# EXPRESSION, PURIFICATION AND CHARACTERISATION OF ANTIMICROBIAL PEPTIDES OF HUMAN AND BOVINE LACTOFERRINS

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## Abstract

Lactoferrin (Lf), a basic, ~80 kDa iron-binding glycoprotein, is a member of the transferrin family. It is present in the milk and other secretory fluids of many, but not all, mammalian vertebrates. Several biological functions have been ascribed to this protein. These include bacteriostastis, modulation of the inflammatory response, iron nutrition, a role as an anti-oxidant and regulation of myelopoiesis.

Full-length human lactoferrin has been expressed in BHK cells, many strains of *Aspergillus* and with limited success in *Saccharomyces cerevisiae*. The main aim at the start of this project was to express full-length human lactoferrin (hLF) cDNA in the yeast *Kluyveromyces lactis* on whey-based media. Yeasts of the genus *Kluyveromyces* have been used for many years in the food industry and are classified as "Generally Regarded As Safe" (GRAS) organisms. *K. lactis* has impressive secretion capabilities and can grow on whey-based media (which is abundantly available in New Zealand). Attempts were made to sub-clone full-length hLF cDNA into the *K. lactis* vector, pEPS1 and the *S. cerevisiae* vector, pYEXS1 and to express the protein. The establishment of stable insert-carrying constructs of these yeast vectors in *E. coli* turned out to be an unattainable goal. Direct transformation of the ligation mix into *K. lactis* produced transformants, which secreted human lactoferrin protein products into the media as assessed by the lactoferrin ELISA assay. The secretion of hLF protein products by recombinant *K. lactis* continued for few generations, but gradually stopped. There are no known reports on the use of these vectors for the expression of any mammalian proteins in yeast.

Lactoferrin has antimicrobial activity against a broad range of Gram-negative and Gram-positive bacteria and against fungi. Originally, the antimicrobial effect of lactoferrin was attributed to its ability to tightly sequester two atoms of iron and hence inhibit microbial growth through nutritional deprivation of iron. Recently, an N-terminal peptide called lactoferricin, isolated from the acid-pepsin hydrolysate of lactoferrin was

shown to have greater antimicrobial activity than the intact protein.

Currently, the only way to obtain native lactoferricins is to isolate the peptides from the acid pepsin-hydrolysate of lactoferrin, which gives very low yields, or to synthesise them by protein chemical methods, which is very expensive on a large scale. So, heterologous expression of both human and bovine lactoferricins in *E. coli* was attempted in this study. Synthetic DNA fragments encoding both human and bovine lactoferricins and 3′-end variants of these fragments were sub-cloned into *E. coli* expression vectors, pPROEXHTa, pET-15b and pGEX-4T1. The constructs were designed to express lactoferricins either as poly-His- or as GST-fusion proteins. In all cases the fusion proteins were expressed as inclusion bodies. The inclusion bodies were urea solubilised and purified on appropriate affinity resins. However, none of the recombinant proteins remained soluble after the urea was removed and therefore could not be further characterised. A similar situation was encountered by other investigators who attempted to express cationic peptides in *E. coli.*

Both lactoferrin and lactoferricin have been shown to bind to the lipid A portion of the bacterial cell wall lipopolysaccharide (LPS), induce the release of LPS and kill the bacteria. In this work, five different *E. coli* strains were shown to have different susceptibility to native lactoferricin B. Transmission electron microscopy studies of the *E. coli* strains treated with lactoferricin B revealed considerable differences in their membrane ultrastructure. The most susceptible *E. coli* strain showed a direct outer membrane dislocation and effect on the cytoplasmic contents. A relatively resistant *E. coli* strain showed an initial formation of 'membrane blisters'. However, after a long exposure to lactoferricin B, a few cells of this strain showed an outer membrane dislocation and effect on the cytoplasmic contents. The formation of 'membrane blisters' might allow the relatively resistant strain of *E. coli* to reduce the lethal action of lactoferricin B.

## List of publications and presentations

This work has been published or presented in part in the following papers.

1. Rallabhandi, P. and Yu, P-L (1996). Production of Therapeutic Proteins in Yeasts: A Review. *Australasian Biotechnology*. Volume 6, No. 4: 230-237.

2. Rallabhandi, P and Yu, P-L (1996). Production of Recombinant Human Lactoferrin in Yeast *Kluyveromyces*. *Proceedings of Third New Zealand Conference of Postgraduate Students in Engineering and Technology*, Christchurch, New Zealand

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## Abbreviations

| | |
|---|---|
| amp or Ap | ampicillin |
| ARS | autonomous replicating sequence |
| ATP | adenosine triphosphate |
| $A_x$ or $OD_x$ | absorbance at x nm |
| BHK | baby hamster kidney cells |
| bLF | bovine lactoferrin |
| bp or bps | base pairs |
| BRL | Bethedsa Research Laboratories |
| BSA | bovine serum albumin |
| cDNA | complementary deoxyribonucleic acid |
| CFU | colony forming units |
| cpm | counts per minute |
| C-terminal | carboxy terminal |
| dATP | deoxyadenosine triphosphate |
| dCTP | deoxycytidine triphosphate |
| DEPC | diethylpyrocarbonate |
| dGTP | deoxyguanosine triphosphate |
| DMSO | dimethyl sulphoxide |
| DNA | deoxyribonucleic acid |
| DNase | deoxyribonuclease |
| ds | double-stranded |

| | |
|---|---|
| DTT | dithiothreitol |
| dUTP | deoxyuridine triphosphate |
| EDTA | ethylene diamine tetra acetic acid |
| EEO | electroendosmosis |
| ELISA | enzyme-linked immunosorbant assay |
| GM | granulocyte macrophage |
| GM-CSF | granulocyte macrophage colony stimulating factor |
| GRAS | generally regarded as safe |
| HEPES | N-2-hydroxy ethyl piperazine-N'-2-ethane sulfonic acid |
| hGH | human growth hormone |
| hLF | human lactoferrin |
| HPLC | high performance liquid chromatography |
| hTf | Human transferrin |
| HSA | human serum albumin |
| Ig | immunoglobulin |
| IPTG | isopropyl-D-thiogalactopyranoside |
| Kan | kanamycin |
| L or l | litre |
| LB | Luria-Bertani |
| Lf | Lactoferrin |
| LFcin-B | Bovine lactoferricin |
| LFcin-H | Human lactoferricin |
| Lfn | the amino terminal half of human lactoferrin |
| LMP | low melting point |
| MCS | multiple cloning site |
| MES | 2 (N-morpholino) ethane sulphonic acid |
| MM | minimal medium |
| MOPS | morpholinopropane sulphonic acid |
| MW | molecular weight |
| MWCO | molecular weight cut off |
| N-terminal | amino terminal |
| NTA | nitrilotriacetic acid |

| | |
|---|---|
| OD | optical density |
| ORF | open reading frame |
| ori | origin of replication |
| PAGE | polyacrilamide gel electrophoresis |
| PB | phosphate buffer |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PEG | polyethylene glycol |
| pfu | plaque forming units |
| psi or $lb/in^2$ | pounds per square inch |
| RIA | radio immuno assay |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| rRNA | ribosomal ribonucleic acid |
| RT | room temperature |
| SDS | sodium dodecyl sulphate |
| SDS-PAGE | sodium dodecylsulphate-polyacrilamide gel electrophoresis |
| ss | single-stranded |
| SSC | standard saline citrate |
| sTf | serum transferrin |
| TAE | Tris acetate EDTA |
| TBE | Tris borate EDTA |
| TCA | trichloroacetic acid |
| TE | Tris (10 mM) EDTA (1 mM), pH 8.0 |
| TEMED | N,N,N′,N′-tetra methyl ethylene diamine |
| Tris | Tris- (hydroxy methyl) amino methane |
| TTP | thymidine triphosphate |
| UV | ultraviolet |
| YEPD | rich glucose medium |
| YEPL | rich lactose medium |
| X-gal | 5-bromo-4-chloro-3-indolyl -D-galactopyranoside |

## Additional Abbreviations

| | |
|---|---|
| APR | Acute Phase Reaction |
| EGTA | Ethylene Glycol-bis (β-aminoethyl Ether) N,N,N',N'-Tetraacetic Acid |
| GST | Glutathione S-Transferase |
| HCMV | Human cytomegalo virus |
| hrLF | human recombinant Lactoferrin |
| IL | Interleukin |
| KN | Korean Native |
| Lbp | Lactoferrin binding protein |
| LFcin | Lactoferricin |
| MIC | Minimum Inhibitory Concentration |
| NK | Natural Killer |
| OPD | O-Phenylene Diamine |
| PIPES | Piperazine N,N'-bis[2-ethanesulphonic acid] |
| PMN | Polymorphonuclear |
| PMNs | Polymorphonuclear leukocytes |
| PVDF | Polyvinylidene difluoride |
| RES | Reticuloendothelial system |
| RNAse | Ribonuclease |
| TB | Tris buffer |
| TNF | Tumour Necrosis Factor |
| YPD | Yeast extract Peptone Dectrose (rich glucose medium) |

## Abbreviations of units

| | |
|---|---|
| °C | degrees Celsius |
| g | gram(s) |
| Hr or hr | hour(s) |
| kb | kilobases |
| kDa | kilodaltons |
| L | litre(s) |
| M | molar |
| min | minute(s) |
| m | metre(s) |
| rpm | revolutions per minute |
| % (w/v) | percentage weight by volume |
| %(v/v) | percentage volume by volume |
| µf | microfarads |
| Ω | resistance in ohms |
| s | second(s) |
| V | volts |