Urinary Excretion of Bowman-Birk Inhibitor in Humans after Soy Consumption as Determined by a Monoclonal Antibody-based Immunoassay[1]

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Abstract

The Bowman-Birk inhibitor (BBI) found in soybeans is a serine protease inhibitor with anticarcinogenic activity. In the present study, an ELISA for BBI was developed with the use of a monoclonal antibody against a reduced form of BBI. This newly developed ELISA method was used to measure the urinary levels of BBI metabolites in nine human subjects after consumption of 36-oz or 60-oz soymilk (containing 105 or 175 mg of BBI) at two time points 36 h apart. The results demonstrate that urinary BBI excretion rates peaked within 6 h and decreased to baseline levels within 12–24 h after soymilk ingestion. The changes in BBI:creatinine ratios in urine closely paralleled the changes in urinary BBI excretion rates after soymilk consumption. These data suggest that BBI ingested p.o. is absorbed and could be bioavailable for cancer chemoprevention in other organs in addition to those in the gastrointestinal tract.

Introduction

The BBI,[3] which is abundant in soybeans, is a low molecular weight (M_r 8000) double-headed serine protease inhibitor that inhibits both trypsin- and chymotrypsin-like proteases (1), *e.g.*, trypsin (1), chymotrypsin (1), cathepsin G (2, 3), elastase (2, 3), and chymase (4). BBI is also a potent anticarcinogenic agent that inhibits chemical carcinogen- and radiation-induced malignant transformation *in vitro* and suppresses carcinogenesis in several organ systems and animal species (5–7). These observed effects of BBI suggest that BBI can be a useful cancer-chemopreventive agent in humans. This is supported by the epidemiological observations demonstrating a strong correlation between the high levels of soybean consumption and low incidence of colon, breast, and prostate cancers in human populations (8). BBI, in the form of BBIC, has achieved Investigational New Drug status with the Food and Drug Administration, and human trials to evaluate BBIC as a human cancer chemopreventive agent are currently in progress. In a recently completed Phase IIa oral cancer chemoprevention trial in patients with premalignant lesions known as oral leukoplakia, treatment with BBIC at daily doses of 200-1066 chymotrypsin inhibition units for 1 month led to a dose-dependent decrease in oral leukoplakia lesion size (9).

In epidemiological studies and clinical trials to evaluate BBI as a human cancer preventive agent, a method for quantifying BBI and its metabolites in human body fluids is essential for monitoring the BBI exposure. Although BBI in soybeans can be quantified by an immunoassay with the use of MAbs developed against native BBI (10, 11), these MAbs do not detect BBI found in human urine samples after BBIC ingestion. A possible explanation is that BBI is excreted as metabolites with different antigenic structure that are not recognized by the MAbs raised against native BBI.

BBI is a small single chain protein that is rich in disulfide content (1). Each BBI molecule contains 14 cysteine residues that form 7 intramolecular disulfide bonds to maintain the native structure (1, 12). In the presence of glutathione and other small molecular weight thiols, which are abundant in tissues and body fluids, the disulfide bonds that maintain the native structure of the BBI molecule may be broken, resulting in changes of the antigenic structure of BBI molecules after ingestion. If one or more disulfide bonds in BBI are indeed reduced during metabolism, the BBI metabolites might be detectable by antibodies that react with reductively modified BBI molecules. We have previously produced MAbs against reductively modified BBI (13). In the present investigation, we developed a MAb-based immunoassay and measured the levels of BBI in urine samples collected from human subjects after ingestion of BBI-containing soymilk.

Materials and Methods

Antibodies, Chemical Reagents, and Microwell Plates. The MAb 5G2 was generated and characterized as previously described (13). It was prepared from hybridoma culture supernatants by ammonium sulfate precipitation, reconstituted in PBS (pH 7.0) to one-tenth of the original volume of the hybridoma culture supernatants and kept at −20°C. BBI was purchased from Sigma Chemical Co. (St. Louis, MO). r-BBI antigen were prepared by a radiochemical procedure consisting of 720 Gy of

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[3] The abbreviations used are: BBI, Bowman-Birk inhibitor; BBIC, Bowman-Birk inhibitor concentrate; MAb, monoclonal antibody; r-BBI, reduced BBI; ELISA-P, ELISA with polystyrene plates; ELISA-M, membrane-coated plates; ELISA-C, competition ELISA; PB, phosphate buffer; RAB, relative antibody binding; ABI, antibody binding inhibition.

γ-irradiation of native BBI in 100 mM deoxygenated formate buffer followed by an additional dose of 2000 Gy of γ-irradiation in 10 mM etanidazole as described previously (13–15). β-Galactosidase-conjugated goat antimouse immunoglobulins (immunoglobulin) were purchased from Southern Biotechnology Associates (Birmingham, AL). Other chemicals were also purchased from Sigma unless specified otherwise. Polystyrene microwell plates and Immobilon-P membrane-coated MultiScreen IP plates were purchased from Becton Dickinson, (Lincoln Park, NJ) and Millipore (Bedford, MA), respectively.

Comparison of Three Methods of ELISA for Quantification of BBI Metabolites in Urine. Three ELISA methods were compared for their capabilities to measure r-BBI in human urine samples. In the first two methods, the r-BBI antigen was diluted in urine, immobilized onto polystyrene microwell plates or Immobilon-P membrane-coated MultiScreen IP plates and detected by the 5G2 antibody. These methods were referred to as ELISA-P and ELISA-M, respectively. In the third method, the r-BBI antigen diluted in urine was mixed with the 5G2 antibody in solution to compete with immobilized r-BBI antigen for the binding of the 5G2 antibody. This method is referred to as ELISA-C.

To prepare antigen-coated plates used for ELISA-P and ELISA-M, native BBI was serially diluted to specified concentrations in a pooled control urine sample, heated at 95°C for 10 min in the presence of 1% β-mercaptoethanol to reduce the disulfides in the BBI molecules, applied onto polystyrene microwell plates or MultiScreen IP plates at 100 μl/well, and incubated at room temperature for 1 h to allow the antigen to attach. The plates were then fixed with 0.1% glutaraldehyde for 10 min, neutralized with 0.1 M glycine for 10 min, and incubated with 1% BSA in 20 mM PB, pH 7.0, for 1 h to block nonspecific binding sites on the plates. To prepare r-BBI-coated plates used for ELISA-C experiments, r-BBI antigen solution (1.0 μg/ml) was applied onto 96-well polystyrene plates at 100 μl/well and incubated at room temperature for 1 h to allow the antigen to attach. The plates were also fixed and blocked as described above. The prepared BBI antigen plates were used immediately or stored at −20°C before use.

For ELISA-P and ELISA-M experiments, 5G2 antibody was diluted 1:500 in BSA-PB, applied to the BBI-coated microwell plates in triplicate, and incubated at room temperature for 1 h. For ELISA-C experiments, 5G2 antibody was diluted 1:1000 or 1:2000 in BSA-PB, premixed with the control or serially diluted r-BBI-containing urine samples at a ratio of 10:1 (v/v) in microcentrifuge tubes, and incubated at room temperature for 30 min. The antibody-urine mixtures were then applied in triplicate to polystyrene plates precoated with r-BBI antigen and incubated at room temperature for 1 h. The antibody solution was similarly mixed with PB without BBI or urine and included in the experiment as a blank control. In all three ELISA methods, the plates were washed three times with PB after the incubation with 5G2 antibody or antibody-urine mixtures and further incubated with β-galactosidase-conjugated goat antimouse immunoglobulin (100 μl/well) at room temperature for 1 h. The plates were again washed three times with PB and incubated with *o*-nitrophenyl-β-D-galactopyranoside substrate (15 mg/ml in 40 mM boric acid-borax buffer, pH 8.5) at 37°C for 1 h. The absorbance was measured at 415 nm with a microplate reader (model 7250; Cambridge Technology). The results of the ELISA-P and ELISA-M experiments were expressed as RAB, which was calculated by dividing the absorbance reading of each sample by the maximum absorbance reading observed in the same experiments. The results of ELISA-C were expressed as ABI, which was calculated by the formula

$$\mathrm{ABI} = \frac{\mathrm{RAB}_{\mathrm{control}} - \mathrm{RAB}_{\mathrm{test}}}{\mathrm{RAB}_{\mathrm{control}}}$$

The RAB for ELISA-P and ELISA-M and the ABI for ELISA-C increase with the increase of r-BBI antigen concentration and the maximum achievable RAB and ABI were 1.0 by definition.

Soymilk Ingestion and Urine Sample Collection. Six healthy men and three healthy women (21–29 years old, 1.5–1.9 m tall, 43.7–83.6 kg body weight, and 18.9–24.3 kg/m^2 body mass index) who resided in Galveston, TX, participated in this study. The subjects were nonvegetarians and included one Indian (from India), one Chinese, one Hispanic, and six Caucasians. These subjects were admitted to the General Clinical Research Center at the University of Texas Medical Branch for a total of 5 days. The procedures were approved by the Institutional Review Board of the University of Texas Medical Branch. Written informed consent was obtained from each subject. Medical histories, physical examinations, blood cell counts, blood chemistry profiles, and serum ferritin concentrations were normal for all subjects. Each subject consumed a basal diet of the individual's choice for all 5 days during the study. The basal diets had no identifiable soy products.

After being placed on basal diets for 24 h and an overnight fasting, all subjects ingested the first dose of three or five 12-oz portions of soymilk (Banyan Food Co., Houston, TX) at 9 a.m. without other foods. Thirty-six hours later and after 9 h of fasting, the subjects ingested the second dose of three or five 12-oz portions of soymilk at 9 p.m. without other foods. Each 12 oz of soymilk was ingested within 10 min. Urine was collected for ~36 h after each dose of soymilk and pooled in fractions of 2 or 3 h. Urine samples were also obtained on admission and during the 12-h period before ingesting the first dose of soymilk. The urine samples were refrigerated during collection and then frozen at −80°C. Samples packed in dry ice were sent overnight from Galveston, TX, to Philadelphia, PA, and stored at −80°C before analyses.

The BBI content in soymilk was analyzed by a double sandwich ELISA method by Dr. David Brandon (United States Department of Agriculture Western Regional Office, Albany, CA). A 12-oz portion of soymilk was estimated to contain 35 mg of BBI.

Quantification of BBI Levels in Urine Samples. The BBI concentrations in urine samples of study subjects were determined by the ELISA-C method as described above except that 5G2 antibody solution was mixed with an equal volume of urine sample to reach a final antibody dilution of 1:1000 in the reaction mixture. The r-BBI antigen was diluted in a control urine sample to various concentrations and included in each run of the ELISA to generate a standard curve. The urine creatinine concentrations were determined by the fuller's earth method (16, 17), and the results were used to calculate the BBI:creatinine ratio in the urine samples.

Data and Statistical Analysis. The relationships between the BBI concentration and the RAB or ABI were analyzed by semilogarithmic regression analyses with the logs of BBI concentration as the independent variable and the RAB or ABI as the dependent variables. The standard curves used for estimating urine creatinine concentration were established by linear regression analysis with the creatinine concentration as the independent variable and absorbance at 509 nm as the depend-

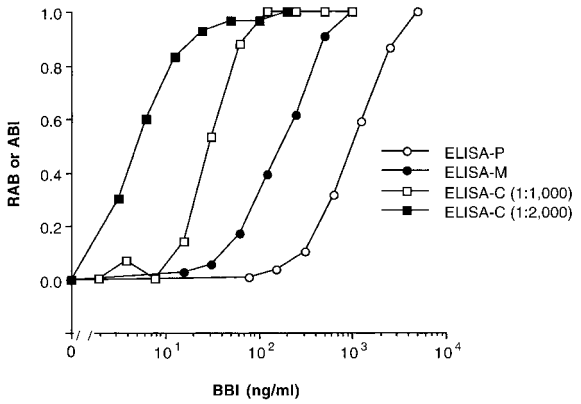

Fig. 1. Comparison of three ELISA methods for measuring BBI concentrations. In the antibody capture assays with polystyrene microwell plates (ELISA-P) or Immobilon-P membrane-coated microwell plates (ELISA-M), purified BBI antigen was diluted in a control urine sample to specified concentrations, reduced by heating at 95°C for 10 min with 1% β-mercaptoethanol, fixed on the plates, and detected with 5G2 antibody. In the antibody-binding ELISA-C, radiochemically reduced BBI was mixed with 1:1000 or 1:2000 diluted 5G2 antibody and the antibody-antigen mixtures were applied to polystyrene microwell plates coated with radiochemically reduced BBI antigen to measure the inhibition by free r-BBI antigen on the binding of 5G2 antibody to the bound r-BBI antigen. The experiments were conducted in triplicate. The results are expressed as RAB for ELISA-P and ELISA-M experiments, and ABI for ELISA-C experiments.

ent variable. The BBI concentrations (ng/ml) in urine of unknown samples were determined using the standard curves generated in each assay. The BBI excretion rate (μg/h) was calculated by multiplying BBI concentration (ng/ml) by urine volume (ml) and divided by the hours of urine collection. The BBI:creatinine ratio (ng/mg) was calculated by dividing the urine BBI concentration (ng/ml) by the urine creatinine concentration (mg/ml). The relationships between urine BBI concentrations, BBI excretion rates, and BBI to creatinine ratios were determined by correlation analyses.

Urinary BBI concentrations, BBI excretion rates, and BBI:creatinine ratios as a function of time before and after soymilk ingestion were graphed for all subjects. The mean values of urine BBI concentration, BBI excretion rate, and BBI:creatinine ratio for the nine human subjects for each of the 6-h intervals were analyzed by the Friedman test followed by Dunn's multiple comparison test. This nonparametric ANOVA method was selected to avoid any possible violations of normality assumptions. The statistical analyses were performed using Prism version 2.0 statistical software (GraphPad Software, San Diego, CA).

Results

We have previously demonstrated that MAbs 3E3, 4H8, and 5G2, which were produced against reduced form(s) of BBI molecules, can detect BBI in human urine samples after BBIC administration (13). The present study was aimed at developing an ELISA method for studying the pharmacokinetics of BBI in human subjects. Among the three MAbs, 5G2 consistently displayed the highest reactivity with r-BBI antigen under various experimental conditions in the preliminary experiments (data not shown) and therefore was selected for the development of the ELISA for measuring BBI metabolites.

The feasibility of using the 5G2 antibody for measuring r-BBI in urine samples was first evaluated in ELISA experiments with the use of polystyrene microwell plates (ELISA-P) to immobilize r-BBI antigen diluted in urine. The results showed that this method could detect r-BBI in urine at concentrations ranging from 200 to 5000 ng/ml (Fig. 1). When diluted in PB instead of urine, r-BBI can be detected with 5G2 by the same method at concentrations as low as 10 ng/ml (data not shown). The difference in the detection sensitivity might have been caused by other proteins in urine that compete with the r-BBI antigen for the antibody-binding sites on polystyrene plates. To increase the amount of r-BBI antigen bound to the wells, the r-BBI antigen diluted in urine was adsorbed onto microwell plates coated with Immobilon-P membrane (ELISA-M), which has a protein-binding capacity several orders of magnitude higher than that of polystyrene plastics. With the use of the membrane-coated plates as the antigen immobilizing matrix, r-BBI diluted in urine could be detected at concentrations ranging from ~30–1000 ng/ml (Fig. 1). This method can measure BBI metabolites in human urine. However, high background readings and interference by other substances in urine were occasionally observed.

To develop a more sensitive and reliable method for measuring BBI metabolites in urine, we experimented with the ELISA-C method. The results show that the ELISA-C method is more sensitive than the ELISA-P and ELISA-M methods for measuring the concentrations of r-BBI in urine. When 5G2 antibody was diluted at 1:1000 for the experiments, r-BBI could be measured by the ELISA-C method at concentrations of 15–125 ng/ml (Fig. 1). By increasing the dilution factor of 5G2 antibody from 1:1000 to 1:2000, r-BBI could be measured at concentrations ranging from 1 to 25 ng/ml (Fig. 1).

The ELISA-C method was used to measure the concentrations of BBI metabolites in urine samples from the nine human subjects after consumption of soymilk containing 105 or 175 mg of BBI. A total of 212 urine samples collected from these subjects were measured in 6 experiments. Each urine sample was assayed in triplicate, and the coefficient of variation (SD/mean) was calculated to assess the precision of the ELISA-C method. The average coefficient of variation for the 212 urine samples was 0.124. To determine the recovery rate of r-BBI added to urine matrix by the ELISA-C method, r-BBI was added into a pooled control urine sample at five concentration levels which ranged from 7.8 to 125 ng/ml and was measured in each of the six experiments. The average recovery rate of r-BBI was 101.40 ± 14.43% (Table 1), suggesting that the ELISA-C method picked up essentially all r-BBI added into the urine matrix. To estimate the assay variation between the experiments conducted on different days (interrun variation), a set of five urine samples was measured six times in the six experiments. The coefficient of the interrun variation (SD/

Table 1 Percent recovery of r-BBI added to urine matrix by the ELISA-C method[a]

r-BBI added (ng/ml)	r-BBI recovered (ng/ml)		% recovery	
	Mean	SD	Mean	SD
7.81	8.13	0.91	104.14	11.60
15.63	17.28	4.27	110.59	27.29
31.25	28.24	5.97	90.38	19.10
62.50	67.46	4.17	107.93	6.68
125.00	117.44	9.32	93.95	7.46
Av.			101.40	14.43

[a] r-BBI was added to a pooled control urine sample at five concentrations and measured six times by the ELISA-C method in six independent experiments.

Table 2 Interrun variation and normalization

Sample	Urine BBI concentration (ng/ml) determined in six experiments[a]								
	1	2	3	4	5	6	Mean	SD	CV
Before normalization									
1	23.84	24.07	17.13	23.82	21.29	30.00	23.36	4.20	0.180
2	18.6	14.39	12.35	18.49	19.79	25.36	18.16	4.53	0.250
3	28.43	22.60	19.04	31.84	39.65	30.55	28.69	7.26	0.253
4	12.66	11.26	9.55	14.00	19.94	24.86	14.55	5.40	0.372
5	19.43	14.41	14.42	17.53	18.57	27.24	18.57	4.76	0.256
Av.	20.59	17.35	14.46	21.13	22.85	27.60	20.66	5.23	0.253
Normalization ratio[b]									
	1.003	1.191	1.429	0.978	0.904	0.749	1.000		
After normalization[c]									
1	23.96	28.67	24.48	23.29	19.26	22.46	23.68	3.06	0.129
2	18.67	17.14	17.65	18.08	17.90	18.99	18.07	0.67	0.037
3	28.53	26.93	27.22	31.13	35.86	22.87	28.76	4.39	0.153
4	12.71	13.41	13.66	13.68	13.51	18.61	14.26	2.16	0.151
5	19.50	17.17	20.31	17.14	16.80	20.39	18.55	1.70	0.091
Average	20.66	20.66	20.66	20.66	20.66	20.66	20.66	2.40	0.112

[a] The BBI concentrations were measured six times in a set of five urine samples by the ELISA-C method. The mean, SD, and coefficient of variation (CV) represent the data from six independent experiments ($n = 6$).
[b] The normalization ratios were calculated by dividing the average urine BBI concentration (20.66 ng/ml) for the five urine samples in six experiments by the average urine BBI concentration for the same urine samples measured in each experiment.
[c] The BBI concentrations of the urine samples measured in each experiment were normalized with the respective normalization ratio.

mean) was estimated to be 0.253 (Table 2). To control and compensate for this interrun variation, the average urine BBI concentration for the five urine samples in the six experiments was divided by the average urine BBI concentration for the same five urine samples in each experiment to calculate a normalization ratio for each experiment. The urine BBI concentrations determined for these five samples in each of six experiments were normalized using the normalization ratios calculated for respective experiments. This normalization step decreased the coefficient of interrun variation from 0.253 to 0.112 (Table 2). The urine BBI concentrations for other samples measured in each experiment were also normalized with the respective normalization ratio so that the urine BBI concentration values determined in different experiments were comparable on the same scale. The results demonstrated that although the urinary BBI concentrations varied considerably among the subjects, the group mean stayed within the 95% confidence intervals of the baseline and did not differ significantly from the baseline levels ($P > 0.05$) in any of the 6-h intervals after the soymilk ingestion (Fig. 2). When the results are expressed as BBI excretion rates, the group mean exhibited a time-dependent increase shortly after the soymilk ingestion (Fig. 3*A*). The BBI excretion rates peaked at 5.8 ± 3.2 and 4.4 ± 5.6 h, respectively, after the first and the second soymilk ingestion. Peaks of urine BBI excretion were detected in all nine study subjects after soymilk ingestion, suggesting that BBI was absorbed by all subjects. The mean hourly rates of BBI excretion during the first 6-h period immediately after the first and second soymilk ingestion were 5.09 and 4.75 μg/hr, respectively, which were 339% ($P < 0.01$) and 309% ($P < 0.01$) higher than the baseline levels (1.16 μg/h). The area under the time course curve and above the baseline for the BBI excretion rate was calculated for each 6-h period to estimate the amount of BBI excreted in the corresponding time period. The amounts of BBI excreted in the first 6-h periods after the first and second soymilk ingestion were 23.6 and 21.6 μg, respectively (Fig. 3*B*).

The discrepancy between the changes in the urine BBI concentration and the BBI excretion rate could be caused by the fluctuation in the urine water content, which is expected to inversely affect the urine BBI concentration. To test this hypothesis, the urine BBI concentrations were normalized to the urine creatinine concentrations and expressed as the BBI:creatinine ratio. The group mean of the BBI:creatinine ratios showed a time-dependent change (Fig. 4), which is similar to that of the BBI excretion rates after soymilk ingestion. The BBI:creatinine ratio during the first 6-h periods immediately after the first and second soymilk ingestion were 156.33 and 147.33 ng/mg, respectively, which represented 225% ($P <$

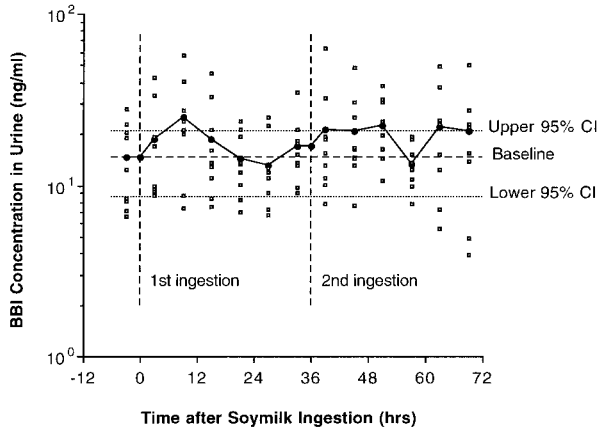

Fig. 2. BBI concentrations in urine after soymilk ingestion. Six men and three women were admitted to a metabolic unit and placed on an identical low soy diet of his or her choice for 5 days. After 1 day on the low soy diet and an overnight fast, each subject ingested three or five 12-oz soymilk servings within 30 or 50 min, respectively. The soymilk ingestion was repeated again 36 h after the first soymilk ingestion. Urinary BBI concentrations were determined by the ELISA-C method. □, mean values of the urine BBI concentrations for each subject; ●, group means.

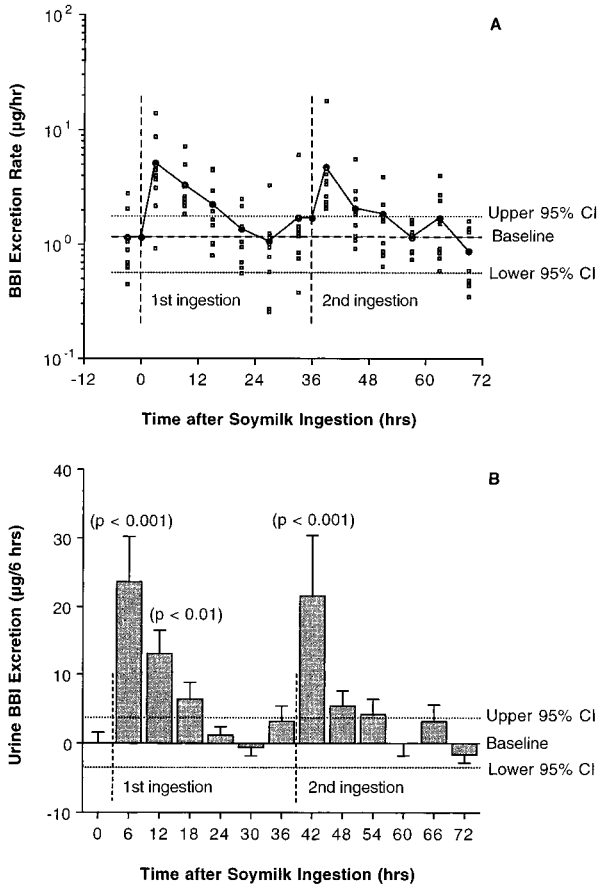

Fig. 3. Time courses of BBI excretion rates in urine after soymilk ingestion. The urine BBI concentration was determined as described in the Fig. 2 legend. The urine BBI concentration was multiplied by the urine volume and divided by the urine collection time to calculate the BBI excretion rate (*A*). □, BBI excretion rates for individual subjects; ●, group means of the BBI excretion rate. The areas between the BBI excretion rate time course curves and the baseline for each 6-h period after soymilk ingestion were calculated to estimate the amount of BBI excreted during the corresponding time period (*B*). The amounts of BBI excreted by the nine subjects in each 6-h period after the soymilk ingestion were expressed as mean + SE.

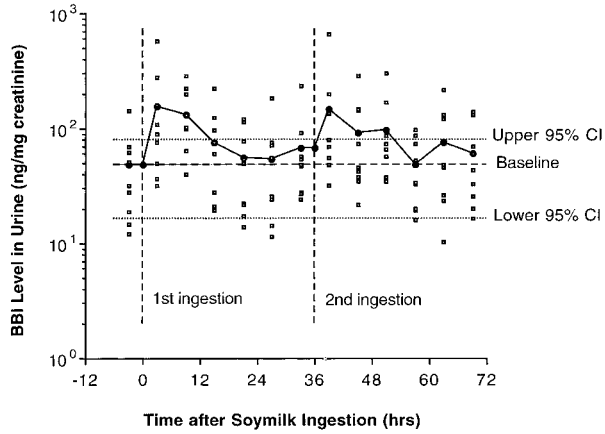

Fig. 4. Time courses of the BBI:creatinine ratio in urine after soymilk ingestion. The urine BBI concentrations were determined as described in the Fig. 2 legend. The urine creatinine concentrations were determined by the fuller's earth method (16, 17). □, mean values of the BBI:creatinine ratio for each subject in each of the 6-h periods before and after soymilk ingestion; ●, group mean.

0.05) and 207% ($P < 0.05$) increases over the baseline level (48.05 ng/mg). The BBI excretion rates correlated well with the BBI to creatinine ratios (Fig. 5*A*, Pearson $r = 0.904$, $P < 0.0001$). Urine BBI concentrations also correlated with the BBI excretion rates (Fig. 5*B*, Pearson $r = 0.446$, $P < 0.0001$) and the BBI:creatinine ratios (Fig. 5*C*, Pearson $r = 0.510$, $P < 0.0001$); however, these correlations were not as good as the correlation between excretion rates and excretion ratios.

Discussion

In the present study, a MAb-based ELISA-C method was developed and used to measure BBI metabolites in urine samples collected from human subjects who had consumed BBI-containing soymilk. The results demonstrate that the urinary BBI excretion rate peaked within hours after the soymilk ingestion, suggesting that p.o. ingested BBI is rapidly absorbed and excreted into urine. This is consistent with the results of previous animal studies in which p.o. administered radioiodinated BBI was detected in the bladder and urine of rats and mice within 2–3 h after BBI administration (see Ref. 7 for a review). In a previous oral cancer prevention study in patients with oral leukoplakia, peaks of urine BBI excretion were also detected within hours after administration of a single dose of 800 chymotrypsin inhibition units of BBIC or less (18). The peaks of urine BBI levels detected by the ELISA method are unlikely to have been caused by a "bulk protein" effect associated with the ingestion of high protein soymilk, because the amount of protein in urine is negligible compared with the amount of BSA present in the ELISA reaction mixture, peaks of urine BBI levels were also detected after BBIC administration in the patients with oral leukoplakia, and these patients did not ingest soymilk.

It has been reported previously that the BBI content in soy flour can be as high as 5.5 mg/g (19). In the present study, each dose of soymilk ingested by the subjects contained 105–175 mg, which is equivalent to as little as 19–32 g of soy flour. Because 19–32 g of soy flour are quite attainable in a usual serving of soy meal, it is expected that the ELISA method described here can be used to monitor the BBI intake in people with high soy consumption. The BBI content is considerably lower in other foods. It has been reported that 1 g of textured soy protein, tofu, dry cereal, or pancake mix contains ~0.48, 0.08, 0.10, or 0.38 mg of BBI, respectively (20). The urine BBI concentrations in the individuals who regularly consume these food items may or may not reach a level that can be detected by the ELISA method. However, the amount of BBI needed to have a cancer-preventive effect is also likely to be well be below the level of BBI intake in the present study because BBI is effective in suppressing the malignant transformation of the cells even at nanomolar concentrations (21).

We have observed relatively large individual variations in the urine BBI levels among the nine subjects, which may hinder the use of this assay for screening individuals with high BBI intakes in the general population. However, this method can be useful for monitoring urine BBI excretion in individuals after BBI administration or soy intake because the individual variations can be corrected for when the urine BBI levels after BBI administration or soy intake are compared with the background levels in the same individuals.

The levels of BBI metabolites detected by the 5G2 anti-

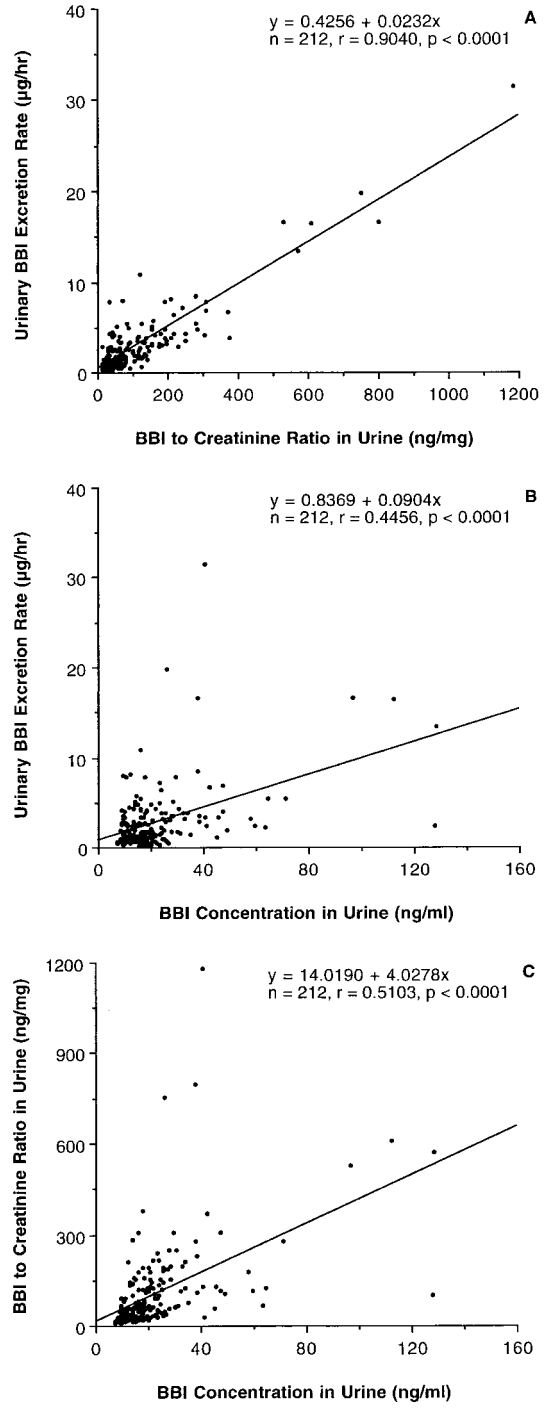

Fig. 5. Correlation analyses of BBI concentrations, BBI excretion rates, and BBI:creatinine ratios. The BBI concentration, BBI excretion rate, and BBI:creatinine ratio were determined in 212 urine samples from 9 human subjects and calculated as described in the legends for Figs. 2, 3, and 4 and analyzed by linear correlation analyses.

body were expressed as urine BBI concentrations, BBI excretion rates, and BBI:creatinine ratios in the present study. Statistically significant increases were detected in the BBI excretion rates and the BBI to creatinine ratio but not in the urinary BBI concentrations after soymilk ingestion. It is expected that the BBI excretion rate and the BBI:creatinine ratio are superior to the urine BBI concentration for monitoring the BBI clearance in urine after BBI ingestion because the urine BBI concentration is inversely affected by the urine water content, whereas the BBI excretion rate and the BBI:creatinine ratio are not affected by the urine water content. This is supported by the close correlation between the BBI excretion rate and the BBI:creatinine ratio as well as the lack of a close correlation between the urine BBI concentration and the BBI excretion rate or the BBI:creatinine ratio.

The close correlation between the BBI excretion rate and the BBI:creatinine ratio suggests that both of these parameters can be used to monitor BBI clearance in urine after ingestion of BBI or BBI-containing food items. To determine the BBI excretion rate, whole urine samples must be collected so that the urine volume needed to calculate the BBI excretion rate can be measured. In contrast, only small aliquots of urine samples are needed for the determination of BBI:creatinine ratio because the urine volume data are not needed for the calculation of the BBI:creatinine ratio. In clinical trials and epidemiological studies, continuous collection of whole urine samples is often impractical, especially when participants are not confined in hospitals. The close correlation between BBI excretion rates and BBI:creatinine ratios suggests that the BBI:creatinine ratio measured in spot urine samples can be used satisfactorily for assessment of treatment or dietary compliance and BBI exposure.

In rat and mouse studies, the amounts of BBI recovered from bladder and urine accounted for ~6–16% of the radioiodinated BBI administered (22, 23). In the present study, the amounts of r-BBI detected in the urine samples collected during the first 6-h period after the first and second soymilk ingestion accounted for <0.02% of the BBI ingested. This recovery rate of BBI is much lower than those reported in the animal studies in which radioiodinated BBI was used (22, 23). Previous animal studies have shown that the BBI recovery rate determined by a radiometric method was 4.5 times of the recovery rate determined by a RIA method in which polyclonal antibodies against BBI are used (24). The discrepancy between the results obtained by the radiometric method and the immunoassay method could result from the inability of any given antibody to recognize all possible forms of BBI metabolites, which may differ in their molecular and/or antigenic structures. Considering the fact that each BBI molecule contains seven pairs of intracellular disulfide bonds (1), which can potentially be rearranged in a number of different ways, it is possible that a significant proportion of BBI metabolites may exist in urine with configurations or three-dimensional structures unrecognizable by the 5G2 antibody. Therefore, the total amounts of BBI metabolites in urine could be much higher than the amounts of r-BBI detected by the 5G2 antibody. Because the antibodies directed against the native form of BBI were unable to detect BBI metabolites in urine, it is assumed that BBI metabolites and native BBI are different in at least some of their three-dimensional structures.

Although the amounts of BBI detected by the radiometric method and the immunological method in chicks were substantially different from each other, the distribution patterns and the time course of BBI appearance in various organs and tissues determined by the two methods were quite similar (24). The time course for urinary excretion of BBI established in the present study by the ELISA-C method is also similar to the previously established time courses of BBI absorption in chicks (24) and urinary BBI excretion in rats and mice (22, 23),

suggesting that the time course for the urinary excretion of BBI detected by the 5G2 antibody may also represent the time course of urinary clearance of other BBI metabolites that are not recognized by the 5G2 antibody. This can be clarified when more antibodies against various reduced forms of BBI are produced.

The increase in BBI excretion rate and the amount of BBI excreted after the second soymilk ingestion were comparable with and not higher than those observed after the first soymilk ingestion. These results suggest that the BBI absorbed after the first soymilk ingestion was probably cleared before the second soymilk ingestion took place. The kinetics of BBI excretion were similar after the first soymilk ingestion in the morning and the second soymilk ingestion in the evening, suggesting that there was no circadian rhythm for BBI excretion. The relatively rapid urinary excretion and the lack of BBI accumulation in human subjects after the ingestion of BBI-containing soymilk suggest that daily intake of BBI may be necessary to achieve optimal cancer chemopreventive effects.

Although probably only a small proportion of BBI metabolites in urine were detected by the ELISA-C method in the present study, the antibodies produced against r-BBI antigen, such as 5G2, remain the only means of detecting BBI metabolites in human urine samples at this time. Using the 5G2 antibody, we have demonstrated that BBI is excreted into urine shortly after oral ingestion, suggesting that BBI is absorbed systemically and could be bioavailable for chemoprevention of cancer in other organs in addition to those in the gastrointestinal tract.

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References

1. Birk, Y. The Bowman-Birk protease inhibitor: trypsin- and chymotrypsin-inhibitor from soybeans. Int. J. Peptide Protein Res., *25:* 113–131, 1985.

2. Larionova, N. I., Gladysheva, I. P., Tikhonova, T. V., and Kazanskaia, N. F. Inhibition of cathepsin G and elastase from human granulocytes by multiple forms of the Bowman-Birk type of soy inhibitor. Biochemistry (Moscow), *58:* 1437–1444, 1993.

3. Larionova, N. I., Gladysheva, I. P., Tikhonova, T. V., and Kazanskaya, N. F. Inhibition of cathepsin G and human granulocyte elastase by multiple forms of Bowman-Birk type soybean inhibitor (in Russian). Biokhimiya, pp. 1046–1052. New York: Plenum Publishing Corp., 1994.

4. Ware, J. H., Wan, X. S., Rubin, H., Schechter, N. M., and Kennedy, A. R. Soybean Bowman-Birk protease inhibitor is a highly effective inhibitor of human mast cell chymase. Arch. Biochem. Biophys., *344:* 133–138, 1997.

5. Kennedy, A. R. *In vitro* studies of anticarcinogenic protease inhibitors. *In:* W. Troll and A. R. Kennedy (eds.), Protease Inhibitors as Cancer Chemopreventive Agents, pp. 65–91. New York: Plenum Publishing Corp., 1993.

6. Kennedy, A. R. Anticarcinogenic activity of protease inhibitors: overview. *In:* W. Troll and A. R. Kennedy (eds.), Protease Inhibitors as Cancer Chemopreventive Agents, pp. 9–64. New York: Plenum Publishing Corp., 1993.

7. Kennedy, A. R. Chemopreventive agents: protease inhibitors. Pharmacol. Ther., *78:* 167–209, 1998.

8. Fontham, E., and Correa, P. The epidemiologic approach to the study of protease inhibitors. *In:* W. Troll and A. R. Kennedy (eds.), Protease Inhibitors as Cancer Chemopreventive Agents, pp. 1–8. New York: Plenum Publishing Corp., 1993.

9. Meyskens, F. L., Jr., Armstrong, W. B., Wan, X. S., Taylor, T. H., Jenson, J., Thompson, W., Nguyen, Q. A., and Kennedy, A. R. Bowman-Birk inhibitor concentrate (BBIC) affects oral leukoplakia lesion size, neu protein levels and proteolytic activity in buccal mucosal cells. Proc. Am. Assoc. Cancer Res., *40:* 432, 1999.

10. Brandon, D. L., Bates, A. H., and Friedman, M. Monoclonal antibody-based enzyme immunoassay of the Bowman-Birk protease inhibitor of soybeans. J. Agric. Food Chem., *37:* 1192–1196, 1989.

11. Brandon, D. L. Antigenicity of soybean protease inhibitors. *In:* W. Troll and A. R. Kennedy (eds.), Protease Inhibitors as Cancer Chemopreventive Agents, pp. 107–129. New York: Plenum Publishing Corp., 1993.

12. Flecker, P. Chemical synthesis, molecular cloning and expression of gene coding for a Bowman-Birk-type proteinase inhibitor. Eur. J. Biochem., *166:* 151–156, 1987.

13. Wan, X. S., Koch, C. J., Lord, E. M., Manzone, H., Billings, P. C., Donahue, J. J., Odell, C. S., Miller, J. H., Schmidt, N. A., and Kennedy, A. R. Monoclonal antibodies differentially reactive with native and reductively modified Bowman-Birk protease inhibitor. J. Immunol. Methods, *180:* 117–130, 1995.

14. Kock, C. J., and Raleigh, J. A. Radiolytic reduction of protein and nonprotein disulfides in the presence of formate: a chain reaction. Arch. Biochem. Biophys., *287:* 75–84, 1991.

15. Lord, E. M., Harwell, L., and Koch, C. J. Detection of hypoxic cells by monoclonal antibody recognizing 2-nitroimidazole adducts. Cancer Res., *53:* 5721–5726, 1993.

16. Haeckel, R. Assay of creatinine in serum, with use of fuller's earth to remove interferents. Clin. Chem., *27:* 179–183, 1981.

17. Meyer, M., Smith, S., and Meyer, R. A., Jr. Creatinine assay by the fuller's earth procedure or by enzymatic determination is adequate for urine but not plasma of mice. Comp. Biochem. Physiol., *106B:* 685–689, 1993.

18. Armstrong, W. B., Kennedy, A. R., Wan, X. S., Atiba, J., McLaren, C. E., and Meyskens, F. L., Jr. Single dose administration of Bowman-Birk inhibitor concentrate (BBIC) in patients with oral leukoplakia. Cancer Epidemiol. Biomarkers Prev., *9:* 43–47, 2000.

19. Brandon, D. L., Bates, A. H., and Friedman, M. ELISA analysis of soybean trypsin inhibitors in processed foods. *In:* M. Friedman (ed.), Nutritional and Toxicological Consequences of Food Processing, pp. 321–337. New York: Plenum Publishing Corp., 1991.

20. DiPietro, C. M., and Liener, I. E. Soybean protease inhibitors in foods. J. Food Sci., *54:* 606–609, 617, 1989.

21. Yavelow, J., Collins, M., Birk, Y., Troll, W., and Kennedy, A. R. Nanomolar concentrations of Bowman-Birk soybean protease inhibitor suppress X-ray induced transformation in vitro. Proc. Natl. Acad. Sci. USA, *82:* 5395–5399, 1985.

22. Yavelow, J., Finlay, T. H., Kennedy, A. R., and Troll, W. Bowman-Birk soybean protease inhibitor as an anticarcinogen. Cancer Res., *43:* 2454–2459, 1983.

23. Billings, P. C., St. Clair, W. H., Maki, P. A., and Kennedy, A. R. Distribution of the Bowman Birk protease inhibitor in mice following oral administration. Cancer Lett., *62:* 191–197, 1992.

24. Madar, Z., Gertler, A., and Birk, Y. The fate of the Bowman-Birk trypsin inhibitor from soybeans in the digestive tract of chicks. Comp. Biochem. Physiol., *62A:* 1057–1061, 1979.

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