Osteoporosis is a serious problem in our current aging society. The main pathogenesis of this disease is calcium intake deficiency and also abnormal type I–collagen degradation in bone. Bone resorption originates from the following two interrelated mechanisms: 1) abnormal stimulation of matrix type I–collagen degradation and 2) deficiency of calcium intake or a disturbance in the calcium setting in bone (1–3). Since cathepsins K and L participate mainly in the degradation of matrix type I–collagen in bone, various cathepsin inhibitors have been investigated to prevent osteoporosis (4).

Milk contains a large amount of calcium and is one of the main sources of dietary calcium; it also contains various inhibitors of collagenolytic cathepsins. Katunuma et al. (5, 6) reported that lactoferrin and β-casein in milk are powerful cysteine protease inhibitors and are present at very high concentrations. However, these inhibitory proteins have relatively high molecular weights, are difficult to absorb through the portal pathway and are absorbed mainly through lymphatic pathways. Some other inhibitory proteins with low molecular weights which are expected to be absorbed directly via the portal vein were supposed to be contained in milk. These inhibitory proteins in milk may act cooperatively with the other proteases inhibitors to suppress the degradation of bone matrix collagen.

In this paper, we show that β-lactoglobulin (LG)-B in milk is a cysteine protease inhibitor and may play a role in the suppression of bone collagen degradation. We discuss the contribution of this protein to the control of bone resorption.

MATERIALS AND METHODS

Materials. Untreated raw bovine milk was provided by a dairy farm in Tokushima City, Japan. Cathepsins K and B were purchased from Calbiochem (San Diego, CA, USA). Cathepsin L, thermolysin, pepsin, trypsin, chymotrypsin, β-LG-A and B, β-casein (bovine milk) and type I–collagen from calf skin (acid soluble) were purchased from Sigma (St. Louis, MO, USA). Z-Phe-Arg-MCA was obtained from the Peptide Institute, Inc. (Osaka, Japan). Lysyl-endopeptidase for the digestion of collagenolytic cathepsins. Using the everted-sac method in rat small intestines, it was found that β-lactoglobulin was more easily absorbed from the intestines of young rats (5 wk-old) than from those of older rats (more than 20 wk-old). The digested products of β-lactoglobulin B with lysyl-endopeptidase showed a similar inhibitory activity against cathepsin K to that of β-lactoglobulin B did. Therefore, peroral intake of β-lactoglobulin in milk and its digested peptides are expected to help protect osteoclastic bone resorption via inhibition of collagenolytic cathepsins K and L.

Summary. It is well known that the collagenolytic cathepsins play an important role in the degradation of bone matrix. Therefore, the purpose of this study was to clarify the prevention effect of bone resorption by milk components. Using double-layer reverse zymography, we found a 20 kDa protein in milk which inhibited cysteine proteases. This inhibitory protein was identified as β-lactoglobulin B. The inhibitory activity of β-lactoglobulin B against cathepsin K was stronger than that of β-lactoglobulin A. β-Lactoglobulin B specifically inhibited papain type cysteine proteases such as cathepsins K and L, but not serine proteases, aspartic proteases or metallo proteases. β-Lactoglobulin B inhibited cathepsin K competitively and the Ki value was 10⁻⁵ M. The formation of osteoclastic pits in the culture system was effectively inhibited by 10⁻¹²–10⁻¹⁰ M β-lactoglobulin B in vitro. Furthermore, we demonstrated that β-lactoglobulin B inhibited degradation of type I–collagen by collagenolytic cathepsins. Using the everted-sac method in rat small intestines, it was found that β-lactoglobulin was more easily absorbed from the intestines of young rats (5 wk-old) than from those of older rats (more than 20 wk-old). The digested products of β-lactoglobulin B with lysyl-endopeptidase showed a similar inhibitory activity against cathepsin K to that of β-lactoglobulin B did. Therefore, peroral intake of β-lactoglobulin in milk and its digested peptides are expected to help protect osteoclastic bone resorption via inhibition of collagenolytic cathepsins K and L.

Key Words. cathepsin K, cysteine protease inhibitor, β-lactoglobulin, pit formation

Inhibition of Collagenolytic Cathepsins by β-Lactoglobulin in Milk and Its Suppressive Effect on Bone Resorption

Naoko OGAWA1, Masae TAKASHI2, Kazumi ISHIDOH3,4 and Nobuhiko KATUNUMA2

1Faculty of Life Science, and 2Institute for Health Sciences, Tokushima Bunri University, Yamanashiro-cho, Tokushima, Tokushima 770–8514, Japan
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E-mail: ogawanao@tokushima.bunri-u.ac.jp
ECL Western blotting were purchased from GE Healthcare UK Ltd. (Buckinghamshire, England). Five-week-old (young) or more than 20 wk-old (old) male Sprague-Dawley rats were used in the uptake study, using the everted-sac method. They were purchased from CLEA Japan, Inc. (Tokyo, Japan). An osteoclast culture kit was purchased from the Primary Cell Co., Ltd. (Sapporo, Japan).

Detection of a new inhibitory protein for papain type cysteine protease. The milk proteins were precipitated in 5% perchloric acid (final concentration) and the acid-soluble low molecular weight proteins were fractionated in a gel filtration column (Superdex peptide 10/300GL). A 15% acrylamide gel was used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (7). The gel was subjected to reverse zymography (8). For reverse zymography, the acid-soluble fractions containing low molecular weight inhibitory proteins were subjected to 15% SDS-PAGE. After removal of SDS in 2.5% Triton X-100 solution, the gel was incubated with papain (0.5 mg/100 mL) at 37°C for 30 min. The slab gel was covered with a cellulose acetate membrane which had been previously treated with 10% glycerol and 100 µM of the target fluorescent peptide MCA substrate, Z-Phe-Arg-MCA. Inhibitor bands were detected under UV light (365 nm). Then, the gel was stained with a silver staining kit.

Characterization of a new inhibitor. Cathepsins and papain activities were determined principally by Barrett’s method with slight modifications (9): cathepsins K, L, B and papain activities were measured using Z-Phe-Arg-MCA as the substrates. Cathepsin (final concentrations of the activities of cathepsins K, L, and B used in this study were 25, 395, and 250 µU/mL), chymotrypsin (300 µU/mL) and β-LG-B (10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M, respectively) were pre-incubated at 37°C for 10 min in 0.1 M Tris-HCl buffer (pH 7.4). The enzyme reactions were initiated by adding either Boc-Phe-Ser-Arg-MCA for trypsin, or Suc-Ala-Ala-Pro-Phe-MCA for chymotrypsin, at a final concentration of 20 µM. After incubation at 37°C for 30 min, the reactions were stopped by the addition of 0.1 M acetic acid buffer (pH 4.0). The released fluorescence was measured at an excitation wavelength of 370 nm and an emission wavelength of 460 nm in a microplate fluorometer.

Determination of the inhibitor amino acid sequences. In order to determine the amino acid sequence of the intact inhibitory protein against papain, the protein was subjected to 12.5% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. The target protein was subjected directly to amino acid sequence analysis for the determination of the amino terminal sequence. In parallel, for the determination of internal amino acid sequences, the protein band in the other gel was excised and subjected to trypsin digestion and amino acid sequence analyses. All procedures, including trypsin digestion of the protein, were carried out by APRO Sciences (Tokushima, Japan).

Assay of osteoclastic bone resorption using a pit formation test. The osteoclast culture kit was used for the bone resorption assays which were carried out according to the manufacturer’s recommendations (10). Briefly, premature osteoclast cells were incubated in culture medium containing M-CSF and RANKL for 3 d on ivory grafts (150 µm thickness) in 96-well culture plates. In order to investigate the inhibition of pit formation by β-LG-B, 10^{-3} or 10^{-4} M β-LG-B was added to the wells. The same amount of saline was added to separate wells as a control. Each experiment was performed in triplicate. After 7 d, the ivory grafts were sonicated in 1 M ammonium solution and observed under a scanning electron microscope (SEM) (HITACHI, Tokyo, Japan). In order to measure the area of pit formation, the electron micrograph was photocopied and the pitted area was cut out of the copy and weighed. The weight of the paper is proportional to the area of pit formation, i.e. a low weight of paper represents a small area of pit formation.

Inhibition of collagenolytic cathepsins by β-LG-B using type I-collagen as a substrate. Cathepsin K or L plus β-LG-B (10^{-4} or 10^{-5} M) was pre-incubated at 37°C for 10 min in 0.1 M acetic acid buffer (pH 5.5) containing 2 mM cysteine. The reaction was initiated by adding 25 µM of the target fluorescent peptide MCA substrate, Z-Phe-Arg-MCA at a final concentration of 10 µM. The enzyme reaction was initiated by adding 20 mM Z-Phe-Arg-MCA at 37°C for 15 min and stopped by the addition of 0.1 M acetic acid buffer (pH 4.0). The released fluorescence was measured at an excitation wavelength of 370 nm and an emission wavelength of 460 nm using a spectrophotometer at a wavelength of 280 nm. Pepsin (6 U/mL) and β-LG-B (10^{-4} and 10^{-3} M, respectively) were pre-incubated at 37°C for 10 min in 0.2 M glycine-HCl (pH 2.0). The enzyme reaction was initiated by adding 2% hemoglobin at 37°C for 30 min and stopped by the addition of 10% TCA. After centrifugation, the concentration of the acid soluble products was measured in a spectrophotometer.
of Laboratory Animals of the Tokushima Bunri University and were approved by the Committee for the Care and Use of Laboratory Animals at Tokushima Bunri University. The excised small intestine (10 cm in length: upper jejunum) was everted and bound the end (11). The everted-sac (inside, serosa-side containing 1 mL of saline) was incubated with 25-fold diluted mature milk or \( \beta \)-LG in saline (outside, mucosa-side) with aeration at 37˚C. After 30 and 60 min of incubation, each 100 µL of the inner solution was pumped out by a capillary pipette to analyse the absorbed \( \beta \)-LG. The samples were subjected to SDS-PAGE, and Western blot analysis (12) for the specific detection of \( \beta \)-LG. The proteins were transferred to PVDF membranes for Western blot. The membranes were reacted with anti-\( \beta \)-LG antibody, and then with peroxidase conjugated goat anti-rabbit IgG antibody. Immunoreactive proteins were detected using ECL Western blotting detection reagents or 3,3′-diaminobenzidine tetrahydrochloride (DAB) and \( \text{H}_2\text{O}_2 \) and observed in a Fuji LAS 3000 mini system (Fuji Film, Tokyo, Japan) (13). The amounts of absorbed \( \beta \)-LG from the small intestine of young (5 wk-old) or older (more than 20 wk-old) rats were estimated from the results of Western blot using the computer package Multi Gauge Ver 5.0 in the LAS 3000 mini system.

Inhibition of collagenolytic cathepsin by the digested products of \( \beta \)-LG-B with lysyl-endopeptidase. Lysyl-endopeptidase (2 mAU) with or without \( \beta \)-LG-B (5 \( \times \) 10\(^{-3}\) M) was incubated in 50 mM Tris-HCl buffer (pH 9.5) at 37˚C for 16 h. These samples were applied to SDS-PAGE using a peptide gel after heating at 100˚C for 5 min to ensure digestion of \( \beta \)-LG-B. Cathepsin K activity was assayed in the presence of 20 mM \( p \)-APMSF and lysyl-endopeptidase which were preincubated at 37˚C for 10 min, as a control. The activity of cathepsin K in the presence of lysyl-endopeptidic \( \beta \)-LG-B peptides which added to 20 mM \( p \)-APMSF was compared with the control.

RESULTS

Detection of a low molecular weight cysteine protease inhibitor in cow’s milk using double-layer reverse zymography

A low molecular weight cysteine protease inhibitor in cow’s milk was detected using Katunuma’s double-layer reverse zymography method (8). The molecular weight

\[
\text{Inhibitor IVTQ} \\
\begin{array}{c}
\text{IVTQTMKGL DQKVQAGTW} \\
\text{SLAMAASDIS LLDAQSAPLR} \\
\end{array}
\]

\[
\begin{array}{c}
\text{VYVEELKTPP} \\
\text{EG} \\
\text{VYVEELKTPP} \\
\text{EGNLEILQK WENGECQKK IIAEKTIPA} \\
\end{array}
\]

\[
\begin{array}{c}
\text{VFKIDALLEN KVLVLDTDYK KYLLFMCENS AEPEQSLACQ} \\
\text{LS FNPTQLE} \\
\text{CLVRTPEDVNEALEKFDKAL KALPMHIRLA FNPTQLEGQC} \\
\end{array}
\]

Fig. 1. Detection of papain inhibition using double-layer reverse zymography. The fraction containing papain inhibitors was subjected to SDS-PAGE and reverse zymography with papain (A: fraction sample a, 2 µL; b, 5 µL; and c, 10 µL), after which the gel was stained with a silver staining kit (B). Arrows indicate the protein band (20 kDa) representing the papain inhibitor.

Fig. 2. Amino acid sequences of the low molecular weight papain inhibitor in milk. Amino acid sequences of amino terminal (I2–Q5) and internal peptides, V41–G52, L49–E157, from the tryptic digest of the purified protein (upper lanes) were determined in an amino acid sequencer and compared with that of bovine \( \beta \)-LG-B (lower lanes, accession number S00132).
Inhibition of Collagenolytic Cathepsins by \( \beta \)-Lactoglobulin

The inhibitory activity against papain increased in a dose-dependent fashion as shown in Fig. 1A and B.

Identification of a new inhibitory protein in milk

We determined the amino acid sequences of the purified inhibitory protein. The amino-terminal four-amino acid sequence was IVTQ and the amino acid sequence of one of the trypic peptides was VYEELKPTPEG (Fig. 2). Both sequences were completely identical to the amino acid sequences of L12–Q3 and V41–G52 of bovine \( \beta \)-LG-B, respectively (14). The amino acid sequence of the other trypic petide was LSFNPTQLE, which was almost identical to that of L149–E157 of bovine \( \beta \)-LG-B. Thus, the purified low molecular weight inhibitor in bovine milk was identified as bovine \( \beta \)-LG-B.

Inhibitory specificities of \( \beta \)-LG-B

The B form of \( \beta \)-LG was a stronger inhibitor against...
cathepsin K than the A form (data not shown). The specificities of inhibitory activity of β-LG-B against cathepsins K, L, B and papain were examined. The inhibitory activities of β-LG-B against cathepsins K and L which are the collagenolytic cathepsins (papain family) were the strongest, but that against cathepsin B was very weak (Fig. 3). β-LG-B did not inhibit serine proteases, aspartic protease or metallo protease (data not shown). Thus, β-LG-B had a specific inhibitory activity against cysteine proteases, and in particular a strong activity against collagenolytic cathepsins.

Mode of β-LG-B inhibition against cathepsin K

The inhibition kinetics of β-LG-B against cathepsin K was analyzed using a Lineweaver-Burk reciprocal plot of the substrate-velocity relationship. As shown in Fig. 4, the inhibition kinetics of β-LG-B against cathepsin K was non-competitive and the Ki value was $10^{-5}$ M.

Suppressions of pit formation and type I-collagen degradation by cathepsins K and L in the presence of β-LG-B

Pit formation was significantly suppressed by the addition of β-LG-B, in a dose-dependent fashion as shown in Fig. 5. The pit area was decreased by 36 and 80% in the presence of $10^{-5}$ and $10^{-4}$ M β-LG-B, respectively. We also determined the inhibition of collagenolytic cathepsins by β-LG-B in SDS-PAGE using type I-collagen as a substrate. As shown in Fig. 6, the degradation of type I-collagen was suppressed by β-LG-B, which inhibited the activities of cathepsins K and L, respectively.

Absorption of β-LG in milk from the rat small intestine using the everted-sac method in vitro

β-LG in the inner solution of the young rat intestine was detected within 30 min, while that of the old rat intestine could be detected after 60 min (Fig. 7A, B and C). When we compared the amount of absorbed β-LG from milk to that from β-LG dissolved in saline, β-LG in milk was effectively absorbed in the everted-sac method (Fig. 7D).

Inhibition of cathepsin K by lysyl-endopeptidic peptides of β-LG-B

The inhibition of cathepsin K activity by the β-LG-B digested products was also assayed. Figure 8A showed that β-LG-B was completely digested with lysyl-endopeptidase to the peptides. The peptides inhibited cathepsin K activity; those from $5 \times 10^{-5}$ M β-LG-B inhibited 40% of cathepsin K activity in the presence of lysyl-endopeptidase plus p-APMSF (Fig. 8B). The inhibitory activity of the peptides was quite similar to that of...
Inhibition of Collagenolytic Cathepsins by β-Lactoglobulin

In this study, we showed that β-LG-B inhibited the activity of cathepsins K and L. After incubation at 37 °C for 16 h, aliquots of the samples were subjected to SDS-PAGE and stained with CBB (lane a, 25 μg of β-LG-B; lane b, 0.7 μg of lysyl-endopeptidase; lane c, β-LG-B plus lysyl-endopeptidase). (B) Cathepsin K activity (control) was compared with that in the presence of the digested products of β-LG-B (peptides from β-LG-B).

FIG. 8. Inhibition of the lysyl-endopeptidic β-LG-B peptides against cathepsin K. (A) After incubation at 37 °C for 16 h, aliquots of the samples were subjected to SDS-PAGE and stained with CBB (lane a, 25 μg of β-LG-B; lane b, 0.7 μg of lysyl-endopeptidase; lane c, β-LG-B plus lysyl-endopeptidase). (B) Cathepsin K activity (control) was compared with that in the presence of the digested products of β-LG-B (peptides from β-LG-B).

DISCUSSION

In this study, we showed that β-LG-B inhibited the activity of cathepsins K and L. After incubation at 37 °C for 16 h, aliquots of the samples were subjected to SDS-PAGE and stained with CBB (lane a, 25 μg of β-LG-B; lane b, 0.7 μg of lysyl-endopeptidase; lane c, β-LG-B plus lysyl-endopeptidase). (B) Cathepsin K activity (control) was compared with that in the presence of the digested products of β-LG-B (peptides from β-LG-B).

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We found that the β-LG-B in milk effectively inhibits the collagenolytic cathepsins and osteoclastic pit formation. However, an important problem is whether orally administrated β-LG-B can be incorporated into the blood from the digestive tract. The absorption of β-LG was investigated using the everted-sac of the rat small intestine. The absorbed β-LG was detected by Western blot analysis as a 20 kDa band using anti-β-LG antibody. Furthermore, the efficiency of β-LG absorption from the everted-sac was dependent on the age of rats; absorption of β-LG from the everted-sac in young rats was significant after 30 min, while that in older rats could be detected only after 60 min. Further, β-LG in milk was taken up faster than β-LG in saline from the everted-sac of the rat small intestine. These results suggest that the absorption of β-LG may be an adaptive event to other milk components than β-LG. This phenomenon may provide a link between aging and the onset of osteoporosis.

Furthermore, peptides resulting from the digestion of β-LG-B with lysyl-endopeptidase inhibited the activities of cathepsin K. Although, β-LG is efficiently absorbed via the portal pathway from the small intestine due to its small molecular weight, the absorption of smaller peptides of β-LG-B origin via the same pathway may be more efficient than that of intact β-LG-B.

These findings suggest that peroral intake of β-LG-B may prevent osteoporosis by inhibition of the collagenolytic cathepsins K and L.

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