8 ng/ml, 19.6 ng/ml, and 39.2 ng/ml lactoferrin added to seminal plasma diluted 5,151-fold with ELISA buffer were $99.8 \pm 5.9\%$ (mean \pm S.D., $n=4$), $103.3 \pm 5.2\%$ ($n=4$), and $101.3 \pm 3.9\%$ ($n=4$), respectively. Intra-assay coefficients of variations from twelve measures of lactoferrin in two equine seminal plasmas were 5.0% ($161 \pm 8 \mu\text{g/ml}$) and 2.9% ($342 \pm 10 \mu\text{g/ml}$). Inter-assay coefficients of variations from nine measures of lactoferrin in two equine seminal plasmas were 4.0% ($149 \pm 6 \mu\text{g/ml}$) and 5.8% ($310 \pm 18 \mu\text{g/ml}$).

Seminal plasma lactoferrin from 23 apparently healthy horses was determined. The lactoferrin concentration varied from 42 to 453 $\mu\text{g/ml}$, with a mean value of $157 \pm 118 \mu\text{g/ml}$.

The molecular mass (80 kDa) of lactoferrin purified from equine seminal plasma in the present study is equivalent to that (76 kDa) of lactoferrin purified from human seminal plasma [16]. Although lactoferrin identified in boar seminal plasma has not been biochemically characterized [15], it was recently found that lactoferrin is first secreted as a 75-kDa glycoprotein and its carbohydrate moieties are gradually digested to form a 70-kDa protein in the porcine cauda epididymis [10]. Lactoferrin purified in the present study was highly iron-saturated. It cannot be excluded that a trace of iron contained in the reagents and distilled water used bound to lactoferrin during the purification process. Iron saturation of lactoferrin in stallion seminal plasma remains to be examined.

Lactoferrin is known to be secreted by the epididymis, but not by the testis or vas efferens in stallions [6]. It is, however, unclear whether the epididymis is the major secretion site of lactoferrin in stallion reproductive organs. In mice, the lactoferrin concentration is much higher in the vas deferens and epididymis than in the testis, seminal vesicle, prostate, and coagulating gland [20]. In contrast, lactoferrin is secreted by the seminal vesicle and prostate, but not by the testis, ductus deferens, or epididymis in humans [19].

Transferrin, which serves as a major iron-binding protein in serum, is known to be present in seminal plasma also, and its origin is Sertoli cells [7, 9]. Lactoferrin and transferrin are homologous [3], but these proteins from cattle have not

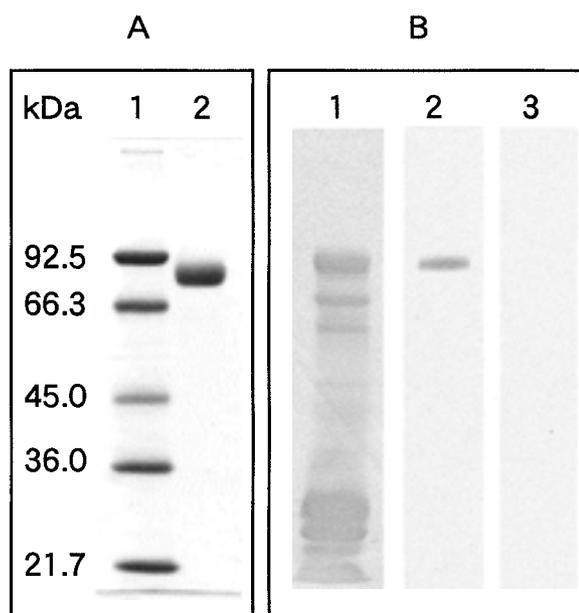


Fig. 1. SDS-PAGE of purified lactoferrin (A) and immunoblotting of seminal plasma proteins (B). (A) Samples are molecular mass standards (1 μg each, lane 1) and purified lactoferrin (2 μg , lane 2). (B) Blotting membranes, to which seminal plasma proteins separated by SDS-PAGE were transferred, were stained for total proteins with Coomassie Brilliant Blue R-250 (lane 1), or immunostained with rabbit antibodies to equine seminal plasma lactoferrin (lane 2) and normal rabbit IgG (lane 3). Anode at bottom.

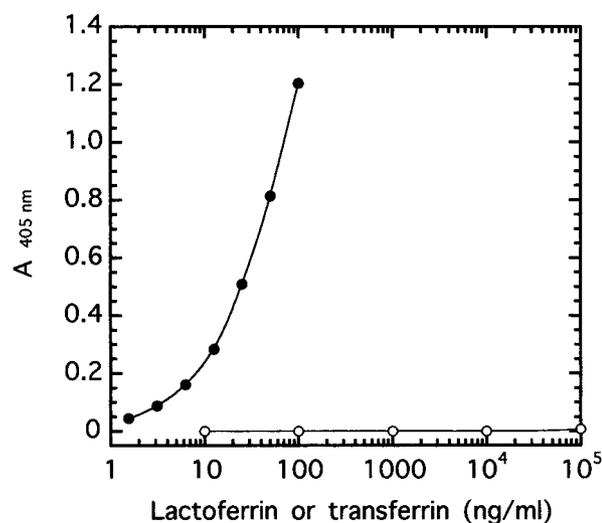


Fig. 2. ELISA standard curve for lactoferrin and cross-reaction of transferrin with anti-lactoferrin antibody. Equine seminal plasma lactoferrin (●) and serum transferrin (○) were used at concentrations ranging from 1.56 ng/ml to 100 ng/ml and from 10 ng/ml to 100 $\mu\text{g/ml}$, respectively.

been found to cross-react immunologically with each other in their native forms [18]. In our ELISA system for equine

lactoferrin, anti-lactoferrin antibodies also showed no immunological cross-reaction with transferrin under the conditions used, indicating the specific measurement of lactoferrin coexisting with transferrin in seminal plasma.

The present study was the first to measure the concentration of lactoferrin in equine seminal plasma. The concentration range of lactoferrin in stallion seminal plasma appears to be the same as that in boar seminal plasma [15]. Buckett *et al.* recently reported increased lactoferrin concentration in semen samples showing oligospermia and oligoasthenospermia compared to normospermic samples in humans [5]. Further studies need to determine whether there is any relationship between seminal plasma lactoferrin concentration and the number of sperm or sperm motility in stallions.

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