JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

In Vitro Inhibition of the Activation of Pro-matrix Metalloproteinase 1 (Pro-MMP-1) and Pro-matrix Metalloproteinase 9 (Pro-MMP-9) by Rice and Soybean Bowman−Birk Inhibitors

HIBA A. BAWADI,[†] TANIA MARIA ANTUNES,[§] FRED SHIH,[#] AND JACK N. LOSSO*[,§]

School of Human Ecology and Department of Food Science, Louisiana State University Agricultural Center, Baton Rouge, Louisiana 70803, and Southern Regional Research Center, U.S. Department of Agriculture, New Orleans, Louisiana 70112

The in vitro inhibitory activity of the rice Bowman−Birk inhibitor (rBBI) or soybean Bowman−Birk inhibitor (sBBI) against trypsin-catalyzed activation of pro-matrix metalloproteinase 1 or 9 (pro-MMP-1 or pro-MMP-9), respectively, was investigated using electrophoresis with silver staining, heparin-enhanced zymography, biotinylated gelatin, Biotrak assay, and fluorescence quenched substrate hydrolysis. rBBI at concentrations of 0.08−0.352 mg/mL dose-dependently inhibited the in vitro activation of 45 μg/mL pro-MMP-1 by trypsin. Heparin-enhanced zymography analysis of pro-MMP-1, trypsin-activated MMP-1, and a mixture of pro-MMP-1−trypsin−rBBI showed clear zones associated with trypsin-activated MMP-1 and the absence of clear zones in lanes containing pro-MMP-1 or a mixture of pro-MMP-1, trypsin, and rBBI. The results of the Biotrak assay also indicated that rBBI dose-dependently suppressed the activation of pro-MMP-1 by trypsin. sBBI dose-dependently inhibited the activation of 100 μg/mL of pro-MMP-9 by trypsin. Biotinylated gelatin assays demonstrated that pro-MMP-9 or pro-MMP-9 in the presence of trypsin and BBI did not hydrolyze gelatin, whereas *p*-aminophenylmercury acetate (APMA)-activated MMP-9 and trypsin-activated MMP-9 caused significant hydrolysis of gelatin. Quenched fluorescence substrate hydrolysis for total MMP activity showed that pro-MMP-1 or pro-MMP-9 did not hydrolyze the substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2; active MMP-1 or MMP-9 hydrolyzed the substrate, but lower substrate hydrolysis was obtained when pro-MMP-1 or pro-MMP-9 was incubated with trypsin in the presence of increasing concentrations of rBBI. The results are discussed in light of the role of MMP-1 and MMP-9 in the process of angiogenesis and the potential of rBBI or sBBI as a functional food ingredient.

KEYWORDS: Metalloproteinase 1; metalloproteinase 9; Bowman−Birk inhibitor; angiogenesis; functional food ingredient

INTRODUCTION

Angiogenesis is the formation of new blood vessels from pre-existing vascular ones. There are two types of angiogenesis: physiological and pathological angiogenesis. Physiological angiogenesis is vital in embryonic development, wound healing, bone repair, and reproduction. Pathological angiogenesis is associated with almost every chronic life-threatening disease, including chronic inflammation, arthritis, osteoporosis, multiple sclerosis, macular degeneration, Alzheimer's disease, Parkinsons's disease, diabetes blindness, Crohn's disease, Lou Gehrig's disease, psoriasis, stroke, AIDS complications, cancer, and many others. Regulation of angiogenesis has been identified and recognized as a target for disease prevention and possibly therapy (*1*, *2*).

Matrix metalloproteinases (MMPs) have been identified as catalysts of the early basement membrane degradation step (*3*). There are more than 20 MMPs reported to date (*4*). MMPs are secreted, to the extracellular matrix, as zymogens and require activation before they can catalyze the process of basement membrane degradation. Serine proteases such as trypsin, chymotrypsin, thrombin, elastase, plasmin, and cathepsin G are endogenous activators of most matrix metalloproteinases (*5*−*7*). Controlling the activity of MMPs by keeping the MMPs as zymogens (pro-MMPs) or inactivating the active MMPs has been recognized as an important approach to control angiogenesis (*3*, *8*).

Pro-matrix metalloproteinase 1 (MMP-1) is a 52 kDa zymogen found in abundance in the lining of the human stomach and gastrointestinal tract wall and the skin of smokers (*9*, *10*).

* Author to whom correspondence should be addressed [telephone (225) 578-3883; fax (225) 578-5300; e-mail jlosso@lsu.edu].
[†] School of Human Ecology, Louisiana State University.
[§] Department of Food Science, Louisiana State University.
[#] U.S. Department of Agriculture.

10.1021/jf034576u CCC: $27.50
Published on Web 07/03/2004

The functions of MMPs in the gut are not known. The zymogen pro-MMP-1 is activated by serine proteases to active MMP-1. The active form of MMP-1 is a 42−44 kDa polypeptide. Active MMP-1 catalyzes the process of angiogenesis and metastasis (*9*).

Pro-MMP-9 (gelatinase B) is a 92-kDa type IV pro-collagenase. MMP-9 hydrolyzes type I and IV collagens and gelatins. The degradation of the extracellular matrix is also dependent upon the concentration of endogenous inhibitors of MMPs, also called tissue inhibitors of metalloproteinases (TIMPs). The presence of more active MMPs at the expense of inhibitors leads to increased susceptibility to angiogenic diseases.

There is strong evidence that MMPs participate in cardiovascular diseases, angiogenesis, tumor growth, and metastasis (*9*−*11*). The literature is well established that in tumors such as bladder, breast, gastric, colon, neck, pancreas, prostate, and lung cancers, MMP-9 and MMP-2 are overexpressed compared to normal tissues (*3*). Preventing pro-MMP-1 and/or pro-MMP-9 activation may delay the onset/and or progression of angiogenesis-dependent diseases.

Rice Bowman−Birk inhibitor (rBBI) is a double-headed 16 kDa water-soluble protein present in bran and endosperm. rBBI consists of 133 amino acid residues with 18 half-cystine residues, which are involved in 9 disulfide bridges in the molecule (*12*). Rice BBI has a duplicated structure of the soybean Bowman−Birk inhibitor (sBBI) because the protein sequence has four domains; domains I and III are homologous to the first domain of sBBI, and domains II and IV are homologous to the second domain of sBBI. Unlike the sBBI, which inhibits trypsin and chymotrypsin, rBBI inhibits only trypsin and many other tryptases.

Soybean contains health-enhancing bioactive compounds such as isoflavones, protease inhibitors, saponins, phytic acid, and oligosaccharides. The sBBI has been identified and recognized by several in vitro studies as a protease inhibitor with the ability to enhance human health (*13*−*15*). Naturally occurring protease inhibitors that can inhibit MMP-9 and MMP-1 are being intensively investigated as efficient bioactive compounds against the onset and/or progression of degenerative diseases and angiogenesis (*16*, *17*). Several naturally occurring MMP and protease inhibitors have entered clinical trials, where some have demonstrated effectiveness (*11*, *14*). The objective of this research was to study the in vitro inhibitory activity of rBBI against pro-MMP-1 activation and the in vitro inhibition of pro-MMP-9 activation by the sBBI.

MATERIALS AND METHODS

Materials. Rice bran was obtained from Riceland, Stuttgart, AR. Rice Bowman−Birk inhibitor was isolated in our laboratory following the procedure of Tashiro et al. (*12*). Pro-MMP-1 (human synovial rheumatoid fibroblast, catalog no. 444208), active MMP-1 (human protein, catalog no. PF 067), pro-MMP-9, *p*-aminophenylmercury acetate (APMA)-activated MMP-9, and the fluorescent substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (catalog no. 03-32-5033) were obtained from Calbiochem (San Diego, CA). Chymotrypsin−trypsin inhibitor (BBI), trypsin (TPCK-treated), heparin, aprotinin, β-casein, Azur A, and Tween 20 were obtained from Sigma Chemical Co. (St. Louis, MO). Gelatin (EIA grade) was from Bio-Rad (Richmond, CA). Biotinylated gelatin was prepared as described by Ratnikov et al. (*18*). Streptavidin-coated microplates were obtained from Becton Dickinson (Bedford, MA). MMP-1 ELISA kit (Biotrak) was from Pharmacia Biotech (Piscataway, NJ). Streptavidin horseradish peroxidase and TMB/E were from Chemicon (Temecula, CA). Gradient 4−12% Bis-Tris gels (NP 0321), 10% zymogram gelatin gels (EC 6175), Silver Xpress silver staining kit (LC 6100), MES running buffer (NP 0002), and lithium dodecyl sulfate (LDS) sample buffer (NP 0007) were obtained from Invitrogen (Carlsbad, CA). The electrophoresis unit was a Hoefer Mini VE unit purchased from Amersham Pharmacia Biotech. All other reagents were of analytical grade.

Electrophoresis of Rice and Soybean BBI. The electrophoretic separation of rBBI or sBBI was carried under the following conditions. BBI at a concentration of 1.0 mg/mL was dissolved in water. Fifteen microliters was mixed with 25 μL of LDS sample buffer and 50 μL of deionized distilled water and boiled for 3 min, and 25 μL was separated on a 4−12% Bis-Tris gradient gel at 399 mA for 35 min. The separated protein was stained and destained following the manufacturer's instructions.

Activation of Pro-MMP-1 and Pro-MMP-9 by Trypsin in the Absence or Presence of Rice BBI or Soybean BBI. The method of Duncan (*5*) was followed with some modifications. Pro-MMP-1 or pro-MMP-9 was incubated with TPCK-treated trypsin in a ratio of 1 to 10 (i.e., 10 μg/mL enzyme/100 μg/mL BBI) in 50 mM Tris-HCl buffer containing 0.5 M NaCl and 5 mM $CaCl_2$ at pH 7.5, in the absence or presence of various concentrations of BBI (0−0.4 mg/mL) for 40 min at 37 °C. Aprotinin (9 μg/mL for rBBI and 20 μg/mL for sBBI, respectively) was added to trypsin-treated pro-MMP-1 samples to inactivate trypsin. The activation reaction was further stopped by removing the samples from 37 °C and adding sample buffer followed by a 3 min boiling and a 10 s centrifugation on high speed using a microcentrifuge. Then 25 μL of sample containing 6 ng of enzyme was loaded on the gel for electrophoretic separation. The samples were separated by electrophoresis under nonreducing conditions using a 4−12% gradient gel containing Bis-Tris according to the manufacturer's instructions. At the end of the electrophoretic separation (∼35 min), the gel was silver stained using the Silver Express kit. The position of the bands on the gel was compared to the position of the band from pro-MMP-1 and active MMP-1 obtained from the supplier.

Heparin-Enhanced Gelatin Zymography for MMP-1. Gelatin zymography of pro-MMP-1, active MMP-1, and pro-MMP-1 incubated with trypsin and treated with aprotinin was carried according to the method of Yu and Woessner (*4*) using gels containing 0.1% gelatin. Briefly, 10 μL of 0.3 mg/mL heparin in sample buffer was added to the lanes 20−30 min after the electrophoresis had begun. Electrophoretic separation was carried at 120 V for 90 min. The gel was treated with 2.5% Triton X-100 and stained with Azur A stainer.

Determination of the Activity of Pro-MMP-9 and Active MMP-9 Using Biotinylated Gelatin. The method of Ratnikov et al. (*18*) was followed with minor modifications. Pro-MMP-9, APMA-activated MMP-9, trypsin-activated MMP-9 (containing aprotinin inhibitor), and a pro-MMP-9−trypsin−BBI mixture (all at enzyme concentrations between 0.15 and 2.5 ng/mL) in 50 mM Tris-HCl buffer containing 150 mM NaCl, 5 mM $CaCl_2$, and 0.005% Brij 35 at pH 7.5 was mixed with equal volumes of 150 ng/mL biotinylated gelatin. The mixture was incubated at 37 °C for 30 min, and the reaction was stopped by the addition of 40 μL of 250 mM EDTA. One hundred microliters of sample was transferred to the wells of streptavidin-coated microtiter plates previously blocked with 1% of β-casein in PBS for 30 min at 37 °C. The plate was incubated at 37 °C for 30 min and washed with PBS-containing 0.05% Tween 20. One hundred microliters of streptavidin−horseradish peroxidase at 1:10000 dilution in PBS was added to wells, and the plate was again incubated at 37 °C for 30 min. The plate was washed three times with PBS−Tween, and 100 μL of TMB/E, as substrate for HRP, was added. The reaction was stopped after 3 min with the addition of 50 μL of 1 N HCl per well, and the color was read at 450 nm in a Spectramax Plus UV−visible microtiter plate reader (Molecular Devices, Sunnyvale, CA).

Quenched Fluorescence Substrate Hydrolysis. The enzymatic activity of pro-MMP-1, APMA-activated MMP-1, trypsin-activated MMP-1 (containing aprotinin to inactivate trypsin), pro-MMP-1−trypsin−rBBI (with rBBi at concentrations of 0.08−0.352 mg/mL), pro-MMP-9, AMPA-activated MMP-9, trypsin-activated MMP-9 (containing aprotinin to inactivate trypsin), and pro-MMP-9-trypsin-BBI (with sBBI at concentrations of 0.0 2−mg/mL) was assayed using the fluorescence quenching substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (*19*). Fifteen microliters of 100 ng of the enzyme was added to 182 μL of assay buffer (0.1 M Tris-HCl containing 0.1 M NaCl, and 10 mM $CaCl_2$ at pH 7.5). The fluorescence substrate (3 μL of 5 μM)

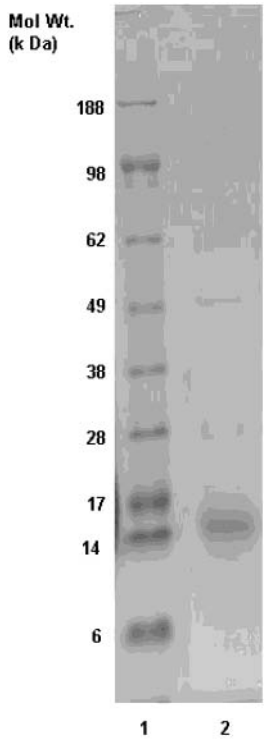

Figure 1. Electrophoretic profile of rice Bowman–Birk inhibitor. The molecular mass marker is on the left lane and the rice bran BBI is on lane 2. BBI electrophoretic separation was carried under nonreducing conditions using a 4–12% Bis-Tris gel as described under Materials and Methods.

was added. The sample was incubated for 3 h at 37 °C, and the activity of the metalloproteinase was determined using a Perkin-Elmer LS 50B luminescence spectrometer (λ_{exc} at 328 nm and λ_{em} at 393 nm). The control consisted of buffer with the substrate and BBI (rBBI at 0.352 mg/mL and sBBI at 0.4 mg/mL).

MMP-1 Biotrak Assay. MMP-1 activity using the Biotrak Assay (Amersham Pharmacia Biotech) was determined following the manufacturer's instructions.

RESULTS

Electrophoretic Profile of Rice BBI and Soybean BBI. The SDS-PAGE profile of rBBI separated under nonreducing conditions is provided in **Figure 1**. The molecular size of rBBI was between 15000 and 20000 Da. Tashiro et al. (*12*) reported a value of 14500 Da for BBI obtained from rice bran. Rice bran BBI has been reported to be a duplicated molecule of the soybean BBI but with a chymotrypsin inhibitory site weaker than the soybean BBI chymotrypsin inhibitory site (*12*). A representative SDS-PAGE profile of soybean BBI is shown in **Figure 2**. The molecular mass of sBBI appeared to be close to 10000 Da. The presence of the band around 20000 Da is associated with the ability of BBI to form dimers in aqueous solutions. The electrophoretic profile of BBI also showed that the protein was pure.

Activation of Pro-MMP-1 or Pro-MMP-9 by Trypsin in the Absence or Presence of rBBI. The molecular mass of pro-MMP-1 is 52 kDa (**Figure 3**, lane 1). **Figure 3**, lane 2, shows the band associated with APMA-activated pro-MMP-1 as obtained from the manufacturer. Incubation of pro-MMP-1 with trypsin for 40 min at 37 °C generated a fully activated MMP-1 at ~43 kDa (**Figure 3**, lane 3). Incubation of pro-MMP-1 with trypsin in the presence of 0.12−0.352 mg/mL of rBBI showed increased inhibition of pro-MMP-1 activation. (**Figure 3**, lanes 4 and 5). In the presence of rBBI, the band associated with the matrix metalloproteinase was located at ~52 kDa, which demonstrated that pro-MMP-1 activation did not occur.

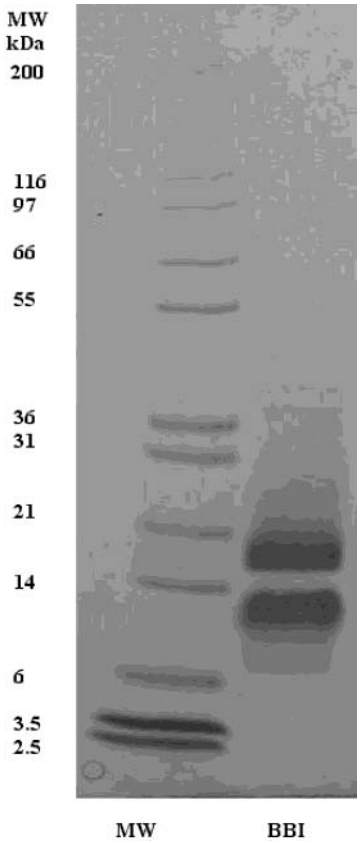

Figure 2. Electrophoretic profile of soybean Bowman–Birk inhibitor. BBI electrophoretic separation was carried under nonreducing conditions using a 4–12% Bis-Tris gel. Molecular mass marker is labeled MW, and the Bowman–Birk inhibitor is labeled BBI.

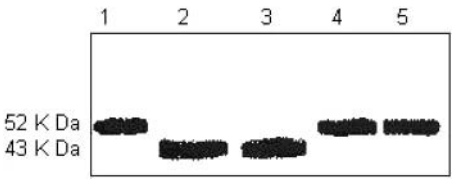

Figure 3. Activation of pro-MMP-1 by trypsin in the absence or presence of rBBI: activation of pro-MMP-1 (lane 1), APMA-activated MMP-1 (lane 2), trypsin-activated MMP-1 (lane 3), and trypsin-activated MMP-1 in the presence of 0.12 mg/mL of rBBI (lane 4) or 0.352 mg/mL of rBBI (lane 5). pro-MMP-1 (45 μg/mL) was activated by trypsin (final concentration = 4.5 μg/mL) in the absence or presence of rBBI for 40 min. The reaction was stopped by the addition of aprotinin (final concentration = 9 μg/mL). The activated enzyme (MMP-1) was analyzed by SDS-PAGE under nonreducing conditions using a 4–12% Bis-Tris gel; the gel was silver stained, and its position on the gel was compared to pro-MMP-1 and active MMP-1 obtained from the supplier.

Pro-MMP-9 is a 92 kDa protein molecule (**Figure 4**, lane 2). Activation of pro-MMP-9 can be initiated with APMA in the presence of Ca^{2+}, followed by autocatalysis to a stable form of 82 kDa or smaller. In this experiment, AMPA was not considered as an activator of pro-MMP-9 in the presence of BBI because APMA is not present in vivo. Activation of pro-MMP-9 can also be initiated with trypsin in the presence of Zn^{2+} and Ca^{2+}. Trypsin, which is an enzyme present in the human body, was used for pro-MMP-9 activation. The activation of pro-MMP-9 by trypsin at 37 °C is time-dependent and generates active MMP-9 polypeptides at 82, 67, and 63 kDa after 20−120 min (*5*). In this experiment, the incubation of pro-MMP-9 with trypsin in the presence of Ca^{2+} and Zn^{2+} resulted

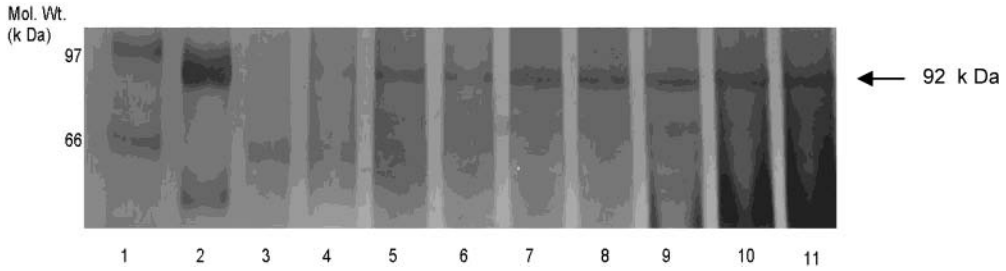

Figure 4. Activation of pro-MMP-9 by BBI. Pro-MMP-9 (100 μg/mL) was treated with TPCK-treated trypsin (10 μg/mL) in 50 mM Tris-HCl containing 0.5 M NaCl and 5 mM $CaCl_2$ at pH 7.5, in the absence or presence of various concentrations of BBI (20–400 μg/mL) for 40 min at 37 °C. The mixture was heat treated, and 6 ng of MMP per lane was separated by electrophoresis using a 4–12% Bis-Tris gel. Lane 1 represents the molecular weight marker, lane 2 represents the pro-MMP-9, and lane 3 represents trypsin-activated MMP-9. Lanes 4–11 represent the mixture of pro-MMP-9 and trypsin in the presence of increasing concentrations of BBI (0.02, 0.04, 0.08, 0.1, 0.16, 0.2, 0.3, and 0.4 mg/mL, respectively).

Figure 5. Heparin-enhanced gelatin zymography. Trypsin-activated MMP-1 (lane 1), pro-MMP-1 (lane 2), pro-MMP-1–trypsin–0.08 mg/mL rBBI (lane 3), pro-MMP-1–trypsin–0.12 mg/mL rBBI (lane 4), and pro-MMP-1–trypsin–0.352 mg/mL rBBI (lane 5) were separated by heparin-enhanced gelatin zymogrpahy as described under Materials and Methods. An active enzyme was identified by a clear zone on the gel after staining and destaining.

in the degradation of pro-MMP-9 with the generation of active MMP-9 of ~63 kDa after 40 min of incubation (**Figure 4**, lane 3). In the presence of sBBI at a concentration as low as 20 μg/mL, pro-MMP-9 was not completely activated by trypsin to active MMP-9 (**Figure 4**, lane 4). Increased concentrations of sBBI (20−400 μg/mL) were associated with inhibition of pro-MMP-9 activation (**Figure 4**, lanes 5−11). As sBBI concentration in the reaction mixture increased, the band at 92 kDa remained unchanged. In **Figure 4**, lanes 4−11, the presence of the pro-MMP-9 band at 92 kDa indicates that sBBI inhibited the activation of pro-MMP-9. There was no band at 63 kDa, indicative of the presence of active MMP-9, as the concentration of sBBI in the reaction mixture increased. sBBI inhibits trypsin and chymotrypsin in a 1:1 ratio. Higher concentrations of BBI preserved the pro-MMP-9 as a zymogen and demonstrated the effectiveness of sBBI as an inhibitor of trypsin-catalyzed activation of pro-MMP-9.

Interaction of BBI with trypsin and chymotrypsin has been at the basis of negative effects ascribed to protease inhibitors in legumes as antinutrients (*20*). However, recent biomedical investigations have shown that the Bowman−Birk inhibitor may have significant health-enhancing properties by virtue of its ability to inhibit serine proteases that have been associated with several malignancies (*13*, *21*−*23*).

Heparin-Enhanced Gelatin Zymography for MMP-1. In the presence of active matrix metalloproteinase, the gelatin substrate embedded in the acrylamide gel is hydrolyzed, and, as a result, a clear zone appears on the gel. Although zymography provides an inexpensive and quick routine screening tool for MMP activity, the technique makes a very sensitive assay for MMP-2 and MMP-9 but is less sensitive to MMP-1 (*4*). Yu and Woessner (*4*) demonstrated that the addition of heparin, after the electrophoretic separation of MMP-1 on zymogram gel has run for 30 min, enhanced the sensitivity of the assay to a level as low as 0.2 ng per lane. **Figure 5**, lane 1, shows the gelatinolytic activity of trypsin-activated MMP-1. The result of heparin-enhanced zymography also showed that active MMP-1

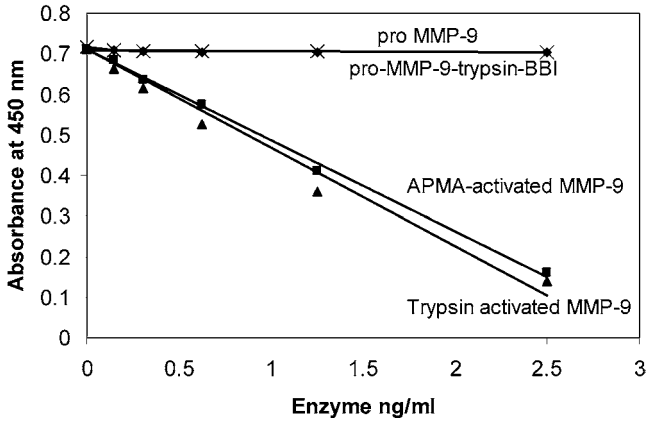

Figure 6. Gelatinolytic activity of pro-MMP-9 and active MMP-9 determined by biotinylated gelatin. Biotinylated gelatin at 150 ng/mL was incubated with different concentrations of pro-MMP-9, APMA-activated MMP-9, trypsin-activated MMP-9, and a mixture of pro-MMP-9–trypsin–BBI (0.15–2.5 ng/mL of enzyme) as described under Materials and Methods. After treatment, the OD at 450 nm was measured.

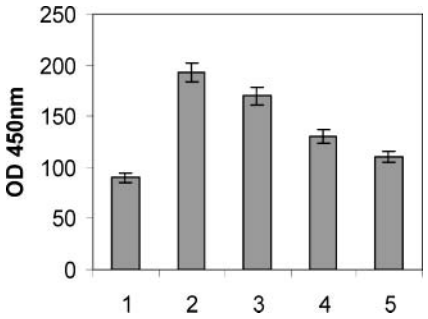

Figure 7. Quenched fluorescence substrate hydrolysis. The activity of pro-MMP-1 (lane 1), APMA-activated MMP-1 (lane 2), trypsin-activated MMP-1 in the presence of 0.08 mg/mL of rBBI (lane 3), a mixture of pro-MMP-1–trypsin–BBI (0.12 mg/mL BBI, lane 4), and a mixture of pro-MMP-1–trypsin–rBBI (0.352 mg/mL BBI, lane 5) (each metalloenzyme at 100 ng/mL) was determined by measuring the increase in fluorescence as a measure of substrate hydrolysis.

was able to digest gelatin embedded in the gel, and, as a result, a clear zone was observed. **Figure 5**, lane 2, shows that pro-MMP-1 did not digest the gel as demonstrated by the absence of a clear zone on the lane where the pro-enzyme was loaded. Data from zymography also showed that as the concentration of rBBI in the reaction medium increased from 0.08 mg/mL rBBI (lane 3) to 0.12 mg/mL rBBI (lane 4) to 0.352 mg/mL rBBI (lane 5), rBBI inhibited the activation of pro-MMP-1. As a result, gelatin hydrolysis did not occur. The effectiveness of

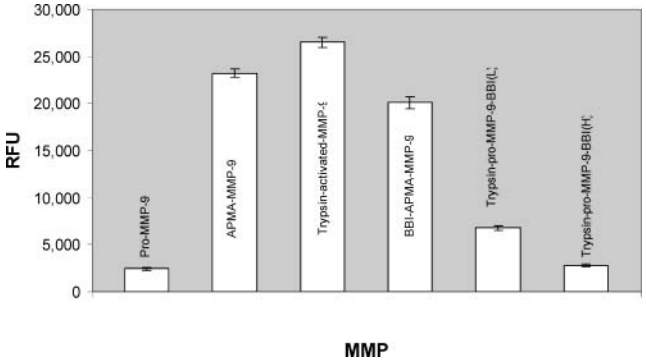

Figure 8. Quenched fluorescence substrate hydrolysis. All enzymes [pro-MMP-9, APMA-activated MMP-9, trypsin-activated MMP-9, a mixture of pro-MMP-9–trypsin–0.02 mg/mL of BBI (L), and a mixture of pro-MMP-9–trypsin–0.4 mg/mL of BBI (H)] at 100 ng/μL were incubated with the substrate in a total volume of 200 μL for 3 h at 37 °C. The increase in fluorescence reading of the reaction medium was read at λ_{328} nm excitation and λ_{393} emission and was associated with the rate of substrate hydrolysis.

rBBI as an inhibitor of pro-MMP-1 activation was again demonstrated.

Biotinylated Gelatin Assay for MMP-9. Gelatin is a substrate for MMPs such as MMP-9 and MMP-2. Gelatin was biotinylated to increase the sensitivity of the detection system (*18*). Following hydrolysis of gelatin by collagenase such as MMP-9, the gelatin fragment containing biotin molecules was added to a 96-well plate containing captured streptavidin. Fragments of gelatin−biotin were captured by the streptavidin on the plate, but the gelatin fragments devoid of biotin did not bind to the streptavidin coated on the plate. At the same time the degradation of gelatin was associated with reduced absorbance at 450 nm. **Figure 6** shows that APMA-activated MMP-9 was active and hydrolyzed gelatin; trypsin-activated MMP-9 was equally effective in hydrolyzing gelatin. The assay was linear in the range of 0.15−2.5 ng/mL. In the presence of pro-MMP-9, the gelatin embedded in the gel was not hydrolyzed, and the transfer of biotinylated gelatin to a streptavidin-coated plate was associated with a higher value of absorbance at 450 nm (**Figure 6**). In the presence of a pro-MMP-9−trypsin−sBBI mixture, gelatin hydrolysis did not occur. As a result, a high absorbance value was obtained regardless of the enzyme concentration used. Absorbance values obtained were similar to the value obtained with pro-MMP-9 and demonstrated that enzyme activation did not occur.

Quenched Fluorescence Substrate Hydrolysis. In the presence of an active matrix metalloproteinase, the substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 is hydrolyzed to Mca-Pro-Leu-Gly (fluorescent) and Leu-Dpa-Ala-Arg-NH_2 (*19*). Therefore, a high hydrolytic activity is associated with high fluorescence reading, and conversely low hydrolytic activity is associated with low fluorescence reading. The results of the interaction of pro-MMP-1, trypsin-activated MMP-1, or a mixture of pro-MMP-1, trypsin, and rBBI with the substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 are shown in **Figure 7**. Higher fluorescence value was obtained when trypsin-activated MMP-1 was incubated with the substrate compared to the fluorescence value of a mixture of pro-MMP-1 and the substrate (**Figure 7**, lane 1 vs lane 2). As the concentration of rBBI in the mixture pro-MMP-1−trypsin−rBBI increased, a low fluorescence value close to the value obtained with pro-MMP-1 was obtained. These data indicated that rBBI suppressed the activation of pro-MMP-1 by trypsin.

Data in **Figure 8** show that active MMP-9 (APMA activated or trypsin activated) hydrolyzed the substrate, but pro-MMP-9 did not. A mixture containing pro-MMP-9−trypsin and 0.02 mg/mL of sBBI showed slight hydrolysis of gelatin. When the highest concentration of sBBI (0.4 mg/mL) was used, the fluorescence value was as low as the fluorescence value associated with pro-MMP-9. These data also showed that BBI effectively prevented pro-MMP-9 activation.

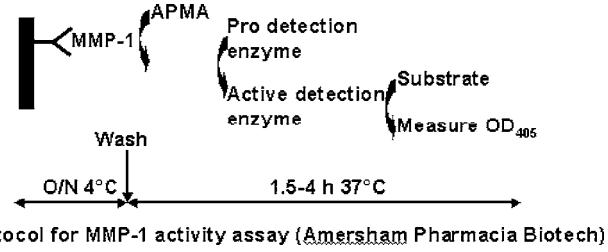

Figure 9. Biotrak assay principle.

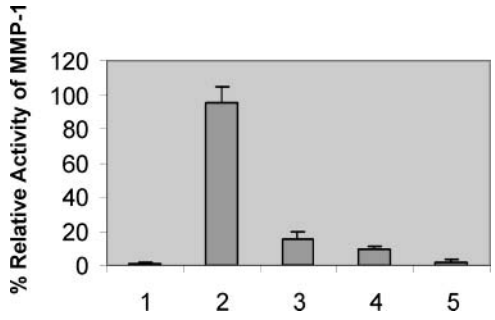

Figure 10. Biotrak assay. The activity of MMP-1 following incubation of the enzyme with different concentrations of rBBI is shown: lane 1, pro-MMP-1; lane 2, active MMP-1; lane 3, trypsin-activated MMP-1 in the presence of 0.08 mg/mL of rBBI; lane 4, trypsin-activated MMP-1 in the presence of 0.12 mg/mL of rBBI; lane 5, trypsin-activated MMP-1 in the presence of 0.352 mg/mL of rBBI.

MMP-1 Biotrak Assay. The principle of the Biotrak assay is depicted in **Figure 9**. Antibodies to active MMP-1 were coated onto a microplate. Any active MMP-1 would activate a prodetection enzyme, which in turn would cleave a chromogenic peptide substrate, followed by color measurement at 405 nm (Amersham Bioscience catalog, 2003). Results in **Figure 10** indicated that as the rBBI concentration in the activation medium increased, the concentration of activated MMP-1 decreased. At an rBBI concentration of 0.352 mg/mL, the activation of pro-MMP-1 did not occur.

DISCUSSION

The removal of the pro-peptide region of the pro-MMP-1 or pro-MMP-9 is the key event in the activation of the zymogen. The removal of the pro-peptide is associated with the release of a residue of amino acids and exposure of the enzyme active site and the zinc atom at the active site as catalyst (*24*). Pro-MMPs are released to the extracellular region, where they are activated to MMPs and become capable of catalyzing the hydrolysis of the extracellular matrix such as collagen. The active MMP-1 degrades fibrillar collagens including type I, II, III, VII, and X collagens. The active MMP-9 degrades native collagens, α2 chains of type I collagen, and collagen types III, IV, and V (*25*). In vivo, MMP-9 and MMP-1 have been associated with the degradation of the extracellular membrane and the progression of several neoplastic and non-neoplastic diseases such as cancer, diabetes and all its complications, arthritis, inflammatory lung diseases, and many others. In particular, MMP-2 and MMP-9 are associated with the invasive malignant potential of several tumors (*26*). MMP-9 has been identified as one of the early catalysts of the onset of angio-

genesis (*4*). Bacterial proteinases such as *Pseudomonas aeruginosa* elastase, *Vibrio cholerae* proteinase, and thermolysin strongly activated pro-MMP-8 and pro-MMP-9 from human neutrophils and pro-MMP-1 from human fibrosarcoma cells and have been associated with tissue injury during bacterial infections (*25*). Inhibition of pro-MMP activation is therefore critical to prevent the onset of basement membrane degradation. The activation of pro-MMP-9 by trypsin requires Ca^{2+}, the role of which has been associated with the stabilization of the metalloprotein conformation and protection of the MMP from extensive degradation by trypsin (*27*). Trypsin removes the NH_2-terminal residue and provides permanent exposure of the enzyme active site. Hashimoto et al. (*28*) demonstrated that human urinary trypsin inhibitor, a serine protease inhibitor, dose-dependently inhibited the production and activation of pro-MMP-3 (stromelysin), pro-MMP-9 (collagenase B) in culture medium containing interleukin-1 (IL-1), and plasminogen. Trypsin activation of pro-MMP-1 was selected because, in vivo, serine proteinases such as cathepsin G, trypsin, chymotrypsin, elastase, plasmin, and thrombin or oxidants such as NaOCl are more likely to catalyze the activation of pro-MMP-1.

Inhibition of pro-MMP-9 activation by BBI prevents potential degradation associated with the active form of the enzyme. The literature is well established that BBI is a bioactive compound with the ability to enhance health. However, BBI, like many other protease inhibitors of food origin, has long been considered to be an antinutrients. Consumption of protease inhibitors is still considered by some researchers as antinutritive. BBI is present in most soy products. Negative effects of BBI should have been observed in Asians whose daily diets contain soy products. Rather, these effects are not observed. The results of this study indicate that trypsin exhibits potent activation of pro-MMP-1 and pro-MMP-9 and that rBBI and sBBI display strong inhibitory activities against the activation process. The potential inhibitory activity of rBBI and sBBI against pro-MMP-1 and pro-MMP-9 activation will provide new insight into the potential in vivo health-enhancing abilities of BBI in degenerative disease onset and/or progression.

Angiogenesis is the result of imbalance between the stimulators (growth factors, hormones, proteins, enzymes, minerals, and oncogenes) and their inhibitors. In the event the inhibitors are depleted, the human body needs a supply of exogenous inhibitors to help re-establish homeostasis. Under these conditions, inhibitors such as rBBI, if effective in vivo, may find applications in human foods for disease prevention. Rice BBI was reported to be hydrolyzed by pepsin in vitro but was resistant to hydrolysis in the presence of a digestible protein such as BSA (*12*). BBI is resistant to acid and alkaline digestion in the gastrointestinal tract and reaches the colon in intact form (*29*). However, slow in vitro hydrolysis of BBI, after prolonged hours, has been reported (*30*). Experiments with laboratory animals have consistently shown that BBI is bioavailable (*31* and references therein). BBI enters the circulation via the intestinal epithelial cells or the paracellular mechanism (*32*, *33*). Billings et al. (*33*) provided evidence that 3 h following ingestion, up to 40% of ingested BBI was found in the bloodstream and ~17% of BBI ingested was found in the bladder (*33*). The excreted BBI maintained its protease inhibitory activity (*29*). One to two percent of ingested radiolabeled BBI was found to be present in all organs except the brain (*31*). In animal models in which 0.01% dietary BBI completely inhibited the induction of liver tumors, up to 5 μg of absorbed BBI was detected in the liver, the breast, and the prostate (*29*, *34*). This minute concentration of BBI was effective in preventing or suppressing in vitro malignant cells transformation (*34*) or liver angiosarcoma induced by dimethylhydrazine in animals (*35*). In studies involving humans, Kennedy (*31*) reported that BBI was detected in the urine within 1−9 h following oral administration, and the baseline was re-established within 24 h. The concentration of BBI metabolites recovered from the urine of human subjects fed 36 or 60 oz of soy milk (containing 105 or 175 mg of BBI) was estimated to be <0.02% of the BBI ingested (*36*). It was suggested that the ingested BBI may have undergone conformational change in the presence of natural reducing agents such as glutathione, a chemical change that made it unlikely to be detected by the antibodies generated against native BBI. Ingested BBI may be bioavailable (*36*). Bioavailable BBI, if still active, may therefore provide protection in organs other than those of the gastrointestinal tract. Work is in progress to establish the in vivo effectiveness of rBBI and sBBI against angiogenesis and provide additional evidence for BBI as a functional food ingredient.

ACKNOWLEDGMENT

We acknowledge Riceland, Stuttgart, AR, for providing the rice bran and rice flour samples.

LITERATURE CITED

(1) Folkman, J. Role of angiogenesis in tumor growth and metastasis. *Semin. Oncol.* **2002**, *29* (6 Suppl, 16), 15−18.

(2) Folkman, J. Tumor angiogenesis: therapeutic implications. *N. Engl. J. Med.* **1971**, *285*, 1182−1186.

(3) Chang, C.; Werb, Z. The many faces of metalloproteases: cell growth, invasion, angio-genesis and metastasis. *Trends Cell Biol.* **2001**, *11*, S37−S43.

(4) Yu, W. H.; Woesnner, J. F., Jr. Heparin-enhanced zymographic detection of matrilysin and collagenase. *Anal. Biochem.* **2001**, *293*, 38−42.

(5) Duncan, M. E.; Richardson, J. P.; Murray, G. I.; Melvin, W. T.; Fothergill, J. E. Human matrix metalloproteinase-9: activation by limited trypsin treatment and generation of monoclonal antibodies specific for the activated form. *Eur. J. Biochem* **1998**, *258*, 37−43.

(6) Theret, N.; Musso, O.; L'Helgoualc'h, A.; Clement, B. Activation of matrix metalloproteinase-2 from hepatic stellate cells requires interactions with hepatocytes. *Am. J. Pathol.* **1997**, *150*, 51−58.

(7) Otani, Y.; Kubota, T.; Sakura, Y.; Igarashi, N.; Ypkoyama, T.; Kimata, M.; Wada, N.; Kameyama, K.; Kumai, K.; Okada, Y.; Kitajima, M. Expression of matrix metalloproteinases in gastric carcinoma and possibility of clinical application of matrix metalloproteinase inhibitor in vivo. *Ann. N. Y. Acad. Sci.* **1999**, *878*, 541−543.

(8) Scappaticci, F. A. Mechanism and future directions for angiogenesis-based cancer therapies. *J. Clin. Oncol.* **2002**, *18*, 3906−3927.

(9) Baker, E. A.; Bergin, F. G.; Leaper, D. J. Matrix metalloproteinases, their tissue inhibitors and colorectal cancer staging. *Br. J. Surg.* **2000**, *87*, 1215−1221.

(10) Hrabec, E.; Strek, M.; Nowak, D.; Hrabec, Z. Elevated level of circulating matrix metalloproteinase-9 in patients with lung cancer. *Resp. Med.* **2001**, *95*, 1−4.

(11) Gingras, D.; Batist, G.; Beliveau, R. AE-941 (Neovastat): a novel multifunctional antiangiogenic compound. *Expert. Rev. Anticancer Ther.* **2001**, *1*, 341−347.

(12) Tashiro, M.; Hashino, K.; Shiozaki, M.; Ibuki, F.; Maki, Z. The complete amino acid sequence of rice bran trypsin inhibitor. *J. Biochem. (Tokyo)* **1987**, *102*, 297−306.

(13) Kennedy, A. R.; Billings, P. C.; Wan, X. S.; Newberne, P. M. Effects of Bowman−Birk inhibitor on rat colon carcinogenesis. *Nutr. Cancer* **2002**, *43*, 174−186.

(14) Lippman, S. M.; Matrisian, L. M. Protease inhibitors in oral carcinogenesis and chemoprevention. *Clin. Cancer Res.* **2000**, *6*, 4599−4603.

(15) Losso, J. N. Preventing degenerative diseases with anti-angiogenic functional foods. *Food Technol*. **2002**, *56*, 78−88.
(16) Tosetti, F.; Ferrari, N.; De Flora, S.; Albini, A. Angioprevention′: angiogenesis is a common and key target for cancer chemopreventive agents. *FASEB J.* **2002**, *16*, 2−14.
(17) Losso, J. N. Targeting excessive angiogenesis with anti-angiogenic functional foods and nutraceuticals. *Trends Food Sci. Technol.* **2003**, *14*, 455−468.
(18) Ratnikov, B.; Deryugina, E.; Leng, J.; Marchenko, G.; Dembrow, D.; Strongin, A. Determination of matrix metalloproteinase activity using biotinylated gelatin. *Anal. Biochem*. **2000**, *286*, 149−155.
(19) Knight, C. G.; Willenbrock, F.; Murphy, G. A novel coumarin-labelled peptide for sensitive continuous assays of the matrix metalloproteinases. *FEBS Lett.* **1992**, *296*, 263−266.
(20) Liener, I. E.; Goodale, R. L.; Deshmukh, A.; Satterberg, T. L.; Ward, G.; DiPietro, C. M.; Bankey, P. E.; Borner, J. W. Effect of a trypsin inhibitor from soybeans (Bowman−Birk) on the secretory activity of the human pancreas. *Gastroenterology* **1988**, *94*, 419−427.
(21) Malykh, E. V.; Larionova, N. I. Study of antiproteinase activity of acylated derivatives of Bowman−Birk soybean proteinase inhibitor. *Biochemistry (Moscow)* **2002**, *67*, 1383−1387.
(22) Rao, C. N.; Mahanam, S.; Puppala, A.; Rao, J. S. Regulation of pro-MMP-1 and pro-MMP-3 activation by tissue factor pathway inhibitor-2/matrix-associated serine protease inhibitor. *Biochem. Biophys. Res. Commun*. **1999**, *255*, 94−98.
(23) Sharma, S.; Ghoddoussi, M.; Gao, P.; Kelloff, G. J.; Steele, V. E.; Kopelovich, L. A quantitative angiogenesis model for efficacy testing of chemopreventive agents. *Anticancer Res.* **2001**, *21*, 3829−3837.
(24) Okada, Y.; Gonoji, Y.; Naka, K.; Tomita, K.; Nakanishi, I.; Iwata, K.; Yamashita, K.; Hayakawa, T. Matrix metalloprotease 9 (92-kDa gelatinase/Type IV collagenase) from HT 1080 human fibrosarcoma cells. *J. Biol. Chem*. **1992**, *267*, 21712−21719.
(25) Okamoto, T.; Akaike, T.; Suga, M.; Tanase, S.; Horie, H.; Miyajima, S.; Ando, M.; Ichinose, Y.; Maeda, H. Activation of human matrix metalloproteinases by various bacterial proteinases. *J. Biol. Chem.* **1997**, *272*, 6059−6066.
(26) Heather, E. I.; Grochow, L. B. Clinical potential of matrix metalloprotease inhibitors in cancer therapy. *Drugs* **2000**, *59*, 1043−1055.
(27) Bu, C. H.; Pourmotabbed, T. Mechanism of activation of human neutrophil gelatinase B. Discriminating between the role of Ca^{2+} in activation and catalysis. *J. Biol. Chem.* **1995**, *270*, 18563−18569.
(28) Hashimoto, K.; Nagao, Y.; Kato, K.; Mori, Y.; Ito, A. Human urinary trypsin inhibitor inhibits the activation of pro-matrix metalloproteinases and proteoglycans release in rabbit articular cartilage. *Life Sci.* **1998**, *63*, 205−213.
(29) Yavelow, J.; Finlay, T. H.; Kennedy, A. R.; Troll, W. A. Bowman−Birk soybean protease inhibitor as an anticarcinogen. *Cancer Res.* **1983**, *43*, 2454−2459.
(30) Jensen, B.; Unger, K. K.; Uebe, J.; Gey, M.; Kim, Y.-M.; Flecker, P. Proteolytic cleavage of soybean Bowman−Birk inhibitor monitored by means of high-performance capillary electrophoresis. Implications for the mechanism of proteinase inhibitors. *J. Biochem. Biophys. Methods* **1996**, *33*, 171−185.
(31) Kennedy, A. R. Chemopreventive agents: protease inhibitors. *Pharmacol. Ther.* **1998**, *78*, 167−209.
(32) Billings, P. C.; Habres, J. M.; Liao, D. C.; Tuttle, S. W. A protease activity in human fibroblasts which is inhibited by the anticarcinogenic Bowman−Birk protease inhibitor. *Cancer Res.* **1991**, *51*, 5539−5543.
(33) Billings, P. C.; St. Clair, W. H.; Maki, P. A.; Kennedy, A. R. Distribution of the Bowman−Birk protease inhibitor in mice following oral administration. *Cancer Lett.* **1992**, *62*, 191−197.
(34) Yavelow, J.; Collins, M.; Birk, Y.; Troll, W.; Kennedy, A. R. Nanomolar concentrations of Bowman−Birk soybean protease inhibitor suppress X-ray induced transformation *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 5395−5399.
(35) St. Clair, W. H.; Billings, P. C.; Carew, J. A.; Keller-McGandy, C.; Newberne, P.; Kennedy, A. R. Suppression of dimethylhydrazine-induced carcinogenesis in mice by dietary addition of the Bowman−Birk protease inhibitor. *Cancer Res*. **1990**, *50*, 580−586.
(36) Wan, X. S.; Lu, L. J. W.; Anderson, K. E.; Ware, J. H.; Kennedy, A. R. Urinary excretion of Bowman−Birk inhibitor in human after soy consumption as determined by a monoclonal antibody-based immunoassay. *Cancer Epidemiol. Biomarkers Prev.* **2000**, *9*, 741−747.

Received for review May 30, 2003. Revised manuscript received March 23, 2004. Accepted May 20, 2004. This research was supported by a grant from the U.S. Rice Foundation and an operating grant from the Louisiana State University Agriculture Experimental Station.

JF034576U