Biosci. Biotechnol. Biochem., 66 (12), 2531–2536, 2002

Cystatin C in Milk Basic Protein (MBP) and Its Inhibitory Effect on Bone Resorption *in Vitro*

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Cystatin C in Milk Basic Protein (MBP) and Its Inhibitory Effect on Bone Resorption *in Vitro*

Yasuhiro MATSUOKA,[1,†] Atsushi SERIZAWA,[1] Tomoe YOSHIOKA,[2] Jun-ichi YAMAMURA,[1] Yoshikazu MORITA,[1] Hiroshi KAWAKAMI,[1] Yasuhiro TOBA,[1] Yukihiro TAKADA,[1] and Masayoshi KUMEGAWA[3]

[1]*Technology and Research Institute, Snow Brand Milk Products Co. Ltd., 1-1-2 Minamidai, Kawagoe, Saitama 350-1165, Japan*
[2]*Sapporo Research Laboratory, Snow Brand Milk Products Co. Ltd., 6-1-1-Naebocho, Higashiku, Sapporo, Hokkaido 065-0043, Japan*
[3]*Department of Oral Anatomy, Meikai University School of Dentistry, 1-1 Keyakidai, Sakado, Saitama 350-0283, Japan*

Received April 12, 2002; Accepted August 9, 2002

A cystein protease inhibitor was identified in the basic fraction of bovine milk. We have reported in our previous study that the milk basic protein (MBP) fraction suppressed osteoclast-mediated bone resorption *in vitro*. Since osteoclasts secreted cystein protease to digest collagen in the bone matrix, we identified the cystein protease inhibitor in MBP. A 12-kDa inhibitor was purified from MBP by papain affinity gel chromatography and subsequent Hi-Load Superdex 75 gel filtration chromatography. The N-terminal sequence of the 18 amino acid residues of the inhibitor corresponded to bovine cystatin C. The 12-kDa cystein protease inhibitor in MBP therefore seemed to be cystatin C. Purified cystatin suppressed bone resorption with the use of isolated osteoclasts *in vitro*. Cystatin in MBP is suggested as one of the factors inhibiting bone resorption.

Key words: milk basic protein; bone resorption; cystein protease inhibitor; cystatin; osteoclasts

Osteoporosis is one of the most serious clinical disorders for elderly people, particularly for postmenopausal women. Bone is regularly formed and resorbed. If, for such reasons as estrogen deficiency in menopause, low calcium intake, and lack of exercise, an imbalance between bone formation and bone resorption occurs so that bone resorption exceeds bone formation, the risk of osteoporosis increases.[1,2] Milk contains not only nutrients such as calcium to support the growth of newborn mammals, but also biological functional components such as lactose and casein phospho-peptide (CPP) that affect bone metabolism. We have previously found that milk whey protein enhanced bone strength in ovariectomized rats.[3] It suppressed osteoclast-mediated bone resorption *in vitro* with an unfractionated bone cell culture system.[4] We have shown that this activity in whey could be fractionated into the basic protein fraction (milk basic protein [MBP]) by cation ion-exchange chromatography.[4] Osteoclasts secrete proteases to digest such bone matrix proteins as collagen.[5] Thus, the suppression mechanism for bone resorption is thought to involve the inhibition of cathepsin K activity. Cathepsin K has been reported to be a major protease secreted by osteoclasts[6] and a member of the cystein protease family. Since whey protein suppresses bone resorption, MBP possibly contains the cystein protease inhibitor (CPI). To certify this hypothesis, we purified CPI from MBP with papain affinity chromatography by following the CPI activity and determined the N-terminal amino acid sequence of the inhibitor. The bone resorption activity of CPI was assayed by using isolated osteoclasts.

Materials and Methods

MBP preparation. Fresh bovine milk was skimmed by centrifugation. The skimmed milk was loaded into a column packed with the cation-exchange resin, sulfonated chitopearl™ (Fuji Bouseki, Tokyo, Japan). The column was sufficiently washed with deionized water, and the adsorbed protein was eluted with 1M NaCl. The eluate was dialyzed against water in a cellulose membrane tube (Sanko Junyaku, Tokyo, Japan) and lyophilized as MBP.

Papain affinity column chromatography. Carboxy-

† To whom correspondence should be addressed. Fax: +81-49-242-8157; E-mail: matsuokayasuhiro@snowbrand.co.jp
Abbreviations: MBP, milk basic protein; CPI, cystein protease inhibitor; α-MEM, α-minimum essential medium; FBS, fetal bovine serum

methyl (CM)-papain immobilized gel was prepared by dissolving 1,800 milligrams of papain (Sigma, MO, USA) in 700 ml of a 0.1 M sodium phosphate buffer containing 5 mM EDTA and 2 mM cystein (pH 6.0). Forty milliliters of 0.1 M monoiodeacetate (pH 6.0, Sigma) was then added, and the mixture stirred for one hour at room temperature. After the solution had been adjusted to pH 7.5, fifty grams of AF-tresyl Toyopearl 650M gel (Tosoh, Tokyo, Japan) was added, and the mixture was gently shaken overnight at room temperature. The gel was washed by decantation with 1,800 ml of 0.5 M NaCl. The active site of the gel was then blocked for two hours at room temperature with 700 ml of 0.1 M Tris-HCl (pH 7.8) containing 0.5 M NaCl. An amount of 170 ml of the CM-papain immobilized gel was successively washed with 2 liters of deionized water, 600 ml of 0.1 M HCOONa (pH 2.8) containing 0.5 M NaCl, and a 20 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl (equilibration buffer). An amount of 122 g of MBP was dissolved in 5 liters of the equilibration buffer, and 170 ml of the CM-papain immobilized gel was added to the MBP solution, the mixture being gently stirred overnight with a propeller mixer (type 600, Heidon, Tokyo, Japan) at 4°C. The gel was washed with 2 liters of the equilibration buffer on filter paper (No. 1, Toyo Roshi, Tokyo, Japan) and packed into a glass column ($5\phi \times 20$ cm high), before being washed with the equilibration buffer at 3 ml/min until the absorbance at 280 nm indicated zero. The adsorbed CPI was eluted with a 0.1 M sodium phosphate buffer (pH 12) containing 1 M NaCl. Each 6 ml of the eluate was fractionated and immediately neutralized with 30% formic acid, before the CPI activity of the fraction was measured. Those fractions that had CPI activity from papain affinity chromatography were pooled and concentrated by using an ultra-filtration membrane (Amicon 8200, molecular weight cut-off of 10,000; Millipore, MA, U.S.A.).

Gel filtration chromatography. The concentrated fraction was loaded into a Hi-Load Superdex 75 16/60 column (Amersham Pharmacia, Little Chalfont, U.K.) that had been equilibrated with a 10 mM sodium phosphate buffer (pH 7.0) at a flow rate of 1.0 ml/min. Aliquots of 5 ml were fractionated. The CPI activity in each fraction was measured, and those fractions that had CPI activity from gel filtration chromatography were pooled and concentrated by using an ultra-filtration membrane (Amicon 8200, molecular weight cut-off of 10,000).

Protein determination. The protein concentration was determined by a DC protein assay (Bio-Rad, Hercules, CA, U.S.A.) based on the Lowry method.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were run on High-Density Gel™ under reduced conditions with a PhastSystem™ (Amersham Pharmacia) as prescribed in the manufacturer's manual. The gel was stained with Coomassie brilliant blue (CBB) or with a silver stain kit (Dai-ichi Pure Chemicals, Tokyo, Japan).

N-terminal amino acid sequence of purified CPI. The purified fractions with CPI activity were electrophoresed and transferred to a PVDF membrane (Immobilon-P, Millipore, Bedford, MA, U.S.A.). The membrane was stained with CBB, and the stained band was cut out. The N-terminal amino acid sequence of the band was determined with a protein sequencer (model 476A, PE Applied Biosystems, U.S.A.).

Assay for CPI activity. According to the method of Barret,[7,8] the CPI activity was determined by the inhibition activity in the protease reaction of papain (EC 3.4.22.2), a cystein protease. Five μl of the sample solution was mixed with 10 μl of solution A (185 mM NaH_2PO_4, 215 mM Na_2HPO_4, 4 mM EDTA, and 4 mM dithiothreitol) and 10 μl of solution B (0.1% Briji 35) in a 96-well plate. Ten μl of 100 nM papain in solution B was added to each well, except for the 'blank' wells, and preincubated for 10 min at 37°C. A 120-μl amount of solution C (solution A: solution B = 1:2) was added, and the mixture was preincubated for 10 min at 37°C. A 50-μl amount of a substrate solution (20 μM Z-Phe-Arg-MCA in solution B, Peptide Institute, Osaka, Japan) was reacted for 15 min at 37°C, before the reaction was terminated with 50 μl of 30% formic acid. The fluorescence intensity (FI) of the plate was measured under the conditions of Ex at 360 nm and Em at 460 nm with a plate reader (CytoFluor II series 4000, Perceptive Biosystems, Framingham, MA, U.S.A.). The wells without a sample were also measured as negative controls. The inhibition rate was calculated as follows:

$$\text{Inhibition rate } (\%) = ((C\text{-}S)/(C\text{-}B)) \times 100$$

C: fluorescence intensity of the negative control well
S: fluorescence intensity of the sample well
B: fluorescence intensity of the blank well

Assay for osteoclast-mediated bone resorption, using isolated bone cells (pit assay). According to the method of Takada,[9] purified protein was examined for its suppression activity toward osteoclast-mediated bone resorption. Briefly, unfractionated bone cells were prepared from the femoral bones of 10-day-old rabbits. The long bones were minced into small pieces in an α-minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS). The separated free cells, which had been collected by decantation, were precipitated by centrifugation. The

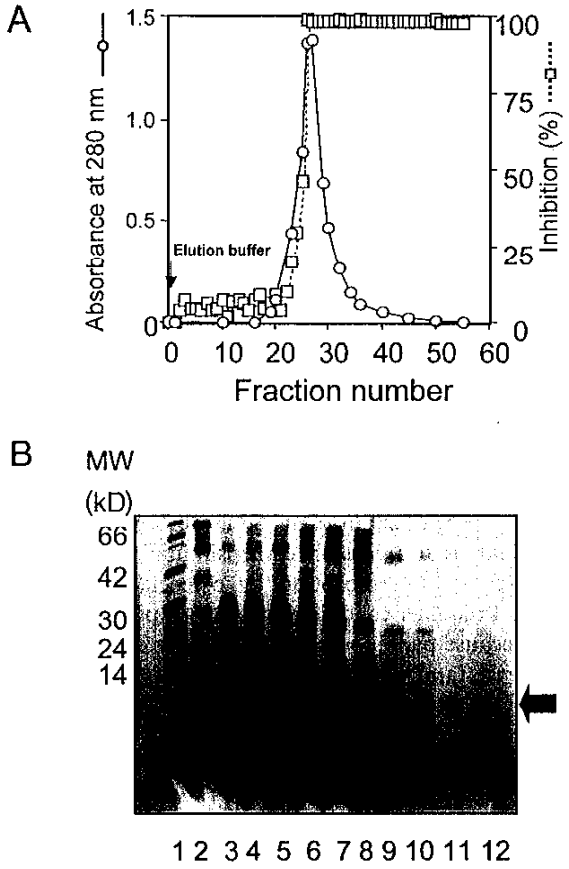

Fig. 1. Papain Affinity Chromatography of CPI from MBP.
(A): Elution profile for the papain affinity chromatography of MBP. The MBP solution was adsorbed to CM-papain immobilized gel. CPI was eluted with 0.1 M NaPB at pH12 and 1 M NaCl. The eluted protein was monitored by its absorbance at 280 nm (left Y-axis), and the inhibition activity was measured (%, right Y-axis). The volume of each fraction was 6 ml. (B): SDS-PAGE profiles of the protein samples fractionated by papain affinity chromatography. Electrophoresis was performed on High-Density Gel™ with a Phastsystem™ (Pharmacia). The proteins were visualized with the silver-staining system. Lane 1: molecular weight marker; lane 2: passed-through fraction; lane 3: fraction 24; lane 4: fraction 25; lane 5: fraction 26; lane 6: fraction 27; lane 7: fraction 28; lane 8: fraction 30; lane 9: fraction 40; lane 10: fraction 50; lane 11: fraction 54; lane 12: pooled fraction eluted with 100 mM NaPB at pH12 and 1 M NaCl. The fraction numbers correspond to those in Fig. 1(A). An arrow indicates approximately 12 kDa.

unfractionated bone cells were incubated on collagen gel in α-MEM with 5% FBS for 4 h. After the non-adhering cells and small bone debris had been washed off with PBS, the culture was treated with 0.001% pronase E containing 0.02% EDTA for 5 min at room temperature to remove any loosely attached cells from the gel. After three washes with PBS, 0.01% bacterial collagenase was added to remove the rest of the stromal cells from the gel. The culture was then washed several times with PBS and digested with 0.1% collagenase for 10 min at 37°C. The released osteoclasts were collected, washed by low-speed centrifugation (500 rpm for 2 min), and suspended in α-MEM with 5% FBS. The isolated osteoclasts were identified by tartrate-resistant acid phosphatase (TRAP) staining with a leukocyte acid phosphatase staining kit (Sigma). The appropriate numbers of isolated osteoclasts were incubated with or without a sample on dentine slices (6 mm in diameter and 0.1 mm thick) in a 96-well plate. After incubating for 48 h at 37°C, the cells were scraped off the dentine surface, and the slices were stained with acid hematoxylin to highlight the 'pits' that had formed. The number of pits was counted under a microscope.

Results

CPI purification from MBP

The MBP solution was loaded into a CM-papain affinity column. After being washed, the CPI adsorbed to papain was eluted with the elution buffer, CPI activity being eluted after fraction 23 (Fig. 1(A)). Reduced SDS-PAGE showed the high-molecular-weight protein bands in fractions 23–40, and an approximately 12-kDa major band were silver-stained after fraction 50 (Fig. 1(B)). Fractions 25–55 were pooled and concentrated by using an ultra-filtration membrane (molecular weight cut-off of 10,000). The concentrated crude CPI fraction was purified by gel-filtration chromatography, in a Hi-Load Superdex 75 16/60 column. The CPI activity was fractionated into tubes 9–12 and 18–25 (Fig. 2(A)). Reduced SDS-PAGE showed that an approximately 12-kDa single band by silver staining was only present in fractions 18–25 (Fig. 2(B)). These fractions were pooled and concentrated by using an ultra-filtration membrane (molecular weight cut-off of 10,000). A 11.4-mg amount of purified CPI was obtained from 122 g of MBP.

Determination of the N-terminal amino acid sequence of purified CPI

The N-terminal amino acid sequence of the purified 12-kDa protein was determined by a gas-phase amino acid sequencer. Eighteen amino acid residues from the N-terminal were determined, the sequence corresponding to that reported for bovine cystatin C[10,11] (Fig. 3).

Inhibition activity of cystatin from MBP to osteoclast-mediated bone resorption in vitro

Purified 12-kDa CPI was added to the medium at the final concentrations of 0.1 μg/ml, 1 μg/ml and 10 μg/ml, and then cultured with isolated osteoclasts on dentin slices. After 48 h of incubation, 12-kDa CPI had significantly inhibited the number of pits formed by osteoclasts in a dose-dependent manner (Fig. 4).

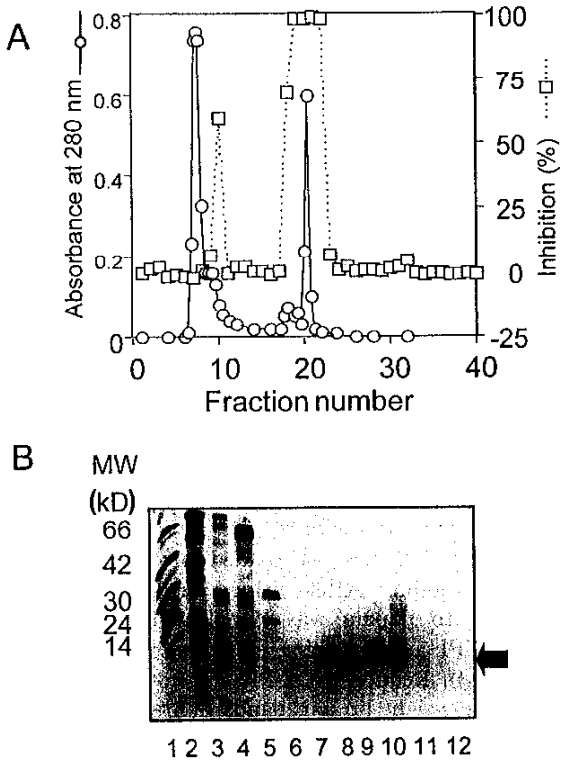

Fig. 2. Gel Filtration Chromatography of CPI from the Papain Affinity Chromatography Eluate.

(A): Gel filtration profile of the eluate from papain affinity chromatography. The concentrated CPI fraction from papain affinity chromatography was loaded into a Hi-Load Superdex 75 16/60 column. The volume of each fraction was 5 ml. Protein was monitored by its absorbance at 280 nm (left Y-axis), and the inhibition activity was measured (%, right Y-axis). (B): SDS-PAGE profiles of the protein samples fractionated by gel filtration chromatography. Electrophoresis was performed on High-Density Gel™ with a Phastsystem™ (Pharmacia). The proteins were visualized with the silver-staining system. Lane 1: molecular weight marker; lane 2: 0.1% MBP solution; lane 3: CPI fraction from papain affinity chromatography; lane 4: fraction 11; lane 5: fraction 13; lane 6: fraction 16; lane 7: fraction 17; lane 8: fraction 18; lane 9: fraction 20; lane 10: fraction 22; lane 11: fraction 24; lane 12: fraction 25. The fraction numbers correspond to those in Fig. 2(A). An arrow indicates approximately 12 kDa.

Discussion

Milk is well known as a good source of calcium and other nutrients. We have previously found that a bovine milk fraction contained factors for bone metabolism. This fraction was eluted from cation-exchange-resin-adsorbed bovine milk, and we named it "milk basic protein" (MBP). MBP showed both bone formation and the suppression of bone resorption *in vitro*[4,12] and *in vivo*.[3,13,14] We have already reported for bone formation that kininogen fragment 1.2[15] and the high mobility group (HMG)-like protein[16] in MBP stimulated the proliferation of osteoblastic MC3T3-E1 cells.

Fig. 3. Alignment of the Amino Acid Sequences of Bovine Cystatin C[10] and Those of the N-Terminal of Purified 12-kDa CPI from MBP.

'X' indicates unidentified residues from sequencing. The identical amino acid residues are common when marked by '*'.

We detected in this present study a bone resorption inhibiting factor from MBP. Bone was resorbed by the following mechanism: Osteoclasts on the bone surface secreted cathepsin K[5,6,17] to digest collagen in the bone matrix. They also secreted protons that dissolved calcium from the bone. Cathepsin K is a member of the cystein protease family. Since bovine milk whey protein suppressed bone resorption,[4,14] we speculate that a possible cathepsin inhibitor in MBP inhibited bone resorption by osteoclasts. We adsorbed the CPI candidate in MBP to papain affinity gel because papain was also a member of the cystein protease family. CPI activity was measured in some fractions eluted from the gel, and crude CPI was obtained. Nevertheless, the chromatogram for the absorbance at 280 nm showed a single peak, and CPI activity was broadly eluted (Fig. 1). One possible reason for this might be that adsorption between CPI and papain was strong against the elution buffer. Crude CPI was therefore further purified by gel filtration chromatography. The chromatogram of protein showed two peaks around fraction numbers 10 and 20; however, the CPI activity in fraction 20 was higher than in 10. The activity in fraction 20 was coincident with the protein peak (Fig. 2(A)). Fraction 20 contained an approximately 12 kDa single silver-stained band by SDS-PAGE (Fig. 2(B)). The activity of fraction 10 had a 'shoulder' protein peak that was presumed to be a minor component in MBP. Thus, to identify this component, further study is needed.

The partial N-terminal amino acid sequence of the purified 12-kDa protein was identical to that of bovine cystatin C (MW12787) which had been identified in bovine colostrum[10,11] (Fig. 3). Since MBP had been fractionated from bovine milk, the purified 12-kDa protein from MBP is likely to have been bovine cystatin C. The first amino acid residue could not identified because of some noise.

Cystatin C is a cystein protease inhibitor and is also found in parotid glands[11] and cerebrospinal fluid.[18] Cystatin C inhibits such proteases as papain and cathepsin B, L and K.[19] Cathepsin K is a major protease secreted by osteoclast cells. Osteoclast differentiation is regulated by osteoblasts, and osteoblasts secrete the receptor activator of NF-κB ligand (RANKL) to stimulate differentiation from precursor to mature osteoclasts. Osteoblasts also produce cystatin C.[20] We have shown in this study

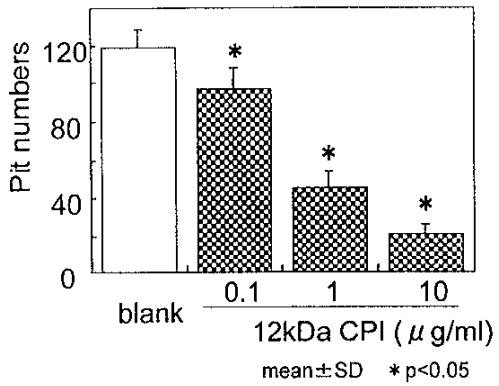

Fig. 4. Inhibition of Pit Formation by 12-kDa CPI, Using Isolated Osteoclasts.

The pit number formed by the isolated osteoclasts on a dentine slice was counted after 48 hrs of incubation in a pit assay with or without purified 12-kDa CPI from MBP. 12-kDa CPI suppressed the pit formation in a dose-dependent manner.

that purified CPI from MBP suppressed bone resorption by isolated osteoclasts *in vitro* (Fig. 4). This result suggests that exogenous milk cystatin also inhibits cathepsin K and suppresses bone resorption by osteoclasts.

We have previously found in a human study that MBP supplementation (40 mg of MBP a day) increased BMD and suppressed the urinary excretion of N-telopeptides of type-I collagen (NTx).[21] NTx excretion is a biochemical marker of bone resorption. This means that MBP suppressed bone resorption and led to a relatively predominant status of bone formation against bone resorption in the bone remodeling process *in vivo*. Our *in vitro* results for the metabolic process indicate that cystatin in MBP played an important role in the suppression of bone resorption.

In conclusion, cystatin C purified from MBP inhibited cathepsin secreted by osteoclast cells and suppressed bone resorption. Milk cystatin is suggested to be one of the components suppressing bone resorption.

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