# Antibacterial activity of peptides homologous to a loop region in human lactoferrin

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**Abstract Human lactoferrin contains a 46 residue sequence named lactoferricin H thought to be responsible for its antimicrobial properties. Synthetic peptides HLT1, corresponding to the loop region of human lactoferricin (FQWQRNMRKVRGPPVS) and HLT2, corresponding to its charged portion (FQWQRNMRKVR), exerted significant antibacterial effects against *E. coli* serotype O111 strains NCTC 8007 and ML35. The corresponding sequences in native human lactoferrin were shown to adopt a charged helix and hydrophobic tail within the N-lobe remote from the iron binding site. Sequence similarities between lactoferricin and dermaseptin and magainins suggest that lactoferricin may act as an amphipathic alpha helix.**

*Key words:* Lactoferrin; Lactoferricin; Antimicrobial peptides

## 1. Introduction

Lactoferrin is an iron-binding glycoprotein of the transferrin family found in the specific granules of neutrophils and in secretions including tears, saliva and milk. It is thought to exert an antimicrobial effect at these sites [1,2] independent of its iron chelating activity [3]. Pepsin digestion of bovine and human lactoferrin releases antibacterial peptides named lactoferricin B and H respectively which may account for this antimicrobial activity [4,5]. Lactoferricin is effective against Gram-positive and Gram-negative bacteria as well as yeasts [6] but its mechanism of action is unknown.

Human lactoferricin comprises two chains from residues 1–46 from the N-terminus of lactoferrin and includes an 18 residue loop formed by one of two internal disulphide bridges [4]. We have synthesised peptides with sequences corresponding to parts of the loop region, assessed their antibacterial activity and compared their structures to those of other known antimicrobial peptides and to the structure of the same sequence in native human apolactoferrin. Throughout this report we adopt residue numbers from the currently accepted structure for human lactoferrin [7]. Residue numbers differ from those in previous publications [4,5] by one residue because of the elimination of one arginine residue between positions 2 and 4 from the previously published sequence [8].

## 2. Materials and methods

Lactoferrin was purified to homogeneity, as assessed by SDS polyacrylamide gel electrophoresis from human milk whey by heparin-Sepharose chromatography [9]. Peptide HLT1, corresponding to the loop region of human lactoferricin (residues 20–35; $NH_2$-FQWQRNMRKVRGPPVS-COOH), was synthesised using *Fast*moc (Applied Biosystems Inc.) chemistry on an Applied Biosystems 431A solid phase peptide synthesizer. The protected peptide was cleaved and deprotected using the cleavage mixture phenol/ethanedithiol/water/trifluoroacetic acid and precipitated using diethyl ether. The peptide was dried and dissolved in water for reverse phase HPLC purification. The product was analysed on a Fisons Instruments VG Platform electrospray mass spectrometer. Peptide HLT2, corresponding to the positively charged portion of the loop region of human lactoferricin (residues 24–35; $NH_2$-FQWQRNMRKVR-COOH), was synthesised commercially (Neosystem Laboratoire, Strasbourg). Peptide HLT5, corresponding to the uncharged portion of the loop region (residues 31–35; $NH_2$-GPPVS-COOH), was synthesised using Fmoc protection on a Rainin PS3 solid phase peptide synthesiser (Protein Technologies Inc. USA) using previously described procedures [10]. A mixture of two peptides, each containing part of the positively charged potentially helical region, was prepared by cyanogen bromide cleavage of peptide HLT1 on the C-terminus of the single methionine residue. Purity of peptides was assessed by HPLC and mass spectrometry. The effectiveness of HLT1 cleavage was confirmed by SDS-PAGE.

Antibacterial activity was tested against *E. coli* serotype 0111 NCTC 8007 and *E. coli* ML35 [11], a species resistant to the action of apo-lactoferrin alone [1]. Suspensions of $10^7$/ml early log phase bacteria, determined spectrophotometrically, were incubated with varying concentrations of lactoferrin and peptides in 1% proteose peptone (Oxoid, Basingstoke, UK) for 2 h at 37°C. Residual viability was assessed by serial dilution and drop counting on nutrient agar plates.

The structure of the loop region of lactoferricin H in native human apo-lactoferrin was examined using coordinates of the apolactoferrin structure [7] and figures were generated using the Evans and Sutherland PS390 graphics system and FRODO software [12]. Sequences were compared for structural similarity by manual alignment of published sequence data for human and bovine lactoferricin [4], dermaseptin [13] and magainins I and II [14].

## 3. Results

Peptides HLT1 (whole loop) and HLT2 (positively charged portion of loop) exhibited potent anti-bacterial activity against *E. coli*. In contrast, native apolactoferrin was inactive (Fig. 1) under the same conditions. Both the peptides and the cyanogen bromide fragments of HLT1 exert a dose dependent effect which is equally potent against both strains. There is no significant difference between the potency of HLT1 and HLT2. The cyanogen bromide cleavage products retain significant antibacterial activity. Peptide HLT5, consisting of the uncharged part of the loop, had no activity (Fig. 1). Killing by HLT1 was time dependent and maximal at 90 min in this model although much more rapid effects are detectable by other methods (data not shown).

In native human apolactoferrin the loop region of human

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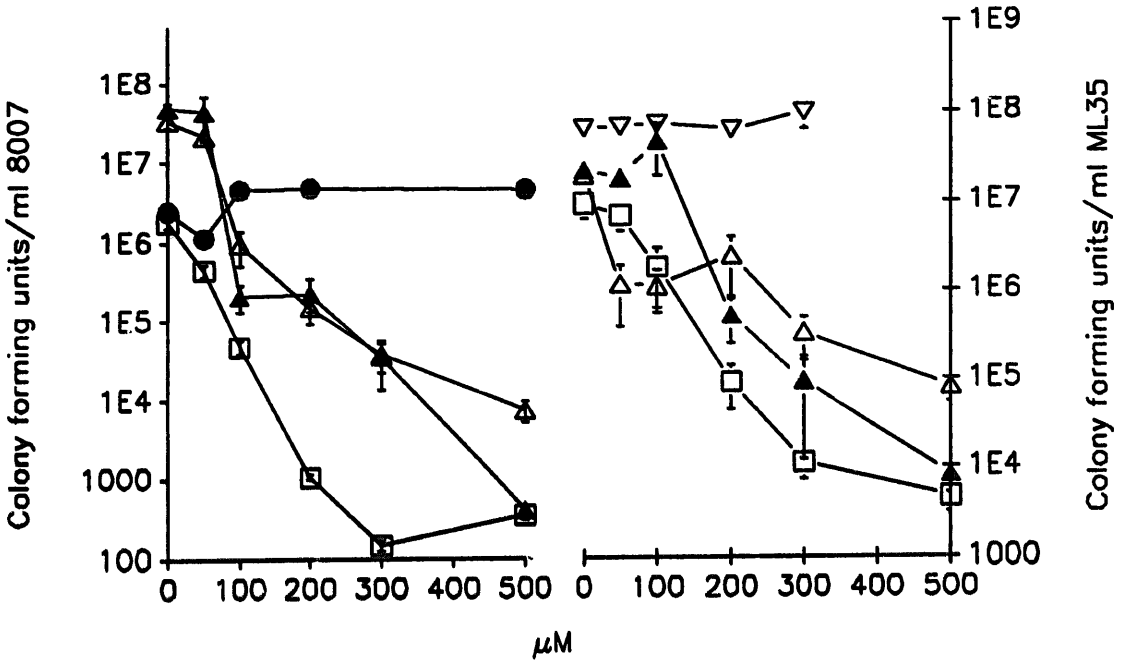


Fig. 1. Antibacterial activity of lactoferrin, peptides and cleavage fragments against *E. coli* strains 8007 (left) and ML35 (right). Mean viability±standard error derived from a minimum of 4 experiments; lactoferrin (●), HLT1 (▲), HLT2 (△), HLT5 (▽) and cyanogen bromide cleaved HLT1 (□).

lactoferricin was found to form an α-helix with a short hydrophobic tail in the N-terminal lobe (Fig. 2). The region corresponding to HLT2 comprises the complete helical portion. This helix is exposed on the surface of the molecule away from the iron binding site with its four basic residue side chains extending outwards (Fig. 3).

A similarity is revealed between the primary structure of various lactoferrins, human and bovine lactoferricins and dermaseptin and magainins, antibacterial peptides from frog and toad respectively. This is enhanced by including insertions and extending the sequences beyond the antimicrobial segments (Table 1).

## 4. Discussion

Two larger peptides corresponding to residues 17–39 of human lactoferricin and residues 17–41 from bovine lactoferricin have previously been shown to be antibacterial [4]. The human peptide comprises two peptide chains linked by a disulphide bridge. Antibacterial activity is known to be independent of a second disulphide bridge which closes the loop region. We have shown that a much smaller 11 residue peptide (HLT2) homologous to just over half the loop region has potent antibacterial activity and may account for all the activity of the larger HLT1 peptide. HLT1 contains four positively charged residues and charge is likely to be an important activity determinant because the antibacterial activity of lactoferrin [1,15] and lactoferricin [5,16] is probably dependent on binding to the bacterial surface. The potency of the killing is slightly less than has been reported previously for the larger human and bovine peptide in a different antibacterial assay system [4]. It is not clear whether this might be due to differences in the target organism, peptide purity, the target : peptide stoichiometry or to the sequences themselves. No antibacterial effect of intact apo-lactoferrin against *E. coli* was demonstrated in our assay system and this is in keeping with previous work indicating that lactoferrin has either no effect [1] or at best a bacteriostatic effect [17] against *E. coli*.

Table 1
Sequence alignment of antimicrobial peptides with lactoferrins including regions beyond the antimicrobial segments

| Peptide | Sequence | Reference |
|---|---|---|
| Dermaseptin I | ALWKTMLKKL-GTMALHAGKAALGA... | [18] |
| Magainin I | EVRGIGKFLHSAGKF-GKAFVGEIMKSKRD... | [14] |
| Magainin II | EVRGIGKFLHSAKKF-GKAFVGEIMNSKRD... | [14] |
| Bovine lactoferrin | EWFKCRRWQWRMKKL-GAPSITCV---RRAF.. | [32] |
| Mouse lactoferrin | EEEKCLRWQNEMRKV-G-PPVSCI---KKS... | [31] |
| Human lactoferrin | EATKCFQWQRNMRKVRG-PPVSCL---KRD | [7] |
| Peptide HLT1 | FQWQRNMRKVRGPPVS | |
| Peptide HLT2 | FQWQRNMRKVR | |

Residues in bold are homologous between one or more lactoferrin sequence and one or more antibacterial sequence. Homologies within each group are not highlighted. Underlined sequences represent bovine lactoferricin and the longer peptide chain of human lactoferricin which contains the loop region. Peptides HLT1 and HLT2, homologous to human lactoferrin, are also shown.

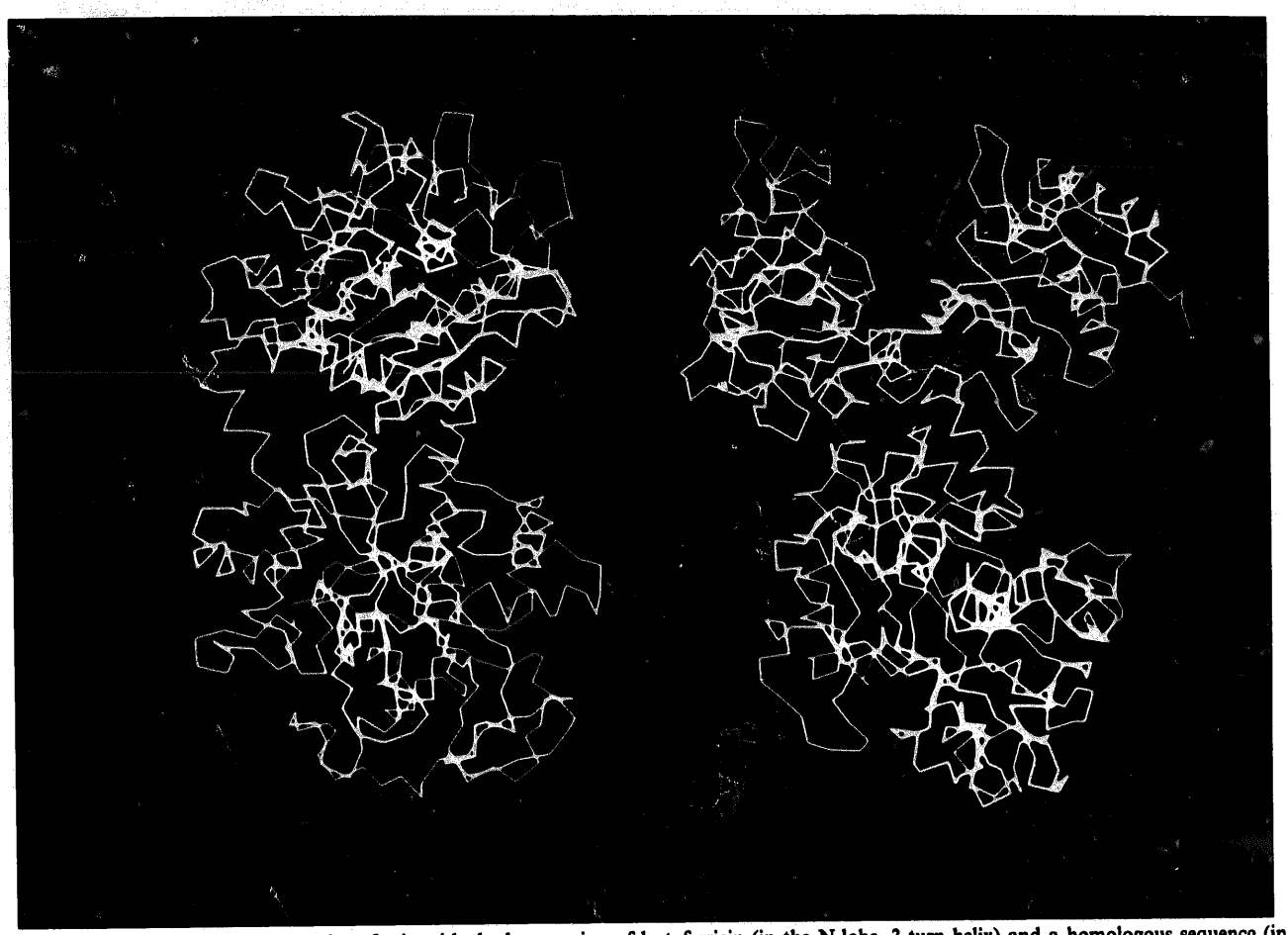

Fig. 2. 3-D structure of human apolactoferrin with the loop region of lactoferricin (in the N-lobe, 3 turn helix) and a homologous sequence (in the C-lobe, 2 turn helix) highlighted. N-lobes upper, two views rotated 90° on a vertical axis. The C-lobes are closed. Co-ordinates from Anderson et al. [7].

Cleavage of HLT1 with cyanogen bromide yields two peptides each with a positive charge and an aromatic terminal residue. The finding that these fragments retained antibacterial activity similar to an equimolar concentration of HLT1 is intriguing and it is not clear whether one or both products might account for the activity. Other peptides based on these cleavage products will be investigated in future work.

It would appear significant that the loop region can adopt a helical conformation in apo-lactoferrin as this is a feature of some antibacterial peptides. An amphipathic α-helical 18 residue region at the amino terminus of dermaseptin [18] and synthetic homologous peptides [19] appears to be responsible for their antibacterial activity. Magainins also adopt a helical structure [20] and are thought to act by forming a tetrameric pore in which the helix lies parallel to the target membrane [21]. Cecropins have a similar hinged helical structure [22] and may also rely on polymer formation for their action [23]. All share the ability to form anion pores and all have a wide spectrum of antibacterial activity [24]. CAP18 [25] and histatin [26] are also partly α-helical. Some, like lactoferricin and HLT1, have their charged residues on one side of the helix and it is their helical nature rather than their chirality which is critical [27,28]. Monomeric HLT2 appears too short to span a bacterial membrane although other synthetic amphipathic α-helical peptides as short as 9 [29] and 15 [30] residues retain bactericidal activity. However, it is possible that these shorter peptides have a different mechanism of action [29,30]. There is no experimental evidence to suggest that HLT1 or HLT2 form helical structures in free aqueous solution and this seems unlikely given their short length.

Further evidence of similarity between these peptides and helical antibacterial peptides is suggested by alignment of their primary sequences. Previously it has been considered that no similarity exists beyond the density of positively charged residues [4]. Similar types of amino acid occur in equivalent positions and these partial homologies will be exploited in the design of peptides for further work. The actual 3D structure of lactoferricin derived peptides remains to be elucidated. Determination of solution structure has not yet been attempted because similar short peptides are known to adopt their helical conformation only when bound to membranes [20].

We have observed that the loop region of human lactoferricin forms a charged α-helix on the surface of lactoferrin in a site remote from the iron binding site. Peptides homologous to the charged helical portion of the peptide exert anti-bacterial activity in vitro and their action may be related to that of other antibacterial peptides of the amphipathic alpha helix family. Further work is in progress to characterise their mechanism of action using peptides designed in the light of our reported sequence similarities.

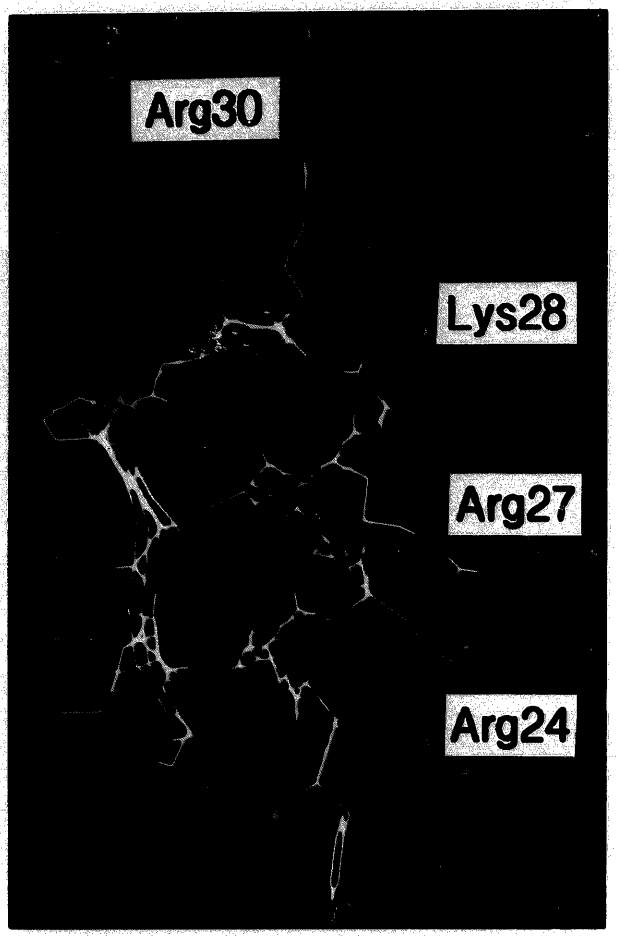


Fig. 3. Ribbon model of lactoferricin showing the helical conformation adopted in native human apolactoferrin. The charged arginine and lysine side chains are clustered on one side of the helix. Note the comments on residue numbering in section 1.

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