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Purification and Characterization of Lactoferrin from Camel Urine

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Abstract

This study reports the isolation, purification, characterization, and antimicrobial activity of lactoferrin derived from camel urine. The lactoferrin was purified through a sequential process involving acetone precipitation, dialysis, ion exchange chromatography, and gel filtration. The partially purified protein was then characterized with respect to its molecular weight, pH and temperature optima, metal ion interaction, and thermal stability. The purification process achieved a 3.23-fold increase in purity with a specific activity of 6.33 U/mg protein and an overall yield of 9.74%. The purified lactoferrin exhibited a molecular weight of 75 kDa and demonstrated optimal activity at pH 7.0 and 40 °C in 50 mM sodium phosphate buffer. Among the metal ions tested, Zn^{2+} enhanced lactoferrin activity, while Mg^{2+} and Al^{3+} acted as strong inhibitors. These findings confirm the potential of camel urine as a novel and viable source of bioactive lactoferrin and lay the groundwork for further biotechnological exploration of its therapeutic applications.

Keywords: Lactoferrin; Camel Urine; Protein Purification; Antimicrobial Activity; Biotechnological Potential

INTRODUCTION

Lactoferrin was first found in mammary secretions in 1939, it is a multifunctional protein that has been found in various secretions, which include milk, saliva, and urine (Avalos-Gómez *et al.*, 2022; Bobreneva and Rokhlova, 2021; Kowalczyk *et al.*, 2022; Levay and Viljoen, 1995). It is a member of the transferrin family and it is known for its ability to bind and transport iron ions (Chasteen and Woodworth, 2024; Ianiro *et al.*, 2023; Shimazaki *et al.*, 1998). Made up of about 700 amino acids stabilized by disulfide bonds, lactoferrin is a large protein with a molecular weight of about 75-80 kDa (Tran *et al.*, 2023). Lactoferrin has been shown to have antimicrobial, anti-inflammatory, and immunomodulatory properties, making it a potential therapeutic agent against various diseases (Lönnerdal, 2009; Lönnerdal and Iyer, 1995; Mayeur *et al.*, 2016; Pan *et al.*, 2021; Sienkiewicz *et al.*, 2022; Tsuda *et al.*, 2002).

Lactoferrin have been traditionally isolated from milk, moreover, it has been identified in various biological fluids, including urine, with Camel urine emerging as a novel and abundant source in arid regions where camels are prevalent (Al-Numair *et al.*, 2022). Camel urine has long been used for medicinal purposes, including the treatment of cancer, certain infectious diseases, and cardiovascular disorders (Alkhamees and Alsanad, 2017).

The purification of lactoferrin from Camel urine present new challenges due to the complexity of the source material, which necessitate robust isolation techniques such as centrifugation, ion exchange chromatography, size exclusion chromatography and subsequent characterization through sodium dodecyl sulfate-polyacrylamide gel electrophoresis to test the proteins purity, molecular weight. Investigating the structural and biological properties of Camel urine-derived lactoferrin, and bioactivity is critical to understanding its potential therapeutic utility.

In this present study aims to detail the methodologies for purifying and characterization of lactoferrin from Camel urine, contributing to the growing body of knowledge on alternative sources of this versatile protein and its application in modern medicine.

MATERIAL AND METHODS

Camel Urine Lactoferrin Assay

Isolation and Purification of Lactoferrin

A modified Isolation and purification procedure by (Yoshida *et al.*, 2000) was used. The procedure involved cation exchange chromatography (CEC) using cation exchanger Diethylaminoethyl Cellulose (DEAE Cellulose) and gel filtration chromatography using Sephadex G-75. The urine was precipitated using acetone. The cold acetone was diluted with distilled water, and added to the filtered urine. The mixture was stirred gently for 30 minutes to allow the protein to precipitate. The supernatant was discarded and the precipitate was retained. The precipitate was transferred to a dialysis bag (10 kDa MWCO). The precipitate was dialized against 50mM phosphate buffer (pH 7.0) for 24 hours at 30 ^{0}C. The buffer was changed after every 6 hours to remove impurities (Simpson and Beynon, 2010; Thongboonkerd *et al.*, 2002).

The dialized sample was loaded to a DEAE-Sepharose column for ion exchange chromatography. The column was equilibrated with 50 mM Tris-HCl buffer (pH 7.5) to remove unbounded proteins. The protein was eluted using a linear gradient elution of 50-300mM NaCl in Tris-HCl buffer (pH 7.5) (Radosavljević *et al.*, 2022).

Sephadex G-75 column was used for gel filtration chromatography. The column was equilibrated using 50 mM Phosphate buffer (pH 7.0). The fractions were eluted using step gradient elution, by using 50-300mM NaCl (Boesman-Finkelstein and Finkelstein, 1982; Wu *et al.*, 1995).

The Lactoferrin Activity

Lactoferrin activity was determined by the modified procedure using a spectrophotometric assay that monitors the oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) by lactoferrin. The enzyme solution (100 µL) was mixed with 1mL of 50mM Phosphate buffer at pH 7.0. After preincubation for 10min at 30 °C, the reaction was initiated by the addition of 200µL of 50mM ferrous chloride solution in 10mM HCl. The change of absorbance at 460nm was measured every 30 s for 4min at 30 °C. One unit of lactoferrin activity was defined as the amount of protein needed to catalyze the oxidation of 2 moles of ferrous iron to 3 moles of ferric iron.(Majka *et al.*, 2013)

Protein Determination

Protein was determined using the Bradford method. A protein determination method that involves the binding of Coomassie Brilliant Blue G-250 to protein. The binding of the dye to protein causes a shift in the absorption maximum of the dye from 465 to 595 nm, and it is the increase in absorption at 595 nm that is monitored (Bradford, 1976).

Camel Urine Lactoferrin Characterization

Molecular Weight Determination by Electrophoresis (SDS PAGE)

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis was performed under reducing conditions using NuPAGE 4-12% Bis-Tris precast gels with 3-(N-morpholino) propane sulfonic acid (MOPS) SDS running buffer at a pH 7.7 (Solarbio, China), following the manufacturer's instructions. The precast gel was rinsed with deionized water and the comb and bottom tape is removed, the gel cassette is inserted into the electrophoresis chamber secure with clamps. 500 µL of NuPAGE antioxidant is added to the inner chamber and 3-(N-morpholino) propane sulfonic acid the outer chamber. The Camel urine lactoferrin is loaded into the gel wells and the pre-stained protein ladder is added in separate well for molecular weight reference. The protein bands were detected by staining with Brilliant Blue Coomassie G-250 (Kiy, 2024; Lewinski *et al.*, 2024).

Effect of pH on Activity and pH Stability of Purified Lactoferrin

A modified procedure by (Liu *et al.*, 2011) was used. The optimal pH for camel urine lactoferrin was determined by incubation of the purified lactoferrin solution within the pH range of 2-10 at 40 ˚C for 10 min. To produce the pH gradient, appropriate buffers were used: 50mM citrate buffer (pH 2- 6), 50mM sodium phosphate buffer (pH 6 – 7.5), 50mM Tris-HCl (pH 7.5 – 9.0), and 50mM glycine-NaOH (pH 9.0 – 10). To determine the effect of pH on the stability of lactoferrin, the protein solution was incubated for 10 minutes at 40 ^{0}C, and the residual activity was measured.

Effect of Temperature on Activity and Thermal Stability

A modified procedure by (Liu *et al.*, 2011) was used. The optimum temperature of the protein reaction was determined in 50 mM phosphate buffer (pH 7.0) over a

temperature range of 20 – 90 ^{0}C for 10 min. Thermal stability was also tested by incubation of the purified lactoferrin in the same buffer over the same temperature range for 1hr.

Effect of Metal ions on Purified Lactoferrin

A modified procedure by (Liu *et al.*, 2011). The purified lactoferrin was incubated with 5mM solutions of salts of metal ions (chlorides of Mg^{2+}, Ca^{2+}, Zn^{2+}, K^{+}, Al^{3+}) at 40 ^{0}C for 10 min and their activities were measured. The activities was measured in the presence of metal ions and calculated relative to the activity in the absence of metal ions.

RESULTS

Camel urine lactoferrin purification

The purification of Camel urine lactoferrin is summarized in Table 1. The effect of acetone precipitation reduces the total protein to 104.78 mg, and a decrease of total activity to 228.3 units, but gave an improvement of specific activity of 2.18 units/mg, and decrease yield of 51.48%. The effect DEAE ion exchanger further reduces the total protein from 104.78 mg to 21.38 mg, and also decreases the total activity to 106.25 units. Specific activity increases to 4.97 units/mg, and a decrease yield of 23.96%. The effect of SG-75 gel chromatography reduces the total protein to 6.83 mg, and a large decrease in the total activity to 43.2 units. However, Specific activity reaches 6.33 units/mg. The overall yield 9.74% was obtained. All these results indicate that Camel urine lactoferrin remained correctly folded in all purification processes.

Approximate molecular weight

Figure 1, showed that camel urine lactoferrin has a molecular weight of 75 kDa.

Optimum pH determination

The results shows a pH levels (2-10) corresponding for the camel urine lactoferrin relative activity and residual activity, which indicated that camel urine lactoferrin was optimally active at a pH of 7.0 and displayed a broad pH profile. Relative activity peaks at pH 7 (100%) and its lowest at pH 2 (46.82%). Residual activity also peaks at pH 7 (94.63%) and is lowest at pH 2 (41.64%). Both activities increase from pH 2 to 7, then decline slightly through pH 10, with relative activity consistently higher than residual activity across all pH levels.

Optimum temperature determination

The effect of temperature evaluated across temperatures from 20˚C to 90˚C revealed that Camel urine lactoferrin relative activity peaked at 40˚C (100%), with the lowest at 20˚C (57.54%). Residual activity followed a similar pattern, highest at 40˚C (93.9%) and lowest at 90˚C (49.7%). Both activities increased from 20˚C to 40˚C, then gradually declined through 90˚C, with relative activity consistently higher than residual activity across all pH levels. This is been summarized in figure 3.

Table 1- Purification of lactoferrin from camel urine

Purification Steps	Volume (ml)	Total Protein (mg)	Activity (units)	Total Activity (Units×ml)	Specific Activity (Unit/mg)	Fold Purification	% Fold
Crude Extract	50	225.88	8.87	443.5	1.96	1.0	100
Acetone Precipitation	30	104.78	7.61	228.3	2.18	1.11	51.48
DEAE Ion-Exchanger	25	21.38	4.35	106.25	4.97	2.54	23.96
S-G75 Gel Chromatography	15	6.83	2.88	43.2	6.33	3.23	9.74

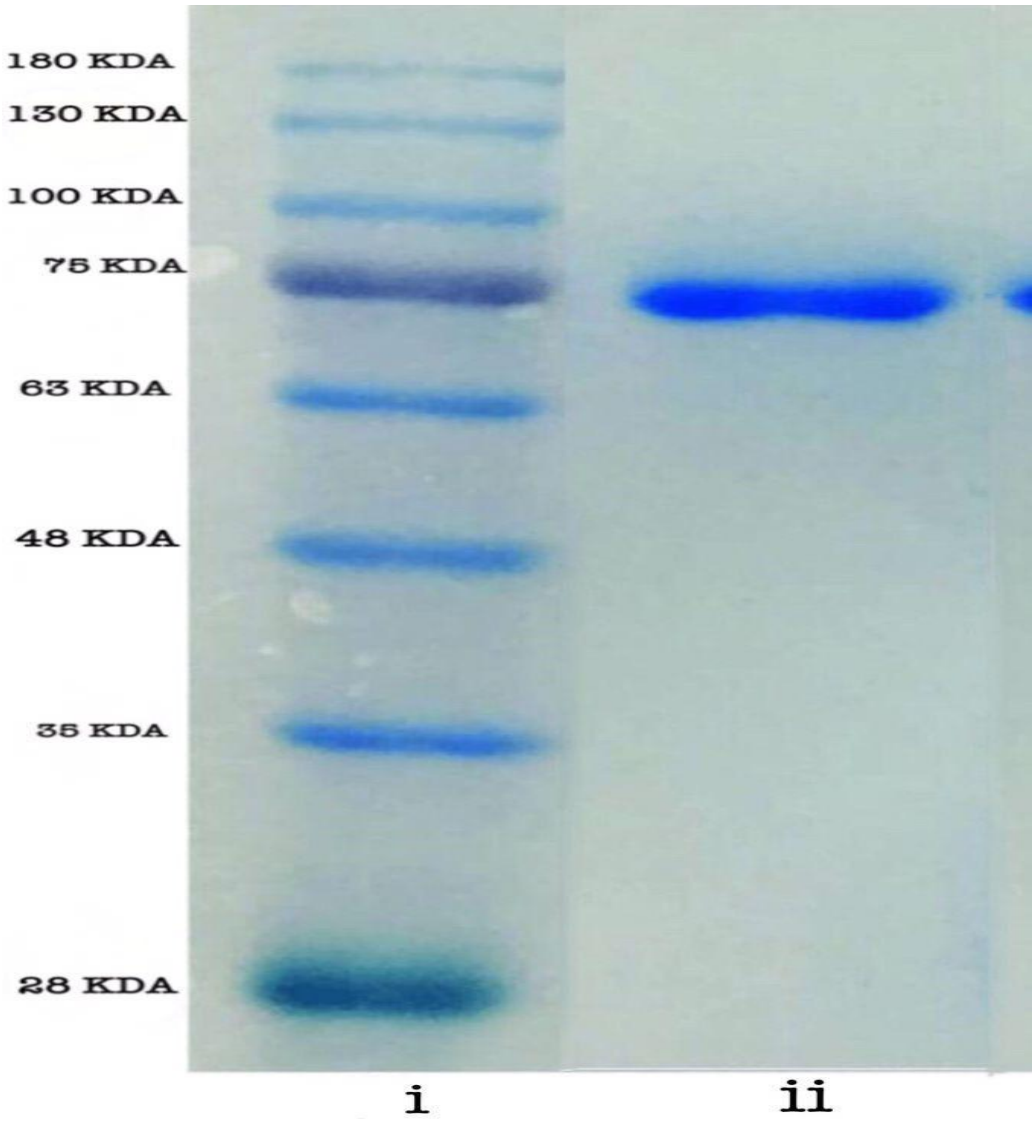

Figure 1- SDS-PAGE analysis of lactoferrin sample under reducing conditions. (i) Molecular mass marker (ladder); (ii) SDS from Camel urine lactoferrin

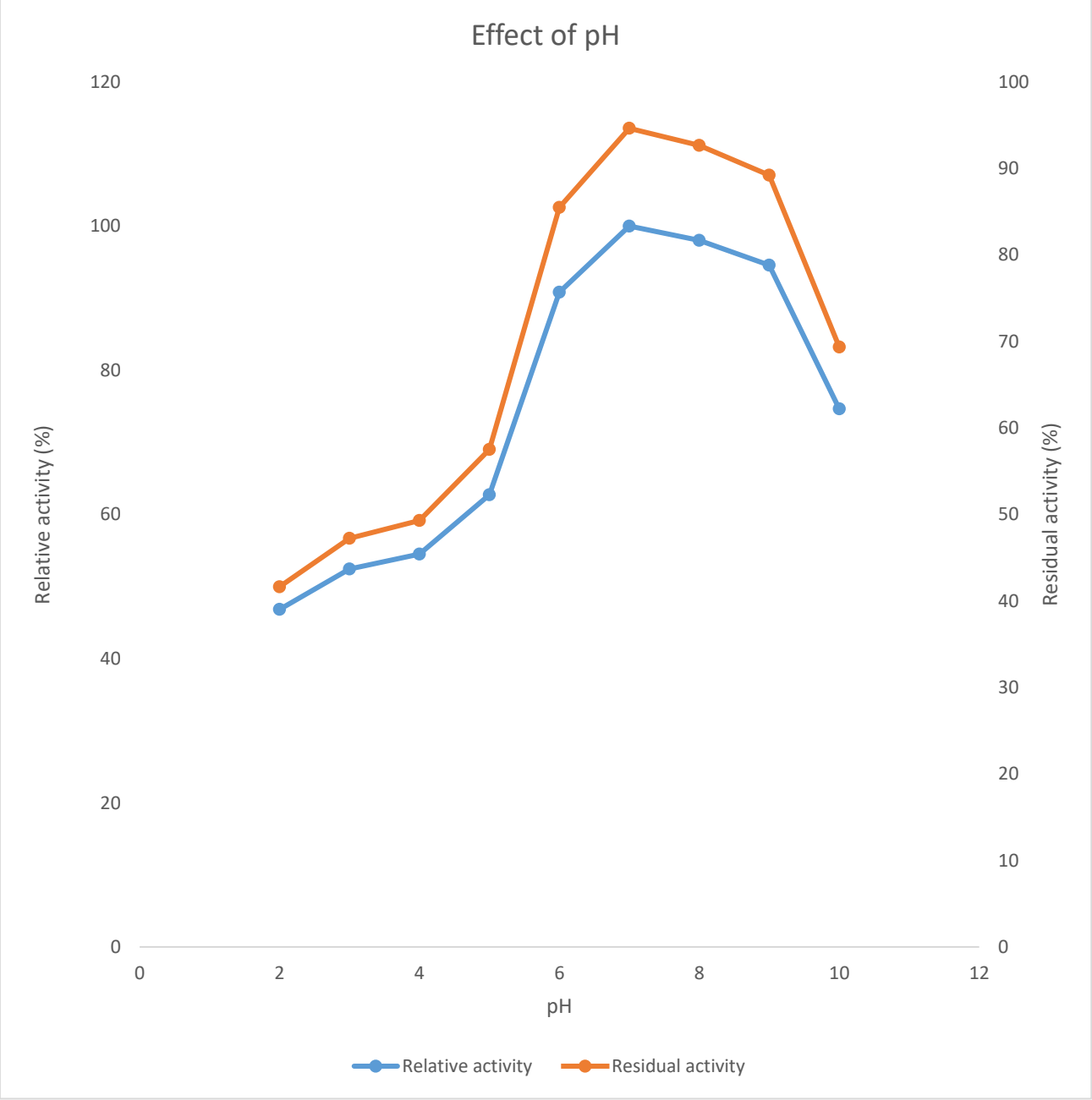

Figure 2- Optimum pH and stability curve of Camel urine lactoferrin. For each pH, the activity was assayed at 40∘C and pressed as relative activity. The pH stability curve represents the residual activity after the preincubation period of 10mins at the indicated pH

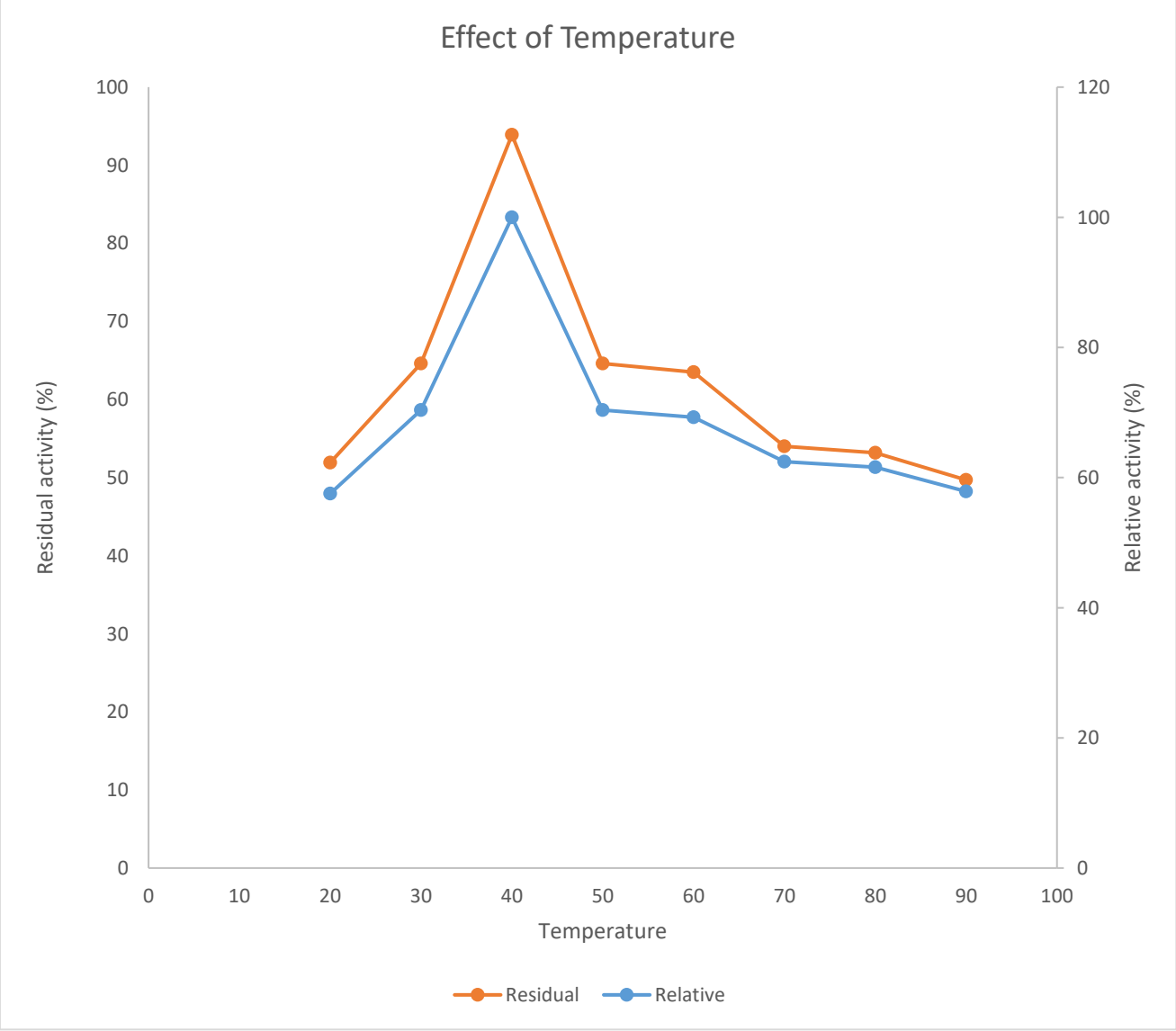

Figure 3- Effect of temperature on the activity and thermal stability of camel urine lactoferrin. Enzyme activity was assayed for each temperature after an incubation period of 10min. For thermal stability experiments, the purified enzyme was prewarmed at the indicated temperature for 1h, and then the remaining activity was determined.

Stability

The effect of pH and temperature on the stability of camel urine lactoferrin shows that the enzyme was stable in the pH range of 3.0 to 10.0. It is therefore relatively stable to alkali pH. At pH values less than 3.0, the enzymatic activity dropped to below 46.82% of maximal activity. As the figure shows, a large decrease of camel urine lactoferrin when the temperature was below 40∘C, but at 60∘C it lost 30.73% of its original activity within

10mins of treatment, and it lost 42.11% at 90∘C after treatment in the same incubation period. This thermal stability clearly shows that camel urine lactoferrin can withstand heat temperatures.

Effect of metal ions on the activity of the enzyme

The results are given in the table below. Among the cations used Zn^{2+} was found to have a stimulating effect on the enzyme activity. This indicates that the purified lactoferrin might belong to the Zn-lactoferrin group. In which zinc acts as the link metal ion in the lactoferrin structure. The results also show that camel urine lactoferrin activity was inhibited by Zn-lactoferrin inhibitors. Ca^{2+} also inhibits the activity of lactoferrin by 67.13%, Mg^{2+} and Al^{3+} were found to be strong inhibitors of lactoferrin and reduce the activity by 71.26% and 69.39% respectively. K^{+} was found not to have any stimulatory or inhibitory effect on camel urine lactoferrin.

Table 2. – Effect of metal ions on the activity of lactoferrin

Reagents	Final concentration	Relative activity (%)
Control	-	100
Ca^{2+}	5 mM	32.87
Mg^{2+}	5 mM	28.74
Al^{3+}	5 mM	30.61
Zn^{2+}	5 mM	108.47
K^{+}	5 mM	99.78

DISCUSSION

The result from the purification table indicates a low yield of lactoferrin in camel urine. The result clearly shows that Camel urine is not a rich source of lactoferrin like Camel milk (Alkhulaifi *et al.*, 2024).The low yield of lactoferrin can be attributed to higher detection of lactoferrin in urine is mostly the cases of urinary tract infection (Rosa *et al.*, 2024), kidney diseases such as nephritic syndrome, and other health issues (Hassan *et al.*, 2021). However, lactoferrin can be higher in camel urine due to hydration and in newborn camels. The reason for the high lactoferrin present in camel newborns could be attributed to maternal transfer from milk, immune system development, kidney development, and stress response (Ho *et al.*, 2024).

The molecular weight indicated that Camel urine lactoferrin has a large molecular weight of 75 kDa. Narmuratova *et al.*, (2024) Also found a single band of 75 kDa in the "In silico determination of physicochemical properties of lactoferrin peptides isolated from equine milk". X. Zhang *et al.*, (2025) in the "Efficient heterologous expression of bovine lactoferrin in Pichia pastoris and characterization of antibacterial activity" found the molecular weight of lactoferrin to be 76 kDa. Abbas *et al.*, (2015) in the "Isolation, purification and characterization of lactoferrin from goat colostrum whey," found the molecular weight of lactoferrin to be 77 kDa. (Elagamy *et al.*, 1996) The "Purification and characterization of lactoferrin, lactoperoxidase, lysozyme and immunoglobulins from camel's milk" found the molecular weight of lactoferrin to be 78 kDa molecular weight. Zumoffen *et al.*, (2013) Reported in "A protein isolated from human oviductal tissue in vitro secretion, identified as human lactoferrin, interacts with spermatozoa and oocytes and modulates gamete interaction" found it to be 79 kDa. Kim *et al.*, (2009) reported in the "Purification and characterization of Mongolian mare lactoferrin" found it to be 82 kDa molecular weight. The variability of the molecular weight with other species of lactoferrin that were isolated in other studies may be due to species, location, glycosylation, and experimental methods (Dyrda-Terniuk and Pomastowski, 2023). However, lactoferrin exhibits heterogeneous glycosylation, meaning the number of sugars attachment sites varies across different species(Piacentini *et al.*, 2024). Furthermore, even within the same species, this variability in glycosylation sites can depend on the tissue where the protein is expressed (Karav *et al.*, 2017; Zlatina and Galuska, 2021). Glycosylation significantly influences lactoferrin's stability and its resistance to being broken down by proteolytic enzymes, primarily by enhancing the solubility of proteins once they are secreted and improving the

ability of lactoferrin to attach to certain types of cells or specific receptors. Nonetheless, this process has a minimal impact on lactoferrin thermal stability or its capacity to bind to and release iron (Piacentini *et al.*, 2024).

The study reveals that the optimal range for Camel urine lactoferrin activity ranges from pH 5.0 to 10.0, and it clearly shows that camel urine lactoferrin works more effectively in an alkaline environment than acidic environment. This can also be attributed to the alkalinity of the Camel urine (Amina et al., 2024). Studies have shown that lactoferrin undergoes conformational change (Chasteen and Woodworth, 2024; Dyrda-Terniuk and Pomastowski, 2023), protonation of histidine residues (Santos-Pereira *et al.*, 2021), disruption of electrostatic reaction and ligand exchange reaction in an acidic pH (below 4.0) (Pryshchepa, Rafińska, et al., 2022). The conformational change exposes the iron-binding site at the C-terminal, which leads to the protonation of the histidine residues. Histidine residues are crucial for iron binding, the protonation reduces the affinity of iron to lactoferrin which disrupts the electrostatic interaction between lactoferrin and iron, facilitating the release of iron (Chasteen and Woodworth, 2024; Ianiro et al., 2023).

The effect of temperature on the activity of the partially purified lactoferrin from camel urine shows that it is optimally active at 40˚ C temperature. Within this range, lactoferrin maintains its native structure, iron-binding affinity, and biological activity (Gruden and Poklar Ulrih, 2021; Jańczuk *et al.*, 2022). This shows that lactoferrin's activity and structure are sensitive to temperature. However, the purified lactoferrin lost most of its activity at a temperature below 20 ˚C this may be due to conformational changes, reduced iron-binding affinity, and increased susceptibility to proteolysis. Studies have shown that lactoferrin undergoes conformational changes in cold temperatures, which reduces its iron-binding affinity, making it less effective at sequestering iron (Dyrda-Terniuk and Pomastowski, 2023; Piacentini *et al.*, 2024). Cold temperatures can make lactoferrin more susceptible to proteolytic degradation, further reducing its activity. The reduction in the activity of lactoferrin at higher temperatures may be due to unfolding and aggregation or loss of iron-binding capacity (Goulding *et al.*, 2021; Zeng *et al.*, 2023).

The effect of metal ions on the activity of partially purified lactoferrin shows that Zn^{2+} has a stimulating effect on Camel urine lactoferrin. Studies have shown that supplementation Zn^{2+} to bovine lactoferrin enhances the antimicrobial effect of lactoferrin (Pryshchepa *et al.*, 2022a; Pryshchepa *et al.*, 2022b) This indicates that zinc serves as a

cofactor of lactoferrin and might belong to the zinc lactoferrin group (Shini *et al.*, 2022). This clearly shows that camel urine-derived lactoferrin can also be inhibited by Zn inhibitors (Ianiro *et al.*, 2023). Ca^{2+} was found to inhibit the activity of lactoferrin, while Mg^{2+} and Al^{3+} were found to be strong inhibitors of lactoferrin respectively. Studies have shown that Ca^{2+}, Mg^{2+} and Al^{3} inhibit Bovine and Camel milk lactoferrin functionality, primarily by competing with iron for binding sites and altering its structural conformation (de Sautu *et al.*, 2024). K^{+} was found not to have any stimulatory or inhibitory effect on camel urine lactoferrin, this may be attributed to the fact that it lacks the chemical properties or binding affinity required to interact meaningfully with lactoferrin. Previous studies with bovine lactoferrin have shown that K^{+} lack stimulatory or inhibitory effects on bovine lactoferrin stems from its monovalent nature, low binding affinity, and inability to induce conformational or functional changes (Pryshchepa *et al.*, 2022a; Pryshchepa *et al.*, 2022b).

CONCLUSION

In the present work, lactoferrin was successfully purified from lactoferrin using a modified procedure of precipitation with acetone and the purified protein was the characterized by SDS-PAGE. The protein activity and protein content in lactoferrin was also determined. The proposed purification method from lactoferrin has a practical value from camel urine lactoferrin production for future use. The purified protein is fully folded, and fully active that can also provide suitable material for further structural and functional analysis.

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Author Contributions

K.A. Ahmad, and A.U. Wurochekke: Conceptualization; K.A. Ahmad: Formal analysis; K.A. Ahmad: Investigation; K.A. Ahmad: Funding; K.A. Ahmad and A.U. Wurochekke: Methodology; A.U. Wurochekke: Project administration; A.U. Wurochekke and M.S. Jada: Supervision; A.U. Wurochekke and M.S. Jada: Validation; K.A. Ahmad: Visualization; K.A. Ahmad: Writing - original draft; A.U. Wurochekke and M.S. Jada: Writing - review & editing. All authors have read and agreed to the final version of this manuscript

Conflict of Interest

The authors have no competing interest to declare.

Data availability

K.A. Ahmad, will provide the data that support the study's findings upon request.

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