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The Fragments of Bovine High Molecular Weight Kininogen Promote Osteoblast Proliferation *In Vitro*

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High molecular weight (HMW) kininogen is known to be a large plasma protein and cleaved by plasma proteinase kallikrein, then it generates four fragments in the blood coagulation cascade: heavy chain, bradykinin, fragment 1·2, and light chain. The fragment 1·2 has also been found in the basic protein fraction of bovine milk as a bioactive protein which promotes osteoblast proliferation. The milk basic protein has been shown to be a multi functional edible protein which promotes bone formation and inhibits bone resorption. In the present study, we purified the fragment 1·2 from bovine plasma and assessed it could promote osteoblast proliferation and posses the activity after pepsin digestion. Purified plasma HMW kininogen did not promote the proliferation, however, the kallikrein-cleaved HMW kininogen promoted the proliferation. The fragment 1·2, purified from the proteolysate, also promoted the proliferation. The pepsin digestion was performed according to the method of the assessment of allergenesity of genetically modified crops. After pepsin digestion, the fragment 1·2 generated resistant fragments and showed the promoting activity of osteoblast proliferation. These results suggest that the enzymatically-digested fragments of bovine HMW kininogen are able to be a naturally occurred active protein that promotes the bone formation by oral administration.

Key words: bone formation, fragment 1·2, kininogen, milk basic protein, osteoblast.

Abbreviations: HMW; high molecular weight; MBP; milk basic protein.

Mammalian plasma high molecular weight (HMW) kininogen has been considered to be a multifunctional protein that releases bradykinin by the action of kallikrein and accelerates the surface-mediated activation of factor XII by forming a molecular complex with prekallikrein or factor XI and binds to negatively charged surfaces in the blood coagulation cascade (*1*). In addition, both HMW and low molecular weight kininogen has been identified as one of the cysteine proteinase inhibitors in plasma (*2*). Bovine HMW kininogen is a single chain glycoprotein with a molecular mass of 76 kDa consisting of four domains that include a heavy chain (48 kDa), a kinin moiety (1 kDa), a fragment 1·2 (14 kDa), and a light chain (16 kDa) (*3*). Its cDNA sequence was reported as a single gene (*4*). Using a large scale preparation and column chromatography technique, the HMW kininogen was found in bovine milk as smaller molecules revealed by a mass spectrometry (*5*).

Recently, we indicated that the fragment 1·2 existed in bovine milk basic protein (MBP) fraction and showed its promotive activity to the proliferation of murine osteoblastic MC3T3-E1 cells, which was used as a bone formation assay system *in vitro* (*6*). In previous study, we have shown that the MBP from whey promoted the proliferation of the osteoblast (*7*) and suppressed the osteoclast-mediated bone resorption and osteoclast formation (*8*). We have also shown dietary MBP prevented bone loss in aged ovariectomized rats (*9*). In human study, we have revealed that MBP increased the bone mineral density at calcaneus in a placebo controlled trial (*10*) and that MBP increased the radial bone mineral density in healthy women (*11*). Using chromatography technique, we purified the active components of MBP that promoted osteoblast proliferation (*12*), then we assigned the kininogen fragment 1·2 by amino-terminal sequence analyses (*6*). Although the kininogen fragment 1·2 is a minor component in MBP, we presume it as one of the responsible components which promote bone formation. Because the MBP promotes the bone formation in mammals by oral administration, the active components are supposed to be resistant to digestive enzymes in their guts. Therefore, we assessed the fragment 1·2 digestion using a simulated gastric fluid containing pepsin. For these experiments, a large amount of the fragment 1·2 was needed. The concentration of fragment 1·2 seemed to be higher in bovine plasma than in bovine milk. Therefore, we purified HMW kininogen from bovine plasma and proteolysed it by plasma kallikrein. Then we prepared the fragment 1·2 for the digestion study.

There are several reports of using artificial digestion of proteins, and then we chose a severe condition of artificial digestion that uses the simulated gastric fluid containing high concentration of pepsin. This method was evaluated by ten laboratories in the world then reported in 2004 (*13*). It was approved by the International Life Science Institution (ILSI) and then has been used in the assessments of digestibility of newly created crops, the genetically modified crops. Therefore, we assessed the bovine kininogen fragment 1·2 digested with the simulated gastric fluid

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and its activity for osteoblast could survive after the digestion, then assumed the oral administration.

EXPERIMENTAL PROCEDURES

Materials—Alpha-modified essential medium (α-MEM) was purchased from Flow Laboratories (Mclean, VA, USA), and fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, USA). DEAE-Sephadex A-50, Sephadex G-150, Chelating Sepharose Fast Flow, and Mono Q HR 5/5 were purchased from Pharmacia (Uppsala, Sweden). HPLC column, Protein-RP (C4) was from YMC (Kyoto, Japan). Kininogen deficient human plasma, Fitzgerald trait, and kallikrein deficient human plasma, Fletzer trait, were purchased from George King Bio-Medical (Overland Park, Kansas, USA). Other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan).

Cell Culture and Proliferation Assays—Osteoblastic MC3T3-E1 cell culture and proliferation assay using [^{3}H]thymidine were described previously (*12*). In brief, the cells were maintained in α-MEM supplemented 10% FBS. The cells was harvested from dishes and cultured in serum-free α-MEM on a 96 well plate and incubated with samples for 24 h then labeled with [^{3}H] thymidine for 4 h. The incorporated [^{3}H] was measured using the Topcount NT microscintillation counter (Packard, USA).

Purification of Plasma HMW Kininogen Fragment 1·2—Plasma HMW kininogen was purified from bovine blood according to the methods of Shimada (*14*) and Hayashi (*15*). Briefly, twelve liters of fresh blood containing EDTA as anticoagulant was corrected at a slaughterhouse. Plasma was prepared by centrifugation and applied to an anion-exchanger DEAE-Sephadex column. Eluates were collected, and kininogen activity was measured by the correction of the prolonged clotting time of Fitzgerald trait plasma (*14*). Fractions contain HMW kininogen were applied to a zinc-chelating column. The column was washed by NaCl and histidine, and bound proteins were eluted with EDTA. The kininogen activity was measured, and active fractions were dialyzed and lyophilized. The protein powder was dissolved and loaded onto a Sephadex G-150 column (*15*). The fractions with kininogen activity were loaded onto a Mono Q column equilibrated with 20 mM Tris-HCl (pH 8.0), 50 mM NaCl. Bound proteins were eluted by linear gradient of 0 to 1.0 M NaCl. HMW kininogen was eluted at 0.4 M of NaCl. The purified protein was dialyzed against 20 mM Tris-HCl (pH 8.0), 50 mM NaCl.

Purification of Kallikrein—Plasma kallikrein was partially purified from the eluate with kallikrein activity of DEAE-Sephadex column, then the eluate was applied to a zinc-chelating column. Bound kallikrein was washed with a buffer used in the equilibration and eluted with 1.0 M of NaCl. Kallikrein activity was measured by the correction of the prolonged clotting time of Fletzer trait plasma (*14*).

Limited Proteolysis of HMW Kininogen and Purification of Fragment 1·2—HMW kininogen was incubated with the kallikrein fraction at 37°C for 16 h. The proteolysate was loaded onto SDS-PAGE and into a C4-column Protein-RP (YMC, Kyoto, Japan) on reversed phase HPLC, and bound proteins were eluted with a linear gradient of 0–80% of acetonitrile. Fragment 1·2 and heavy chain–light chain complex were eluted at 20% and 30% of acetonitrile, respectively.

Polyacrylamide Gel Electrophoresis—Purified fractions and limited proteolysis samples were electrophoresed under non-reducing condition on a 16% polyacrylamide gel with 0.1% SDS in Tris-Tricine system (*16*) by using a Peptide-PAGE mini precast gel (TEFCO, Tokyo, Japan). In pepsin digestion experiments, 10–20% gradient Tris-Tricine gels (Invitrogen, Carlsbad, CA, USA) were used in reducing condition.

Pepsin Digestion—The standardized method for allergenesity assessment of genetically modified crops by Thomas *et al.* (*13*) were used to assess the effect of digestive enzyme degradation of the fragment 1·2. In brief, a ratio of 10 U of pepsin (Sigma, MO, USA, P-6887 porcine stomach) activity/mg of purified fragment 1·2 was used. The pepsin/protein ratio in the digestion reaction was approximately 2.2:1 (w:w). A single tube containing simulated gastric fluid (SGF; 0.084 N HCl, 35 mM NaCl, pH 1.2 and 4,000 U of pepsin) was preheated to 37°C prior to the addition of test protein solution (5 mg/ml). The tube contents were mixed and placed in a 37°C water bath. The samples were removed at 0.5, 2, 5, 10, 20, 30, and 60 min after initiation of the incubation and then quenched by addition of alkali solution (200 mM $NaHCO_3$, pH 11) and 5× Laemmli sample buffer (40% glycerol, 5% 2-mercaptoethanol, 10% SDS, 0.33 M Tris-HCl, 0.05% bromophenol blue, pH 6.8). Quenched samples were immediately heated to more than 75°C for 10 min. Samples from each time point and control reactions were applied to SDS-PAGE electrophoresis in reducing conditions using 10–20% Tris-Tricine gels. The gels were fixed with 40% methanol, 10% acetic acid for 30 min and stained with Coomassie brilliant blue G-250 (Bio-Rad, Richmond, CA; Cat. #161–0786) for 1 h.

Protein Sequencing—The amino-terminal sequence of excised protein bands on PVDF membrane were analyzed by a gas-phase protein sequencer Model 492 cLC (Applied Biosystems, Foster City, CA, USA).

RESULTS

Preparation and Linited Proteolysis of HMW Kininogen—The single chain of HMW kininogen was purified from twelve liters of bovine blood by anion-exchanger columns, a chelating column, and gel filtration column showed a relative molecular weight approximately 100 kDa on a SDS-PAGE (Fig. 1, lane S). The kallikrein proteolysis of HMW kininogen formed the fragment 1·2 (23 kDa) and the lower molecular weight band, heavy chain–light chain complex (80 kDa). The band intensity of HMW kininogen (100 kDa) decreased and lower molecular weight band (80 kDa) and the band of fragment 1·2 (23 kDa) increased in a time-dependent manner (8 and 16 hours). A crude kallikrein fraction (lane E) and the HMW kininogen (lane S) did not contain any fragment 1·2 bands.

Osteoblast Assay of the Cleaved HMW Kininogen—The time course samples were tested for the osteoblast proliferation assay. Although the HMW kininogen and the kallikrein did not show any activity of promotion of the [^{3}H] incorporation, the cleaved kininogen showed

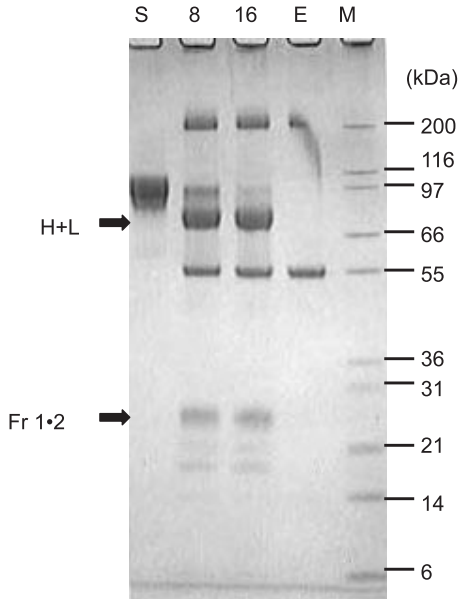

Fig. 1. **SDS-PAGE analysis of kallikrein proteolysis of bovine high molecular weight (HMW) kininogen.** The HMW kininogen was purified from bovine plasma by chromatography according to the method of Shimada. Eight micrograms of substrate HMW kininogen (S) was incubated with 3 μg of partially purified bovine plasma kallikrein (E) at 37°C for the time indicated (8, 16 h). Each sample and blank contained only enzyme (E) were loaded to a SDS–polyacrylamide gels (10% T) under non-reducing condition. After the electrophoresis the gel was stained with Coomassie Brilliant Blue. The HMW kininogen liberated the fragment 1·2 and a nonapeptide, bradykinin, as Kato and Hayashi reported. The position of the heavy and light chain complex (H+L, 70 kDa) and the 23 kDa fragment 1·2 were indicated by arrows.

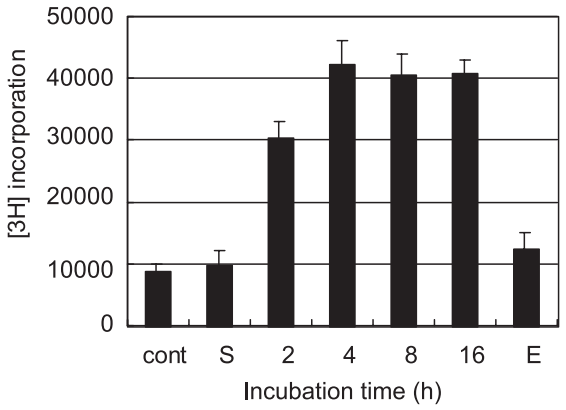

Fig. 2. **The osteoblast proliferation assay of kallikrein-cleaved high molecular weight kininogen.** The substrate HMW kininogen (S) was incubated with partially purified bovine plasma kallikrein. At 2, 4, 8 and 16 h in the incubation the samples were frozen. These samples were added into the osteoblastic MC3T3-E1 cells, then [^{3}H]thymidine incorporation was measured. The buffer control (cont) and the kallikrein alone (E) were also assayed. The bars indicate means and standard deviations of four wells each.

increased activity according to the incubation time. The growth promoting activity reached to the maximum at 4-h incubation (Fig. 2).

Purification and Digestion of the Fragment 1·2—Then we purified the fragment 1·2 from the cleaved kininogen using RP-HPLC, and it showed the molecular weight of 23 kDa on a Tricine SDS-PAGE (Fig. 3). Then we assessed the stability of the fragment 1·2 to a digestive enzyme. Using the method of ILSI standardized assessment of pepsin resistant study, the fragment 1·2 showed relatively resistant to pepsin digestion, moreover, generated two resistant fragments (Fig. 3). The 23 kDa band of fragment 1·2 lasted up to 10 min and as trace in 20–60 min in the digestion. Newly formed fragments, estimated 13 kDa and 8 kDa by the mobility of Tricine SDS-PAGE, Fr 13k and Fr 8k increased in a time dependent manner. The bands of pepsin appeared just above the 36 kDa molecular weight marker band (lane M). The digest were electrophoresed and blotted onto a PVDF membrane, and the Fr 13k and the Fr 8k were excised for the amino acid sequence study. The analyses of these amino-terminal sequences revealed that the Fr 13k and the Fr 8k were consistent with the internal sequence of fragment 1·2, ^{26}Glu- (EERDSGK-), and ^{48}Ile- (IKLHGLG-), respectively. A schematic primary structure of HMW kininogen and its fragments were shown in Fig. 4.

Osteoblast Assay of the Digested Fragment 1·2—The osteoblast promoting activity of the pepsin digests were assessed using the MC3T3-E1 cells. Figure 5 shows that the activity of the fragment 1·2 (column S) was survived after pepsin digestion in the simulated gastric fluid (column D). The simulated gastric fluid did not show the activity in the assay (lane E).

DISCUSSION

MW of Bovine Milk Kininogen Fragment 1·2—The molecular weight of the fragment 1·2, released from kallikrein-cleaved kininogen, on the SDS–polyacrylamide gel was 23 kDa in our study. Kato *et al.* reported it was 14 kDa estimated by a disc gel electrophoresis (*3*). After a decade, they reported rat kininogen compared to that of bovine (*15*). In the article, they showed a slab gel SDS-PAGE, and the MW of bovine fragment 1·2 was shown around 20 kDa on the gel. Han *et al*. reported the fragment 1·2 had 4 kDa of carbohydrate chain (*17*). The microheterogeneities of HMW kininogen and the fragment 1·2 were reported (*18*, *19*). The difference seems to due to the methods of electrophoresis and carbohydrate chain moiety.

The molecular weight of HMW kininogen was estimated as about 76,000 by sedimentation equilibrium and the Archibald method (*20*). However, Hayashi *et al.* reported that the band of HMW kininogen migrated as a band of 100 kDa on a slab gel SDS-PAGE (*15*). It is consistent with our result. They discussed that the molecular weight seemed not to be correct, as abnormal behavior of bovine HMW kininogen on SDS-PAGE and gel filtration had been described by Komiya *et al.* (*20*). The difference was probably due to the glycoprotein nature of the material. The HMW kininogen contains 12.6% carbohydrates, consisting of hexose (4.5%), hexosamines (3.65%), and sialic acid (4.35%) (*20*).

Limited Proteolysis of HMW Kininogen by Kallikrein—It is well known that the heavy chain and the light chain were connected with a disulfide bond (*18*). In our preparation, we applied the kallikrein-cleaved HMW kininogen

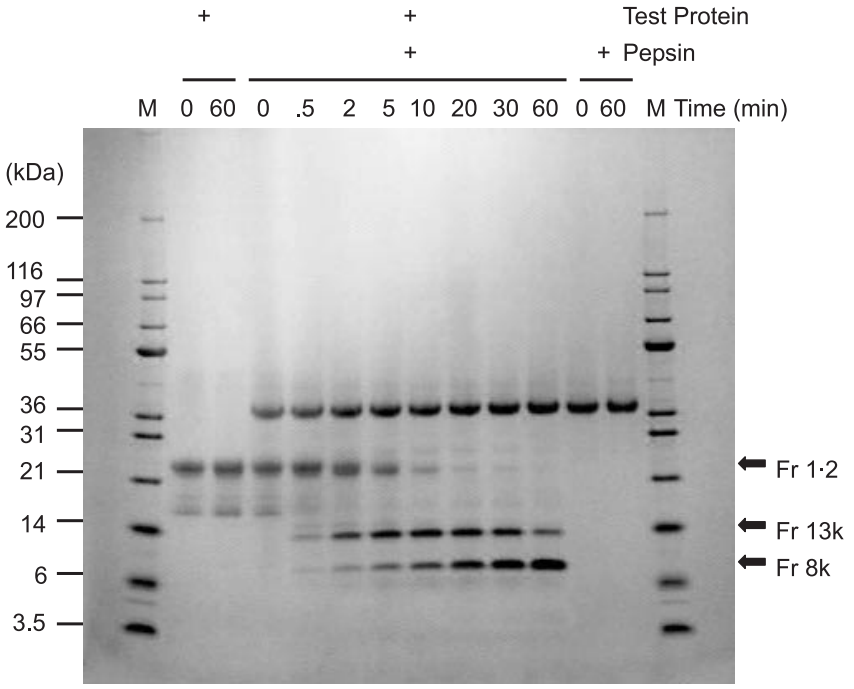

Fig. 3. **The bovine high molecular weight kininogen fragment 1·2 was digested at different rates in a standardized *in vitro* pepsin digestion assay.** The gels were loaded in the same order with same amount of digestion reaction mix or markers. Molecular weights (kDa) are indicated for lanes labeled "M." All other lanes contained 15 μl of quenched reaction mix sampled at the times indicated. The reactions contained either test protein alone in SGF (simulated gastric fluid; 0 and 60 min); test protein in SGF with pepsin (0, 0.5, 2, 5, 10, 20, 30, and 60 min); or SGF with pepsin alone (0 and 60 min). These 10–20% polyacrylamide gels were fixed and stained with CBB-G250. The kininogen fragment 1·2 was relatively resistant and also produced resistant fragments. The migrated position of the fragment 1·2 and the resistant fragments were indicated by arrows.

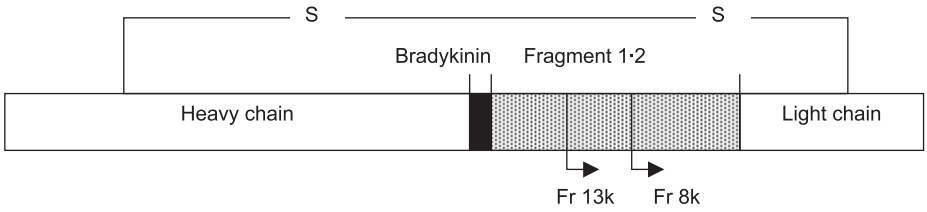

Fig. 4. **The schematic representation of primary structure of bovine HMW kininogen.** The specific plasma enzyme, kallikrein, cleaves three sites of kininogen polypeptide, then generates four fragments. The heavy chain is known to connect to the light chain by a disulfide bond. The nonapeptide, bradykinin and the fragment 1·2 liberate from the heavy–light chain complex. In simulated gastric fluid, the fragment 1·2 is cleaved at two sites by pepsin and generates 13-kDa fragment (Fr 13k) and 8-kDa fragment (Fr 8k). According to the amino-terminal sequence analyses, the position of those two fragments are shown in the scheme.

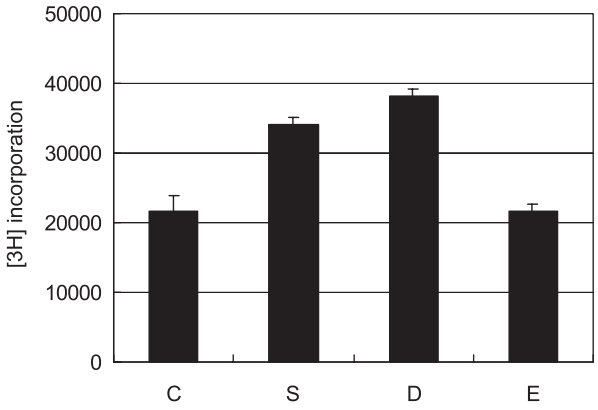

Fig. 5. **The osteoblast proliferation assay of the kininogen fragment 1·2 incubated in the simulated gastric fluid (SGF).** The substrate fragment 1·2 (S) was incubated with pepsin in acidic condition for 60 min. These samples were added into the osteoblastic MC3T3-E1 cells, then [^{3}H]thymidine incorporation was measured. The buffer control (C) and the SGF alone (E) were also assayed. The bars indicate means and standard deviations of four wells each.

onto a SDS-PAGE under non-reducing condition to elucidate the release of the fragment 1·2 from HMW kininogen. As we expected, there were two major bands appeared on the gel, the heavy chain–light chain complex and the fragment 1·2. The molecular weight of the complex was accounted to 64 kDa, the mobility of the band corresponded to it, when carbohydrate chain moiety was added. We have confidence in the preparation of the fragment 1·2 with RP-HPLC, because the N-terminal amino acid sequence was corresponded to reported sequence in SWISS PROT database (gi 125505). Although the plasma kallikrein fraction was not purified, the HMW kininogen was proteolysed and released the fragment 1·2. Bands of bovine serum albumin can be seen in Fig. 1, however, the fragment 1·2 was purified from the proteolysed mixture using an HPLC.

Pepsin Resistant Fragments—There are some food proteins that is resistant for the pepsin digestion. For example, soy bean trypsin inhibitor and concanavalin A are reported to resistant (*13*). Most of food proteins are considered to generate no resistant fragments, because they are eaten by mammals as nutrients. The resistant fragment could act as biologically active peptide or protein.

Therefore, we tested the pepsin digest of the fragment 1·2 to the osteoblast assay. The fragment 1·2 possess a lot of basic amino acid residues, especially histidine and consists an unusual histidine-rich region. Han *et al*. digested the fragment 1·2 by trypsin and chymotrypsin and assigned eight and seven major peptide, respectively (*19*). The pepsin resistant fragments Fr 13k and Fr 8k have a lot of histidine residues, 20 and 17 residues, respectively.

In human, HMW kininogen has been cloned (*21*) and reported as a thiol proteinase inhibitor (*22*), a cell adhesion inhibitor (*23*), and an angiogenesis inhibitor (*24*). There are several reports that domain 5 of HMW kininogen are responsible for these activities (*25*, *26*). In the same manner as bovine, human HMW kininogen is cleaved by plasma kallikrein and liberates bradykinin (domain 4) and results active cofactor, cleaved HMW kininogen (HKa), consists of a heavy chain (65 kDa) containing domains 1, 2, and 3 and a light chain (55 kDa) containing domains 5 and 6. The domain 5 contains the histidine-rich region and is a homologue of bovine fragment 1·2. Colman found significant sequence homologies in human domain 5 and bovine fragment 1·2 (*27*). The residues 443–475 HG-rich region is 55% homologous, and a second area rich in HGK is 61% homologous. These HG-region and HGK-region are located in the C-terminal half of the domain 5 in human and of the fragment 1·2 in bovine. Kawasaki reported the HGK-motif showed suppression of cancer metastasis by inhibition of cell adhesion and haptoinvasion using synthetic peptides, and lysine was responsible to the activity (*28*). Replacement the lysine to arginine diminished the inhibitory activity. In our results, these regions are considered to exist in the Fr 8k. We presume that bovine fragment 1·2 is one of bioactive protein such as human HMW kininogen domain 5.

About Osteoblast Activity of the Pepsin-Digest—We showed the pepsin-digested fragment 1·2 promoted the proliferation of osteoblasts. By PAGE analysis, the digest contained two resistant fragments Fr 13k and Fr 8k. According to molecular weight, these fragments are considered to overlap each C-terminals. In other words, the Fr 13k contains the Fr 8k. In the time course experiment (Fig. 3), the band intensity of the Fr 8k increased during the digestion, while the Fr 13k decreased after 30 min. These results suggests the Fr 8k is more resistant than the Fr 13k and may be the final fragment of the fragment 1·2 in the digestive system.

The osteoblast assay is a very simple *in vitro* test to assess the activity of water-soluble substance to bone cells, and small amount of test sample is needed. If the test samples were assessed by oral administration in animal experiments, large amount of sample and long time period would be needed for replacement of bone cells in animals' body. In our research group, the effectiveness of milk protein on bone biology have been elucidated in both animal experiments and these osteoblast assay. We have reported that milk basic protein showed the activity to prevent the bone loss in ovariectomized rats (*9*) and to increase the bone mineral density in healthy women (*10*, *11*). We have also reported the responsible components of the milk basic protein. We identified the HMW kininogen fragment 1·2 (*6*) and high mobility group like protein (*12*) as one of responsible components for osteoblast growth, and the cystatin C for osteoclast inhibition (*29*) in milk basic protein. The results of the present study could support that orally-administrated kininogen fragment 1·2 produced resistant fragments from digestion and acted as a growth promoting substance for osteoblast *in vivo*. It may be a first step to elucidate that milk promotes the bone growth of infant not only by calcium but by active proteins. Our results show a minor component of milk basic protein is resistant to digestion and is not only amino acid source. The HG-region and HGK-region are specific motif in the bovine fragment 1·2 and human domain 5. After digestion and absorption, these specific motifs might be an analogue of degradation products and a synthesis signal of collagen type 1 in bone, which is extremely rich in glycine. However, there is not much information about the collagen metabolism. The present study showed a possibility that orally-administrated kininogen fragment 1·2 could be a naturally occurred active protein which promotes the bone formation.

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