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Inhibitory Effect of Heparin Inhibitor on Camel Urine Lactoferrin

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Abstract

Lactoferrin, an iron-binding protein of the transferrin family, is a basic protein known for its interaction with acidic biomolecules, including heparin proteoglycans. These interactions can influence lactoferrin's biological functions. This study investigates the inhibitory effect of heparin on lactoferrin activity, revealing a dose-dependent inhibition correlating with increased heparin concentration. Kinetic analysis yielded a V_{max} of 7.01 U/min, K_m of 1037.66 μM, and K_{at} of 8.73 × 10^{-18} s^{-1}, reflecting the enzyme's catalytic efficiency. Inhibition studies showed that heparin acts as a non-competitive inhibitor, with an IC_{50}, K_i, and Kd all equal to 102.06 μM, indicating moderate affinity for lactoferrin. A binding constant (K_β) of 0.0098 μM^{-1} further supports this moderate binding interaction. These findings suggest that heparin binds to the N-terminal region of lactoferrin, modulating its function through non-competitive inhibition. The study provides insights into the biochemical regulation of lactoferrin and its interaction with glycosaminoglycans, with potential implications for therapeutic applications involving inflammation and host defense mechanisms.

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Keywords: Lactoferrin; Heparin; Non-Competitive Inhibition; Protein–Ligand Interaction; Binding Affinity

INTRODUCTION

Lactoferrin is iron binding protein closely related to transferrin, which is found in tears, saliva, and it is primarily found in secondary granules of neutrophils (Avalos-Gómez *et al.*, 2022; Bobreneva and Rokhlova, 2021; Kowalczyk *et al.*, 2022). Studies have suggested that lactoferrin may have an anti-inflammatory role, either through binding and inhibiting the pro-inflammatory effects of bacterial lipopolysaccharide or by scavenging `free' iron at sites of inflammation, thus preventing the catalytic production of toxic oxygen species such as hydroxyl radicals (Ahmed *et al.*, 2021). Another lactoferrin notable effect is its ability to neutralize the anticoagulant effect of heparin, which is widely used glycosaminoglycan used in treatment and preventing thrombotic disorders (Meher *et al.*, 2024). Heparin have shown to exerts its anticoagulant effect by enhancing antithrombin`s inhibition of thrombin and factor Xa, which are critical components of the coagulation cascade(Koirala *et al.*, 2025; Yu, 2025). However, excessive heparin activity can lead to bleeding complications, necessitating agents that can modulate or neutralize its effect(Holail *et al.*, 2025).

Lactoferrin is a highly basic protein, and it usually interacts with many acidic molecules, and such interactions may modify the biological properties of lactoferrin, which made it a potent heparin-neutralizing agent (Weiss, 2015). In particular, lactoferrin is known to interact with the acidic glycosaminoglycan heparin and to neutralize its anticoagulant activity(Bolesławska *et al.*, 2025; Capitani, 2025). The inhibitory effect is also attributed to lactoferrin cationic nature, which allows it to bind strongly to negatively charged sulfate groups of heparin, this process disrupts heparin`s ability to form complexes with antithrombin(Kumar *et al.*, 2012; Sultana and Kamihira, 2024). It has been reported that this interaction involves the basic N-terminus, a region that is also important in binding to cell surfaces and for its antimicrobial activity (Hu *et al.*, 2025).

This study aims to investigate the inhibitory effect of heparin on Camel urine lactoferrin, exploring the potential mechanisms underlying the interaction. A deeper understanding of this interaction will provide valuable insights into the biological and

therapeutic applications of Camel urine lactoferrin, and may give insights on the development of novel anticoagulant therapies.

MATERIALS AND METHODS

Material

The lactoferrin was isolated from Camel urine using acetone precipitation, DEAE ion exchange chromatography, and gel filtration using Sephadex G-75.

Standard drug

Caprin Heparin injection of 25,000 IU/5 mL was obtained from Novomed pharmaceuticals Zoo road, Kano. The heparin was used as the inhibitor of the partially purified lactoferrin from Camel urine.

Kinetics studies

Effect of substrate concentration and determination of Km and Vmax

The effect of substrate concentration was determined by adding different concentrations of ferrous chloride (200 µL – 1.0 mL) into the assay mixture to study the effect on lactoferrin activity. The kinetic constants Km and Vmax was determined graphically with initial velocity measurements obtained at various substrate concentrations as reported by (Copeland, 2000). This was determined from the Lineweaver-burk plot. A plot of 1/V vs 1/[S] Plot was generated.

Determination of Turnover number (Kcat) and Catalytic efficiency

Kcat was determined using the formula: $Kcat = Vmax/[E_T]$. The catalytic efficiency was determined using the formula: Kcat/Km = Vmax/Kcat

Camel Urine Lactoferrin Kinetics Studies

Inhibitory Effect of Heparin on Purified Lactoferrin

This was determined using the Lineweaver-burk plot as reported by (Copeland, 2000; Glumoff, 2009). At first, 100 µL of the purified enzyme was put in the test tube, in a fixed concentration of the heparin inhibitor, the substrate concentration ranging from (200 µL, 400 µL, 600 µL, 800 µL, and 1000 µL) was added to the various test tubes, and finally 50Mm phosphate buffer was added to the reaction and the activity in the presence of inhibitor was measured. A plot of 1/V vs. 1/[S] in the absence of an inhibitor was plotted using the data of

the effect of substrate concentration, and on the same graph a plot of 1/ [VI] was plotted using the reciprocal of the substrate concentration of the same values, in the absence of an inhibitor and the presence of an inhibitor.

Determination of IC_{50}, Inhibitor constant (K_i), Dissociation constant (Kd), Binding constant (Kb)

The IC_{50} was determined from the logarithmic equation of the dose-response graph of percentage inhibition on the y-axis and the logarithmic inhibitor concentration on the x-axis (1/Vi vs $\log_{10}$ C) (Sebaugh, 2011). The inhibitor constant (Ki) was determined using the (Cheng and Prusoff, 1973) equation of the relationship between IC_{50} and Ki

Kd was determined from the value of the Ki, since Ki = Kd (Cheng and Prusoff, 1973)

Kb was determined using the reciprocal of Kd, where, Kb = 1/Kd

RESULTS

Effect of Substrate Concentration

The result shows that as the substrate increases from 200µl to 1000µl the activity of camel urine lactoferrin increase from 1.13 ms^{-1} to 3.37 ms^{-1}, indicating higher reaction rates. Correspondingly, reciprocal of the substrate concentration decreases from 0.005 to 0.001 μM^{-1}, and reciprocal of the activity decreases from 0.887731 to 0.29698 ms^{-1}, reflecting the inverse relationship.

The Vmax value shows that 7.012723 U/min is the maximum rate of the enzymatic reaction, while the km value of 1037.658 µM represents the substrate concentration at which the reaction is half of Vmax.

The result also gave a turnover number (Kcat) is 8.73×10^{-18} $min^{(-1)}$ and catalytic efficiency of 8.42×10^{-21} $M^{-1}s^{-1.}$

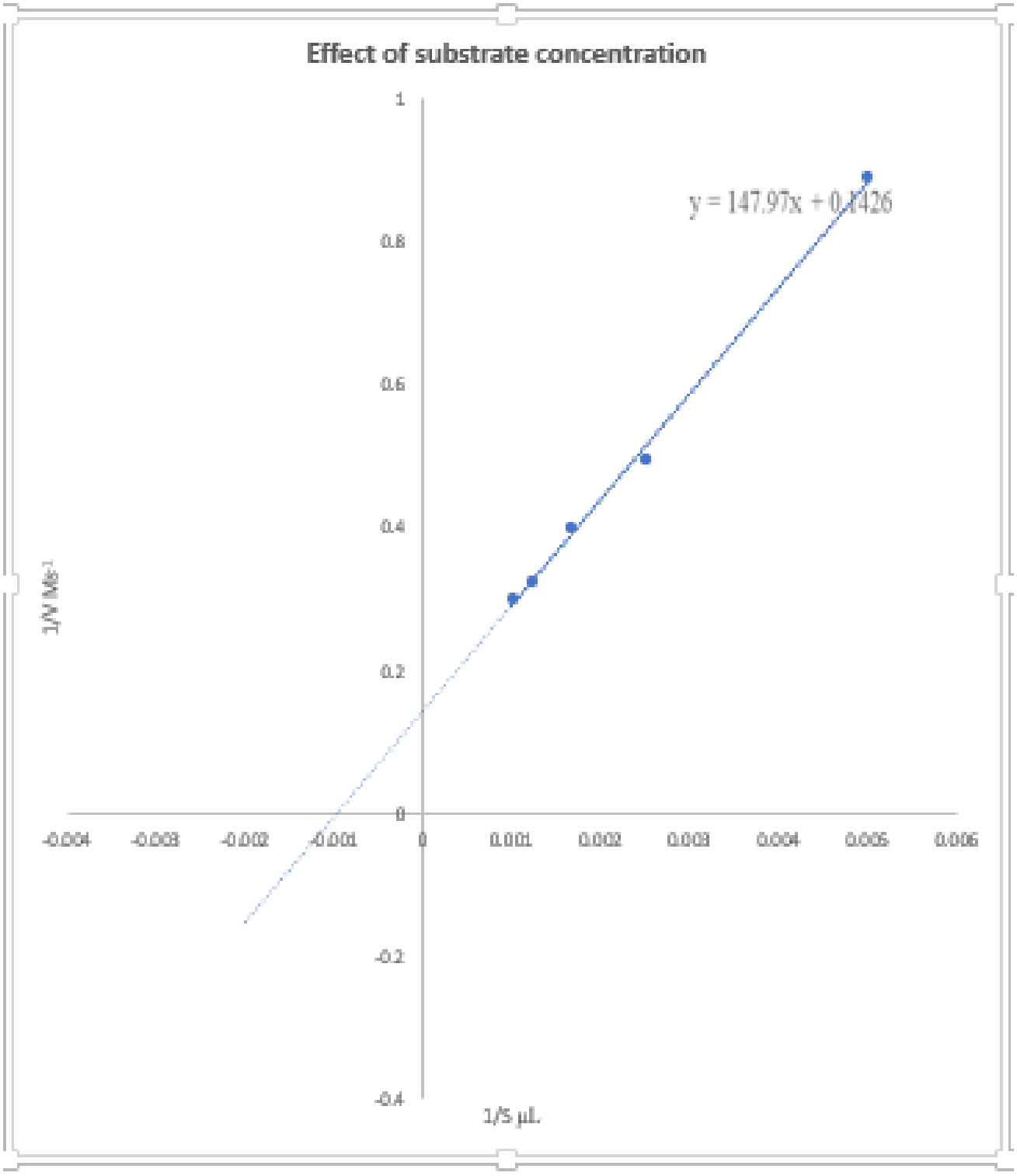


Figure 1- The Lineweaver Burk plot of 1/V against 1/S on the effect of substrate concentration.

Inhibition of Camel Urine Lactoferrin by Heparin

Figure 2 shows a non-competitive inhibition. The slope from the equation on the graph of $1/V_0$ (substrate) is 147.97, and the slope from $1/V_i$ of the graph is 70.811. The intercepts on the 1/V axis are (0.1426, and 0.2926), there is lowering of the Vmax, and the Km of substrate remain unchanged. The intercepts on the 1/V and 1/Vi axis of the graph are different, and the slopes of the lines are different. At concentrations of 200 µL and 400 µL shows an activation of the purified lactoferrin by 1.52 ms^{-1}, and 2.265834 ms^{-1} respectively. The increase in the concentration of the substrate at 600 µL, 800 µL, and 1000 µL decreases the activity of Camel urine lactoferrin by 2.431835 ms^{-1}, 2.561205 ms^{-1} and 2.689995 ms^{-1} respectively.

Half-maximal inhibitory concentration (IC_{50}) of Camel urine lactoferrin

Figure 3, shows the IC_{50} Camel urine lactoferrin. These results indicate that at 200 µL and 400 µL the inhibitor appears to activate the activity, as indicated by the negative percentage inhibition values. At 600 µL, 800 µL, and 1 mL, the heparin exhibits inhibitory activity, with percentage inhibition values ranging from 2.4% to 20.1%.

There is an increase in the percentage inhibition values with increasing concentrations of the substrate, which indicates a concentration-dependent effect. The final result from the graph gave and IC_{50} of 102.06 µM.

Inhibitory constant (Ki) of Heparin Inhibitor on Camel Urine Lactoferrin

From the IC_{50} obtained, using the Cheng and Prusoff relationship between inhibitory constant (Ki) and IC_{50}, the Ki for non-competitive would be 102.06 µM

Dissociation constant (Kd) of Heparin Inhibitor on Camel urine lactoferrin

From the IC_{50} obtained, using the relationship between Ki and Kd, the Kd is 102.06 µM

Binding Constant (Kb) of Heparin Inhibitor on Camel Urine Lactoferrin

The reciprocal of the dissociation constant gives a binding constant (Kb) of 0.0098 μM^{-1}

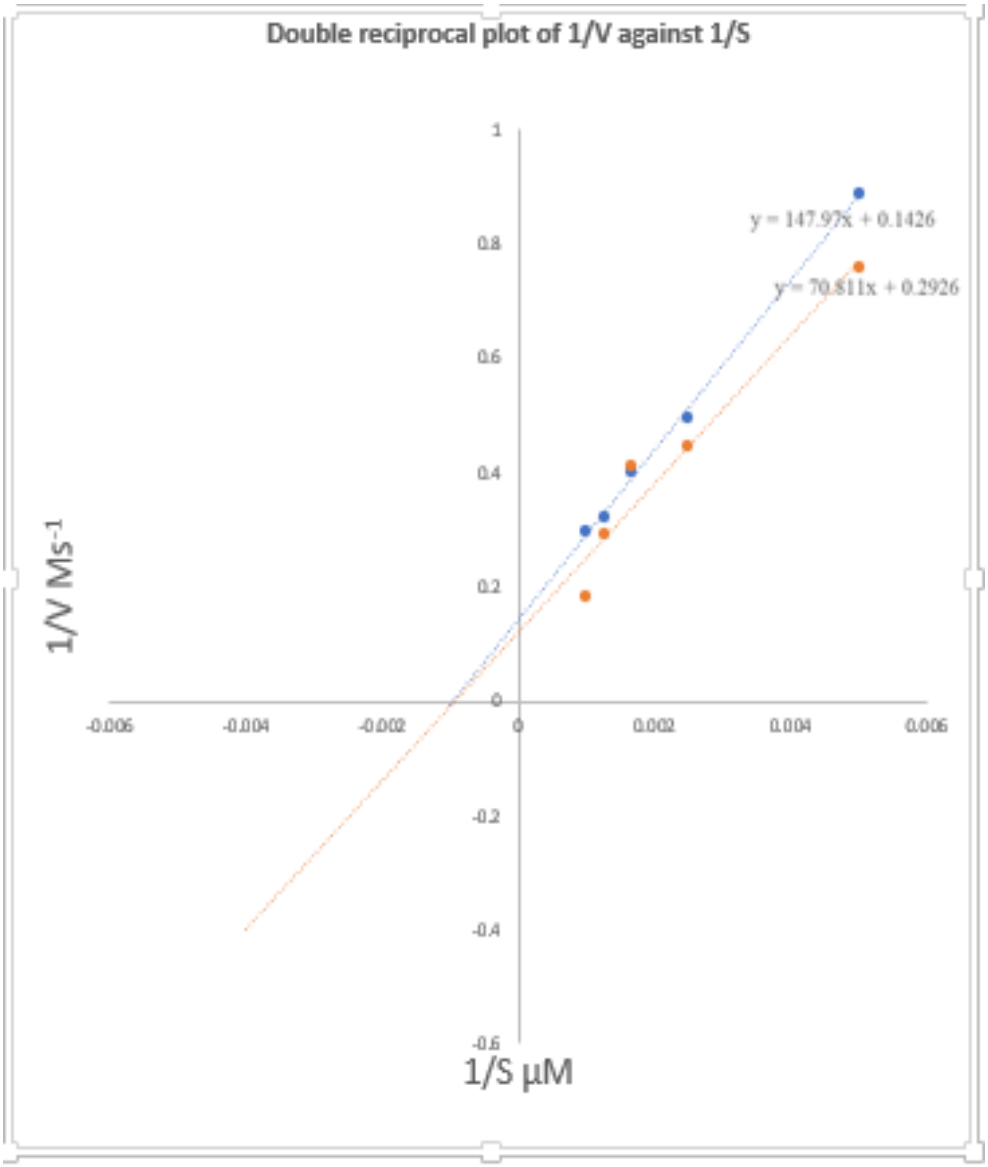


Figure 2- Effect of substrate concentration and heparin inhibitor concentration on camel urine lactoferrin

Table 1 – IC_{50} (50% inhibition)

Inhibitor concentration (log10)	Percentage inhibition (%)
0.68	-35.2
2.11	-11.9
2.29	2.4
2.41	17.3
2.51	20.1

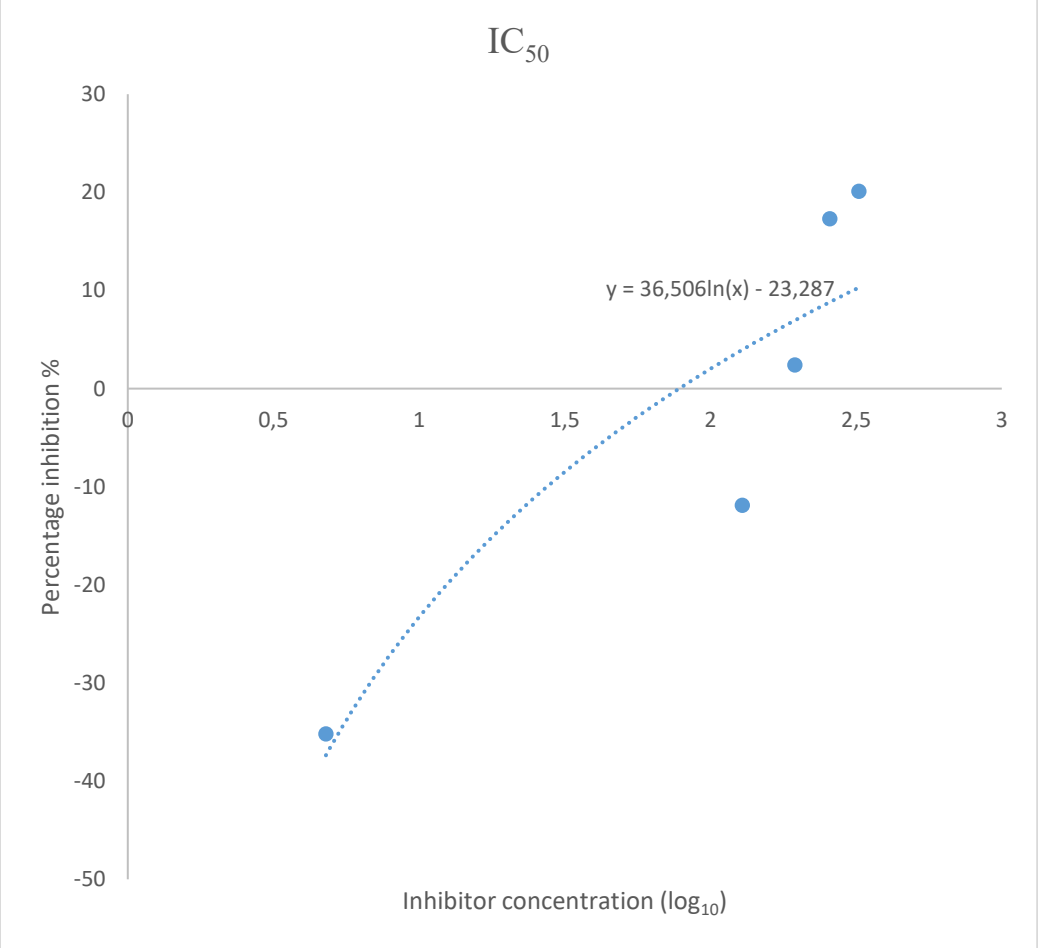


Figure 3 – Logarithmic Curve (IC_{50}) of 50% Inhibition of Lactoferrin by Heparin

DISCUSSION

The effect of substrate concentration on the activity of the partially purified lactoferrin result shows that the activity increases with increased substrate concentration, reaching a maximum (Vmax) of 7.01 U/min. This shows that the concentration of lactoferrin in the solution cannot exceed 7.01 mg/ml, and at this concentration, the solution became saturated and no more substrate can bind to the Camel urine lactoferrin. The value also suggested that the binding site is being filled at that concentration, and any attempts to increase the concentration may lead to precipitation, aggregation, or unwanted effects. The Km value indicates a relatively low affinity of the lactoferrin for the ferrous chloride, as a higher Km value suggests that a higher substrate concentration is required to achieve half-maximal velocity. This also shows the interaction between lactoferrin and ferrous chloride is relatively

weak, which may be beneficial in regulating the lactoferrin activity or allowing for more efficient iron release.

The catalytic efficiency of Camel urine lactoferrin determined suggests that camel urine lactoferrin has a relatively low value compared to its Km value, which indicates that the protein is not very efficient at converting substrate to product, and it requires a relatively high substrate concentration to achieve its maximum velocity. This may also suggest that lactoferrin has a high affinity for iron and it may likely be a transport protein. The Kcat value suggests that the Camel urine lactoferrin has a relatively slower turnover rate, meaning it takes a longer time for the protein to convert substrate to product. Studies have shown that any catalytic activity for bovine lactoferrin (e.g., RNase like or protease like) is generally low compared to dedicated specialized enzymes like RNase A or trypsin. Also specific values like Kcat and Km also indicate weak values (Soboleva et al., 2019)

The result of inhibition of lactoferrin by heparin shows a non-competitive inhibition. Heparin is a well-known anticoagulant and its interaction with lactoferrin has been studied in numerous research (Hoxha and Hodaj, 2021; H. H. Hwang *et al.*, 2023). The result of this research also shows that there is activation of Camel urine lactoferrin at a lower concentration of the heparin, this also suggested that a higher concentration of heparin is needed to inhibit the activity of Camel urine lactoferrin. Studies have also suggested that heparin has an allosteric binding site at the N-terminal different from the binding site of the substrate and it also has a moderate affinity to lactoferrin (Wang *et al.*, 2019). Lactoferrin structure has a binding site at both the N-terminal and C-terminal, which could allow simultaneous binding of heparin and iron. However, an increase in the absorbance of the sample was observed in this research, which suggests that binding of the heparin at the N-terminal binding site causes a conformational change to the C-terminal binding site of iron in lactoferrin structure, which results in excess ferrous chloride in the reaction.

Heparin is a highly charged polysaccharide that interacts with lactoferrin and potentially modulates its activity(Meng et al., 2024; Wang et al., 2019). The interaction between lactoferrin and heparin has been attributed to the electrostatic attraction between the negatively charged heparin and the positively charged lactoferrin (Dyrda-Terniuk and Pomastowski, 2023; Hong *et al.*, 2024; Y. Zhang *et al.*, 2024). This interaction affects lactoferrin's ability to bind to iron, which is essential for its antimicrobial activity. The binding

of heparin to lactoferrin may also influence lactoferrin's interaction with other proteins or molecules.

The IC_{50} signifies the amount of heparin inhibitor required to inhibit 50% of lactoferrin activity. This also suggested that the heparin inhibitor would have an effect on lactoferrin activity. Studies on Camel milk lactoferrin and bovine lactoferrin have reported that a higher IC_{50} inhibits the antiviral function of lactoferrin by forming complexes with Camel milk and bovine lactoferrin, thereby preventing its interaction with viral receptors like heparan sulfate on cell surfaces (Akdaşçi *et al*., 2024; Kaplan *et al*., 2024; Mohammadabadi, 2021). Moreover, at a concentration below the IC_{50} value, the inhibitor would have a lesser effect, and at a concentration higher than the value the inhibitor would have a greater effect on the lactoferrin activity. This implies that heparin is effective in inhibiting lactoferrin activity, but it would require a relatively higher concentration to achieve significant inhibition. This could have implications for the design of experiments or therapeutic applications, where the concentration of heparin may need to be optimized to achieve the desired level of inhibition. The Ki value indicates that heparin is a moderate inhibitor which implies that heparin has a moderate binding affinity for lactoferrin. This also suggested that at a concentration of 102.06 μM, heparin occupies half of the lactoferrin binding sites. The Kd value of heparin suggests that heparin may not be highly specific and binds to lactoferrin with moderate affinity. The binding affinity is strong enough to be biologically relevant but not strong enough that it won't be reversible. The value also suggests that heparin can bind to other proteins or molecules with similar affinity, potentially leading to off-target effects. The Kb value suggests that iron and heparin can have a weak binding with lactoferrin, and also suggests a low binding affinity and they can also be reversible.

CONCLUSION

The result from this study suggest that heparin is a potent inhibitor of camel urine lactoferrin. The finding suggest that heparin binds to lactoferrin with moderate affinity, thereby inhibiting its activity.The inhibition of camel urine lactoferrin by heparin may have important implications for the understanding of the biological functions of lactoferrin and its interactions with sulfated glycosaminoglycan's. This study provides a foundation for future research on the application of lactoferrin inhibitors for therapeutic purpose.

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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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