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Progress in the study of the molecular structure of lactoferrin and its bioactive peptides

Wei Tan, Genna Ba, Yanjun Xia

Inner Mongolia Yili Industrial Group Co., Ltd., Hohhot, Inner Mongolia, 010080, China

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Abstract: Lactoferrin is a natural iron-binding glycoprotein widely distributed in mammalian whey and most biological fluids. Lactoferrin ingested by the human body is hydrolysed by proteases and is mainly digested and absorbed in the form of peptides, thus exerting its physiological functions. In recent years, domestic and foreign studies have reported a variety of lactoferrin bioactive peptides with broad-spectrum antimicrobial, antitumour, antihypertensive, anti-inflammatory and immunomodulatory functions, which have the dual effects of regulating the physiological functions of the organism and providing nutrients to the organism, and thus play an important role in human health. This paper mainly reviews the structure of lactoferrin as well as the types, structures and their mechanisms of action of lactoferrin bioactive peptides, and discusses their application studies in contributing to bone activity, with a view to providing theoretical references for the study of lactoferrin-derived bioactive peptides and the development of related functional dairy products.

1. Introduction

Lactoferrin is an iron-binding glycoprotein consisting of about 700 amino acids with a molecular mass of about 80 kDa, which is mainly expressed and secreted by neutrophils and mammary epithelial cells, and is widely distributed in mammals, and possesses a variety of physiological activities such as antimicrobial, anti-inflammatory, antitumor, and immunomodulatory activities[1]. Lactoferrin contains only one polypeptide chain, folded into two basically symmetric and highly homologous globular leaf structures, called N and C leaf, whose biological activities are closely related to lactoferrin, and also have multiple biological functions such as antitumor, immunomodulation and antioxidant. Compared with lactoferrin, lactoferrin bioactive peptides have the advantages of small molecular mass, good digestion and absorption, and higher bioactivity. Therefore, based on the current research results, this paper reviews the types, structures, and mechanisms of action of lactoferrin bioactive peptides.

2. Lactoferrin structure

Lactoferrin is a molecule consisting of a single peptide chain of approximately 700 amino acids folded into two spherical structures connected by α-helical residues. The two spherical structures carry a glycosylation site and an iron binding site, respectively[2], allowing each lactoferrin

molecule to bind two Fe3+. Lactoferrin exists in two forms, the iron-free form called apo-LF and the iron-rich form called holo-LF, which differ in tertiary structure. In addition to binding Fe3+, lactoferrin has been observed to bind a range of other compounds such as lipopolysaccharides, heparin, DNA and metal ions (including Cu2+, Zn2+, Mn2+, Al3+, etc.). Lactoferrin molecules from different species are highly homologous and have different antibacterial activities with only minor structural differences[3].

3. Types of lactoferrin bioactive peptides

Lactoferrin can be hydrolysed by proteolytic enzymes to obtain a variety of active peptides with different physiological functions, mainly including anti-microbial peptides, blood pressure-lowering peptides, anti-tumour peptides, and immunomodulatory peptides[4]. In this study, we summarized the lactoferrin-derived bioactive peptides reported in the literature in recent years, among which anti-microbial active peptides dominated, accounting for about 46.7% of the total number of peptides; followed by blood pressure-lowering peptides related to the prevention of vascular diseases, accounting for about 21.0%; anti-tumour peptides, anti-inflammatory and immunomodulatory peptides accounted for 14.5% and 11.3%, respectively; in addition to this, there are still some antioxidant peptides and cell proliferation-promoting active peptides derived from lactoferrin. In addition, some antioxidant peptides and cell proliferation promoting peptides derived from lactoferrin were reported, accounting for about 6.5% of the total peptides. For the bioactive peptides derived from lactoferrin, the number of amino acids of most peptides mainly ranged from 10 to 20. Among the anti-microbial active peptides, the number of amino acids was distributed over a wider range, ranging from oligopeptides with less than 10 amino acids to polypeptides with up to 47 amino acids. As for blood pressure-lowering peptides, oligopeptides with less than 10 amino acid molecules mainly dominate.

4. Classification of lactoferrin bioactive peptides and their mechanism of action

With the continuous development of lactoferrin bioactive peptide research, the study of its mechanism of action has been deepened. The lactoferrin peptides reported so far have different mechanisms of action depending on their bioactivities. Anti-microbial active peptides derived from lactoferrin are mainly concentrated at the N-terminus of lactoferrin and have strong cationic properties. Its mechanism of action is different from that of lactoferrin in that the peptide cannot competitively bind iron ions with microorganisms, but due to its strong cationic properties, the peptide can cause damage to the cell membrane of microorganisms, and because of its small molecular mass it is easier to be absorbed by the body, which makes its inhibitory effect on microorganisms stronger. Angiotensin I can be catalytically converted by angiotensin I-converting enzyme (ACE) into angiotensin II, which promotes vasoconstriction, thereby inactivating bradykinin, which has vasodilatory effects, leading to hypertension[5].

4.1 Lactoferrin anti-microbial active peptides

Lfcin is a peptide produced by the hydrolysis of lactoferrin by pepsin under acidic conditions. It is derived from the N-terminus of lactoferrin and is closely related to the function of lactoferrin. Although Lfcin cannot bind iron ions, its antibacterial activity is more than 400 times higher than that of lactoferrin.Lfcin was first found in cow's milk, and its homologues have been found in humans, mice, pigs and other animals. The primary structures of Lfcin from different species have high homology and they include different amino acid lengths (Table 1). Among them, Lfcin B from bovine and Lfcin H from human are the two most important research subjects, because Lfcin B is

the most active and Lfcin H is the most closely related to human.Lfcin B contains 25 amino acid residues, with a molecular mass of about 3 125.80 Da and an isoelectric point of more than 8.5.Lfcin B consists of amino acid residues 17 to 41 from bovine lactoferrin, of which the amino acid residues 17 to 41 are the same as those of bovine lactoferrin, with the isoelectric point of more than 8.5. Lfcin B consists of amino acid residues from 17th to 41st position in bovine lactoferrin, including five tryptophan, three lysine and several aromatic amino acid residues, two of which are cysteines that give the Lfcin B molecule an incomplete cyclic shape through the formation of intramolecular disulfide bonds. In contrast, Lfcin H contains 47 amino acid residues, consisting of amino acid residues 1 to 47 in human lactoferrin, with a molecular mass of approximately 3,021.56 Da. Its primary structure is similar to that of Lfcin B, linked by disulfide bonds, both in a cyclic form, and is divided into two fragments, Lfcin H1-11 and Lfcin H12-47[6].Lfcin possesses a broad-spectrum of anti-bacterial Lfcin has a broad spectrum of antimicrobial activity, including many G+ and G- bacteria, such as Salmonella enteritidis, Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa.Lfcin has a strong inhibitory effect on Mycobacterium tuberculosis and other strains of bacteria that cause lung infections, and also has an inhibitory effect on a number of lactoferrin resistant fungi such as Candida albicans, viruses, and Toxoplasma gondii[7]. However, Lfcin has a selective effect on the inhibition of intestinal bacteria, and has poor or no antimicrobial effect on beneficial intestinal bacteria (such as Bifidobacterium, Pseudomonas fluorescens, and Lactobacillus, etc.).The antimicrobial mechanism of Lfcin is thought to be related to the interaction with cell membranes.Prokaryotic cell membranes contain a large number of negatively charged phosphatidylglycerol, which gives a net negative charge to the cell membrane, and the strongly alkaline Lfcin can interact with negatively charged groups on the surface of the cell membrane via the cell membrane[8]. The strongly basic Lfcin can bind to the negatively charged groups on the surface of the cell membrane by electrostatic attraction. In the process of interaction with microbial cell membranes, amino acid residues in positions 4 to 9 in the Lfcin B sequence are considered to be the active centre of Lfcin B. Among them, the side chains of the three arginine residues are attracted to the cell membrane components through positive and negative charges, while the aromatic structural ring of tryptophan interacts with the glycerol in the head of phospholipids to attach the peptide to the surface of the outer membrane through hydrophobicity, and the use of the hydrophobic structure enables the antimicrobial peptide to enter the cell through transmembrane, or to achieve multiple antimicrobial peptides through transmembrane into the cell, or to achieve the aggregation of multiple antimicrobial peptides into the cell membrane to form an ion channel, which increases the permeability of the cell membrane and causes autolysis of the cell[9].

In addition to its antimicrobial activity, Lfcin likewise inhibits the replication and multiplication of hepatitis viruses, herpes I and II viruses, influenza viruses, etc., as well as the pathogenic degenerative effects of human immunodeficiency virus-1 on MT4 cells and fibroblasts. Viral infection of host cells generally goes through different stages, including adsorption, penetration, decapsidation, nucleic acid replication, transfer of transcription, packaging, etc. Blocking any of these steps can inhibit viral infection.In their study of the inhibitory effect of Lfcin B on feline cupripoxviruses, McCann et al[10] found that cultured cells were bound on the surface of the cells by immunofluorescence observation, which Revealing that Lfcin B may act on the surface of infected cells and interfere with the interaction between the virus and host cell surface receptors. And further studies also showed that Lfcin could bind to heparan sulfate phospholipids and mucopolysaccharides on the host surface, thus preventing the virus from adsorbing to host cells[11].

Lf (1-11), which is also derived from the N-terminus of bovine lactoferrin, also has strong cationic properties with an isoelectric point greater than 11 and has therapeutic effects on a wide range of bacterial-induced infections.The results of a study by Brouwer et al[12] showed that the minimum inhibitory mass concentrations of Lf (1-11) ranged from 1.6 to 6.3 μg/mL against G+

bacteria such as Staphylococcus and Streptococcus, and from 6.3 to 12.5 μg/mL against G. Although similar to Lfcin, the Lactoferrampin molecule also has amphiphilic nature, high antimicrobial activity, and strong cationic properties, it differs from Lfcin in amino acid composition and chain length, and has a very different structure.The positions of Lfcin, Lf (1-11) and Lactoferrampin in lactoferrin are concentrated in the N-terminal part of lactoferrin (Figure 1). Lactoferrin itself not only has a high isoelectric point, but also has multiple positively charged alkaline regions on its surface, which are unevenly distributed and mainly concentrated at the N-terminus, which is not only the key to its anti-microbial activity, but also the basis for the binding properties of lactoferrin with other biomolecules.

Table 1: Comparlson of amino acld sequences o antlmicrobial peptides derived from lactoferrin

lactoferrin peptide	species	sequences	Molecular mass/Da	Electric charge(pH7)
Lfcin	cow	FKCRRWQWRMKKLGAPSIICVRRAF	3125.80	8+
	Human	TKCFQWQRNMRKVRGPPVSCIKRDS	3021.56	6+
	horse	KCAKFQRNMKKVRGPSVSCIRKTS	2753.33	8+
	goats	SKCYQWQRRMRKLGAPSICVRRTS	3012.55	7+
	ninny	KKCAQWQRRMKKVRGPSVTCVKKTS	2934.57	9+
	mice	EKCLRWQNEMRKVGGPPLSCVKKSS	2861.38	4+
Lactoferampin	cow	WKLLSKAQEKFGKNKSR	2048.39	5+
	Human	WNLLRQAQEKFGKDKSP	2045.30	2+
	horse	WKLLHQAQEEFGRNKSS	2.058.26	1+
	goats	WELLRKAQEKFGKNKSQ	2.090.38	3+
	ninny	WKLLVKAQEKFGRGKPS	1972.34	4+
Lf(1~1l)	cow	APRKNVRWCTI	1343.60	3+
	Human	GRRRSVQWCAV	1317.53	3+
	horse	APRKSVRWCI	1316.58	3+
	goats	APRKNVRWCAI	1313.58	3+
	ninny	ASKKSVRWCTT	1266.47	3+

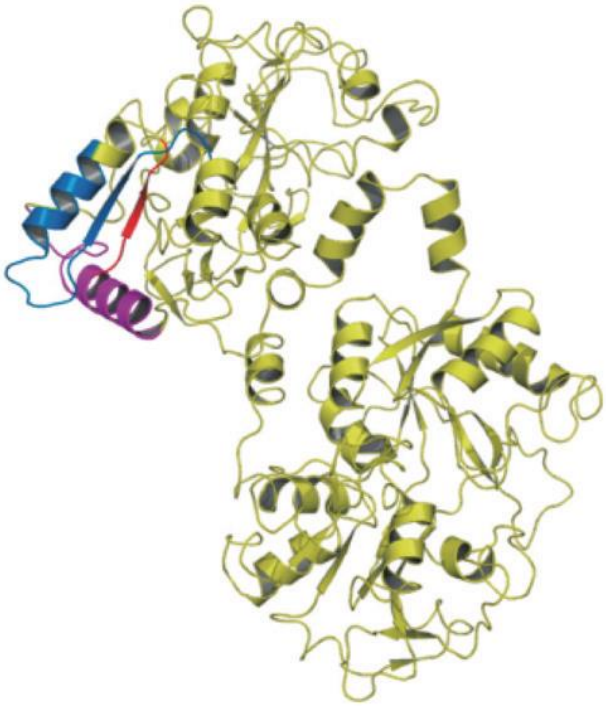

Figure 1: Location of anti-microbial peptides in the structure of bovine lactoferrin

4.2 Lactoferrin blood pressure lowering active peptide

With the change of people's diet and lifestyle, hypertension has become a common health hazard nowadays. As a chronic disease, hypertension is the cause of many diseases such as coronary heart disease, peripheral arterial disease, and stroke, and most of the patients need long-term medication.ACE is a dipeptide carboxypeptidase, which plays an important role in the blood pressure regulation system. The renin-angiotensin system is an antihypertensive system, and the kinin-releasing enzyme-kinin system is an antihypertensive system, which are antagonistic to each other in the regulation of blood pressure. Angiotensin I released from renin catabolism in the renin-angiotensin system can be excised by ACE from its C-terminal dipeptide (H-L) to produce angiotensin II, which causes vasoconstriction. It can also promote the secretion of aldosterone, leading to sodium retention and causing an increase in blood pressure. Therefore, inhibition of ACE activity can reduce the production of angiotensin II, thus effectively lowering blood pressure and preventing and hypertension-related diseases[13]. Currently, ACE activity inhibiting drugs have significant antihypertensive effects in clinical aspects, such as captopril, enalapril and lynopril[14]. However, long-term administration of such antihypertensive drugs often causes side effects such as sore throat, kidney damage, taste disorders, and skin rashes. Food-derived antihypertensive peptides have no side effects and are well absorbed compared to chemically synthesised drugs.Ruiz-Gim énez et al[15] prepared ACE inhibitory peptides by hydrolysis of bovine lactoferrin using porcine pepsin. Ultrafiltration of the hydrolysed fractions revealed high ACE inhibitory activity for hydrolysates with molecular mass below 3 kDa. For the hydrolysates with molecular mass below 3 kDa, three new ACE inhibitory peptides were found after isolation, purification and identification by mass spectrometry with the sequences LIWKL, RPYL and LNNSRAP, and the semi-inhibitory concentrations were 0.47, 56.50 μmol/L and 105.30 μmol/L, respectively. Garc á-Tejedor et al[16] isolated and identified four peptides in bovine lactoferrin yeast hydrolysate, DPYKLRP, PYKLRP, YKLRP, and GILRP, all of which had significant inhibitory effects on ACE activity in vitro.

4.3 Lactoferrin anti-tumour active peptide

For normal cells, lactoferrin promotes their proliferation, but lactoferrin has a significant inhibitory rather than stimulatory effect on tumour cell proliferation. Various in vitro experiments have shown that lactoferrin inhibits the growth of breast, colon, and cervical cancer cell lines. It is now believed that lactoferrin may inhibit cancer development by modulating immunity as well as the activity of enzymes (e.g. glutathione S-transferase) during the tumourigenic phase.Pan Weiru et al[17] also reported the interaction of Lfcin B with GST-P1 in the breast cancer cell line, MDA-MB-231, which showed that Lfcin B binds to the active sites G and H of GST-P1 and inhibit the activity of GST-P1, thus inhibiting the growth of breast cancer cells. In addition, it was found that Lfcin also showed significant inhibitory effects on various tumour cells, such as ovarian cancer cells, nasopharyngeal carcinoma, colon cancer, and breast cancer cells, etc., but had no toxic effects on normal human cells[18].

It is currently believed that the mechanism of selective action of Lfcin on tumour cells is generally due to the loss of the phospholipid head component of the plasma membrane of cancer cells, exposing the net negatively charged phosphatidylserine, which is only present in the plasma membrane vesicle fluid in normal cells. Therefore, Lfcin can bind to net-negatively charged phosphatidylserine in the membranes of tumour cells and inhibit the growth of tumour cells, but does not act on normal cells. In addition, the strong cationic properties, stable secondary structure and amphiphilic molecular properties, as well as the cyclic structure relying on disulfide bond formation are all necessary conditions for the antitumour activity of Lfcin. Studies on the

anti-tumour mechanism of Lfcin have shown that, on the one hand, after binding to tumour cells, Lfcin can disrupt the cell membrane of tumour cells and inhibit cell proliferation; on the other hand, Lfcin promotes apoptosis by initiating Ca2+/Mg2+-dependent nucleic acid endonuclease activity and oxygen-dependent apoptotic mechanism.Mader et al[19] confirmed that Lfcin B can contribute to the production of reactive oxygen species by leukaemia Jurkat T cells, which induces mitochondrial transmembrane potential dissipation, leading to apoptosis of leukaemia cells.Amino acid residues 4-9 (RRWQWR) in the sequence of Lfcin B are considered to be the active centre of Lfcin B.

4.4 Lactoferrin anti-inflammatory and immunomodulatory peptides

Lactoferrin is a component of the innate non-specific immune system of the human body, located on the surface of secretion fluid and epithelial cells, and plays an anti-microbial role by inhibiting microbial reproduction, adsorbing or killing microbes, and participates in anti-inflammatory response of the body, as well as playing an immunomodulatory role by acting on immune cells and cytokines. In the study of the anti-inflammatory and immunomodulatory mechanism of lactoferrin, it is found that, on the one hand, lactoferrin can prevent the activation of Toll-like receptor signalling pathway by binding to lipopolysaccharide, thus inhibiting the secretion of pro-inflammatory factors, such as interleukin (IL)-1β, IL-6, and IL-8, or promoting the secretion of anti-inflammatory factors, such as IL-10, IL-11, and IL-14; on the other hand, lactoferrin can indirectly play a role in anti-inflammatory responses. Indirectly, on the other hand, lactoferrin may also play an immunomodulatory role by promoting the maturation and activation of T cells, B cells and macrophages. van der Does et al[20] reported that hLF-11 derived from human lactoferrin stimulated the release of anti-inflammatory factors from human and mouse monocytes, thereby enhancing the immune response. It was shown that hLF-11 binds to human monocytes and subsequently penetrates the monocyte membrane, thereby exerting an immunomodulatory effect by inhibiting myeloperoxidase activity. LFP-20 (KCRQWQSKIRRTNPIFCIRR) is a peptide derived from the N-terminus of porcine lactoferrin. In addition, Jiang Qin et al[21] modified LFP-20 and successfully expressed the peptide LF-6 with the sequence KWRQWQSKWRRTNPWFWIRR in E. coli, which also possesses immunomodulatory activity.In vivo immunological experiments showed that recombinant LF-6 significantly reduced the levels of pro-inflammatory factors in the plasma and intestines of mice infected with enterotoxin-producing E. coli - k88 and also reduced the levels of pro-inflammatory factors in mice infected with enterotoxigenic E. coli - k88. factor levels and inhibited intestinal mucosal damage in infected mice. Fei-Fei Han[22] also modified the porcine lactoferrin peptide LFP-20 and prepared the modified peptide LF-2, which has a higher ability to disrupt cell membranes than the template peptide LFP-20, and can control the abnormal changes in the immune parameters of the E. coli-k88 infected mice by improving the thymus index, the proportion of B cells in the peripheral blood, and stimulating the transformation of splenic lymphocytes, which can also control the immune parameters of E. coli-k88 infected mice. indices due to E. coli infection. In addition, a study by Miyauchi et al[23] demonstrated that hydrolysates of bovine lactoferrin hydrolysed by pepsin significantly increased the proliferative activity of B cells compared to T cells. Thus, the mechanism of action of lactoferrin peptides on anti-inflammatory and immunomodulatory effects is similar to that of lactoferrin in that both promote the activity of immune cells and inhibit the expression of pro-inflammatory factors.

4.5 Other lactoferrin-activated peptides

In addition to the above reported bioactive peptides derived from lactoferrin, some anticoagulant active peptides and lactoferrin peptides promoting cell proliferation have also been reported. Shi

Pujie et al[24] used computer simulation to screen a segment of peptide LFP-C (FKSETKNLL) derived from the C-terminus of lactoferrin in bovine lactoferrin pepsin hydrolysates, which had a significant promotional effect on the proliferation of osteoblasts MC3TC-E1 and could modulate the cell cycle, inducing cell transformation from G0 to G2/M phase.

5. Progress in the study of bone-enhancing activity of lactoferrin

LF has a variety of biological activities such as antimicrobial, anti-inflammatory, antitumour and immunomodulatory activities, but the most recent findings now indicate that LF has received increasing attention as it also has a significant promotional effect on bone regeneration[25].The results of a series of in vitro experiments have demonstrated that LF has the ability to promote osteoblast survival, proliferation and differentiation as well as inhibit osteoclast-mediated bone resorption. The effects of LF on osteoblasts were dose-dependent, and it was demonstrated that LF at concentrations of 1-10 μg/mL significantly promoted the proliferation of rat osteoblasts, as well as of human osteoblasts SaOS-2. Similarly, at this concentration, LF also stimulated bone formation and chondrocyte proliferation, and the observed proliferative effects were significantly higher than those of IGF-1 and TGF-β. In addition, the results also showed that LF significantly increased alkaline phosphatase expression and calcium deposition in the extracellular matrix after 3 days in human osteoblast-like MG 63 cells, suggesting that LF also has a significant promotional effect on osteoblast differentiation.

6. Conclusions

To date, many studies have been conducted on lactoferrin-derived anti-microbial active peptides, blood pressure-lowering peptides, anti-tumour peptides, and immunomodulatory peptides, including peptides such as Lfcin, Lactoferrampin, and LFP-20, which have higher bioactivity than intact lactoferrin. In this paper, the types, structures and mechanisms of lactoferrin bioactive peptides are reviewed, but the different amino acid types, quantities and structures of the peptides lead to large differences in the mechanisms of their activities, which are still in the research stage. As research continues, the effects of lactoferrin-derived bioactive peptides on osteogenesis are being explored, and it is expected that a variety of functional products related to lactoferrin bioactive peptides will be developed.

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