Note

Inhibition of Iron/Ascorbate-Induced Lipid Peroxidation by an N-Terminal Peptide of Bovine Lactoferrin and Its Acylated Derivatives

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Bovine lactoferrin (LF) and lactoferrin C (LFcin B), an antimicrobial peptide derived from bovine LF, inhibited thiobarbituric acid-reactive substance (TBARS) formation in an iron/ascorbate-induced liposomal phospholipid peroxidation system. The inhibition of TBARS formation occurred with N-acylated 9-mer peptides with a core sequence of LFcin B and, compared to LFcin B, their antioxidant effect was clearly observed at a concentration almost 100 times lower.

Key words: lactoferrin; lactoferricin; antioxidant; TBARS; acylation

Lactoferrin (LF), an iron-binding protein belonging to the transferrin (TF) family, is present in many exocrine secretions of mammals and in the secondary granules of neutrophils. It has a variety of biological activities in vitro and in vivo including antimicrobial activity, immunomodulatory effects, and antioxidant activities.1,2 Antimicrobial peptides much more effective than LF were found to be generated upon digestion of this protein with gastric pepsin or other aspartic proteinases.3 The active peptides of human LF (lactoferricin H) and bovine LF (lactoferricin B, LFcin B) were isolated, sequenced, and found to be derived from the homologous N-terminal regions of the molecules.6 Later, it was reported that both LFcin B and LF effectively inhibit tumor metastasis7 as well as the LPS-induced IL-6 response in monocytes,8 and stimulate IL-8 release from neutrophils.7 Other studies have shown that an N-terminal region of the LF molecule overlapping the LFcin sequence is responsible for the antimicrobial activity of LF9 and the binding of LF to several biological substances and cells.9,10 LFcin may be a peptide or a region with the integrated functions of LF.

Previous studies have shown that LF inhibits lipid peroxidation11,12 with an assay system of liposomal phospholipid peroxidation induced by Fe3+ ion and ascorbic acid. The mechanism of lipid peroxidation in this system could be assumed as follows. Fe3+ is reduced to Fe2+ by ascorbic acid (a), where LF chelates Fe3+ in a noncatalytic form, and thereby blocks this and the following reactions. When Fe2+ is present, O2 produces O2- or H2O2, and H2O2 and LOOH (lipid hydroperoxide) are converted to HO· and LO·, respectively (b)(c).13,14

(a) Ascorbic acid + Fe3+ → Dehydroascorbic acid + Fe2+
(b) H2O2 + Fe2+ → HO· + OH· + Fe3+
(c) LOOH + Fe2+ → LO· + OH· + Fe3+

These products eventually yield cytotoxic lipid aldehydes, detected as thiobarbituric acid reactive substances (TBARS) or malondialdehyde (MDA). Matsue et al. reported that LF hydrolysates treated with proteases such as pepsin, as well as LF, inhibit MDA formation in a solution containing DNA, bleomycin, and Fe2+ or ascorbic acid.15

We studied the effects of LFcin B on TBARS formation in the iron/ascorbate-induced liposomal phospholipid peroxidation system, because LFcin is likely to be a multi-functional peptide, but it does not have the iron-binding motif of LF. In addition, 9-mer peptides from LFcin B were chemically acylated to confer hydrophobicity and were tested for antioxidant activity in an effort to develop more potent peptide derivatives.

Bovine LF was produced by Morinaga Milk Industry Co. (Tokyo, Japan) and its iron-saturation was almost 14%. LFcin B was prepared from bovine LF pepsin hydrolysate as described previously.4 Using a 9-fluorenylmethoxycarbonyl (Fmoc) strategy and solid-phase methodology, peptides were synthesized on a 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl) phenoxacetamido-ethyl resin or preloaded HMP resin (Perkin-Elmer Japan Co. Applied Biosystems Division, Chiba, Japan) using an automated synthesizer (433A, Perkin-Elmer). For N-acylation of the peptides, the resin was treated with the desired fatty acid activated in situ as a HOBt/HBTU ester. Crude peptides were purified by reverse-phase HPLC. The synthesized peptides are shown in Table 1. Assay of TBARS formation was done by the method of Gutteridge(20) with some modifications. Liposomes were prepared by vigorous agitation of a mixture consisting of 5 mg/ml egg yolk lecithin in 10 mM HEPES and 0.15 M NaCl (pH 7.4) for 5 min at 37°C under oxygen-free nitrogen and the mixture was then left at 4°C for 1 h. The supernatant in 0.5-

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Abbreviations: LF, lactoferrin; LFcin, lactoferricin; TBARS, thiobarbituric acid-reactive substances; MDA, malondialdehyde; SOD, superoxide dismutase
ml portions was dispensed into a number of tubes. Then, 50 μl of 112 μM FeNH4(SO4)2, 50 μl of 532 μM ascorbic acid, and 100 μl of test compound solution were added to the tubes followed by vigorous mixing for 5 s. The samples were incubated at 37°C for 1 or 2 h. Three ml of 2.9 M HCl containing 1.0% sodium arsenite was added to each tube. The tubes were mixed and centrifuged at 2,500g for 15 min, 3 ml of the clear supernatant was carefully removed and added to 1% thiobarbituric acid in 0.05 M NaOH, and each sample was heated for 15 min in a boiling water bath. After cooling, the turbidity was removed by filtration, and TBARS formed in each sample was measured photometrically at 532 nm. Statistical analysis was done using the unpaired two-tailed t test to compare two groups.

Effects of LF-related compounds on TBARS formation were assayed in the system of liposomal phospholipid peroxidation during 2 h of incubation (Fig. 1). Fe3+ ion and ascorbic acid added to this system caused considerable TBARS formation. In the presence of Fe2+ and ascorbic acid, LF and a positive control, DL-α-tocopherol, had an inhibitory effect on the reaction as shown in previous studies,11,12 LFcin B also decreased the levels of TBARS formation with a lesser effectiveness compared with that of LF.

To examine the effects of LFcin-derived peptides on oxidation reactions, the synthesized peptides shown in Table 1 were tested. Table 2 shows the effects of these peptides and reference compounds on TBARS formation.
Antioxidant Activity of Lactoferrin Peptides

reference compound, Ac-RRWWCR-NH₂, also inhibited TBARS formation.

In this study, LFcin B and LF had similar antioxidant effects, consistent with those shown in other biological activities. This supports the hypothesis that the LFcin region is a multi-functional domain of LF. One molecule of LF binds two Fe³⁺ ions at binding sites different from the LFcin region. However, it has been reported that LF effectively solubilizes excess Fe²⁺ ions in test solutions, suggesting binding of higher numbers of Fe²⁺ ions to LF and that LF pepsin hydrolysate inhibits Fe²⁺-induced MDA formation in a DNA and bleomycin system. These findings imply that LFcin B interacts with Fe²⁺ ions not in a strict chelating manner and inhibits lipid peroxidation. Matsue et al. proposed that LF also binds ascorbic acid and this is one of the antioxidant mechanisms of LF in the iron/ascorbate-induced oxidation system. To clarify the antioxidant mechanism of LFcin B, investigations involving interactions of the peptide with iron ions or ascorbic acid are necessary. As a reference peptide, a cationic antimicrobial peptide Ac-RRWWCR-NH₂ was tested and inhibited TBARS formation. This peptide has been identified as the most potent 6-mer antimicrobial peptide in synthetic peptide combinatorial libraries and shares sequence similarity with RRWQWR in LFcin B. We adapted hydrophobic motifs to a highly cationic sequence, RRWQWRMKK, of LFcin B and examined the activity. The N-acylated peptides examined significantly inhibited TBARS formation. Acalyptation of the peptide may result in a higher affinity for liposomal phospholipids. A number of physiologically important peptides and proteins are known to have a covalently linked fatty acid which is often essential for their biological activity and for stable associations with cellular membranes. It has been reported that introducing an acyl group into a non-acylated peptide led to strong interaction with a phospholipid membrane. Our observations revealed a novel biological role for acetylated peptides.

References