JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

In Vitro Digestibility of the Cancer-Preventive Soy Peptides Lunasin and BBI

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Lunasin and BBI (Bowman Birk protease inhibitor) are bioactive soy peptides that have been shown to be effective suppressors of carcinogenesis in *in vitro* and *in vivo* model systems. Since they are subject to digestion in the gastrointestinal tract, we investigated here the stabilities of lunasin and BBI to digestion *in vitro* by simulated intestinal fluid (SIF) and simulated gastric fluid (SGF). Samples containing lunasin and BBI of varying purities were subjected to *in vitro* digestion by SIF and SGF at different times and analyzed by Western blot. While the pure BBI reaction is stable after SIF and SGF digestions, the purified lunasin from soybean and synthetic lunasin are easily digested after 2 min in both *in vitro* digestions. In contrast, lunasin from soy protein containing BBI is comparatively stable after SIF and SGF digestions. Both lunasin and BBI are able to internalize into the cell and localize in the nucleus even after digestion, suggesting that some of the peptides are intact and bioactive. These data suggest that BBI plays a role in protecting lunasin from digestion when soy protein is consumed orally. The role of other soy protease inhibitors such as Kunitz Trypsin Inhibitor (KTI) cannot be excluded from these experiments.

KEYWORDS: Lunasin; BBI; *in vitro* digestibility; SIF; SGF digestion

INTRODUCTION

Epidemiological evidence suggests that dietary factors play an important role in the etiology of different kinds of cancer (*1*). Soy products are associated with decreased risk for prostate (*2*–*4*), breast (*5*), and endometrial cancer (*6*). Soybeans, a common source of bioactive peptides, contain about 40% protein. Bioactive peptides may exist naturally or be derived from soy protein hydrolysates. These peptides may act as physiological modulators during the gastrointestinal digestion of soy products (*7*). Many bioactive peptides have certain structural properties in common such as relatively short peptide residue length, i.e., 2–9 amino acids, and the presence of hydrophobic amino acid residues in addition to proline, lysine, or arginine groups (*8*). In particular, bioactive peptides isolated from soybeans such as lunasin and the protease inhibitor Bowman Birk inhibitor concentrate (BBIC) are now being intensively studied as cancer chemopreventive agents.

Lunasin, a 43-amino-acid peptide, is a unique and novel cancer-preventive peptide originally isolated from soybean and now also found in barley. Lunasin's carboxyl end contains 9 asp (D) residues, an –RGD− cell adhesion motif, and a predicted helix with structure homologous to a conserved region of chromatin-binding proteins (*9*). Lunasin has been found to suppress transformation of mammalian cells induced by carcinogens and viral oncogenes E1A and *Ras* (*9*–*13*). Soybean-derived Bowman Birk inhibitor (BBI), used as BBIC, has been shown to suppress carcinogenesis in *in vitro* and animal models and is now the subject of promising clinical trials in cancer patients (*14*, *15*). BBI is a 71-amino-acid protein with 7 disulfide bonds, which stabilize its active configuration, and has a double-headed structure with the well-characterized trypsin inhibitory domain on one head and the chymotrypsin inhibitory domain on the other (*16*). BBI is absorbed and widely distributed in the different organs of mice following oral administration (*17*).

We have shown that lunasin in lunasin-enriched soy (LES) is protected from *in vitro* digestion by pepsin and that lunasin extracted from the liver and blood of rats fed LES is intact and bioactive (*18*). However, there have been no systematic studies on *in vitro* digestion of lunasin and BBI in various forms. Here, we present data on simulated intestinal fluid (SIF) and simulated gastric fluid (SGF) digestion of lunasin and BBI of different degrees of purity.

MATERIALS AND METHODS

Extraction of Soybean Protein. Ten grams of ground soybean (*Glycine max* cv. Taekwangkong) were extracted with 50 mL of phosphate-buffered saline (0.1 M PBS, pH 7.0) supplemented with fresh protease inhibitor cocktail (Sigma). The mixture was centrifuged at 12 000 × *g* for 30 min, and the supernatant protein extract was used for subsequent experiments.

Pure Lunasin from Soybean. The protein extracts from soybean were initially purified by ion-exchange chromatography (IC) on Biogel

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10.1021/jf072107c CCC: $37.00
Published on Web 11/27/2007

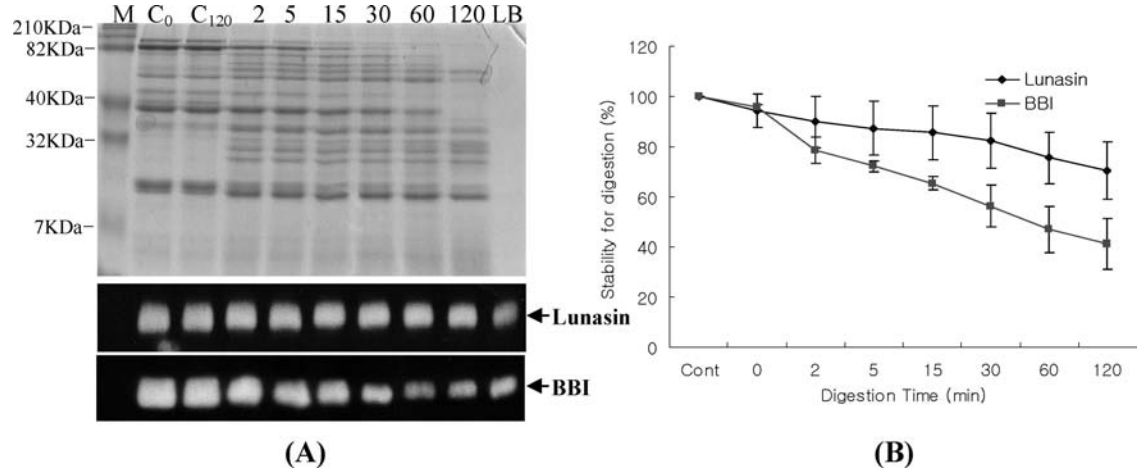

Figure 1. Variation of soy lunasin and BBI contents upon SIF digestion. (**A**) SDS-PAGE and Western blot (**B**), % remaining relative to the original. (**A**) C0: Control (no pancreatin) 0 min. C120: Control 120 min. The numbers correspond to times (min) of digestion; LB refers to lunasin standard (200 ng) and BBI (4 μg).

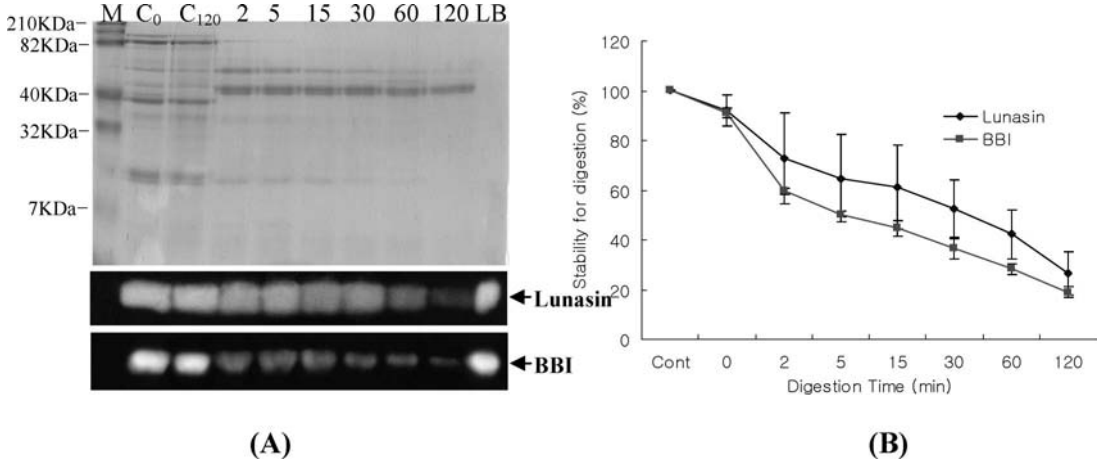

Figure 2. Variation of soy lunasin and BBI contents upon SGF digestion. (**A**) SDS-PAGE. (**B**) Western blot. % remaining relative to original. (**A**) C0: Control (no pepsin) 0 min. C120: Control 120 min. The numbers correspond to times (min) of digestion. LB: Lunasin (200 ng). BBI (4 μg).

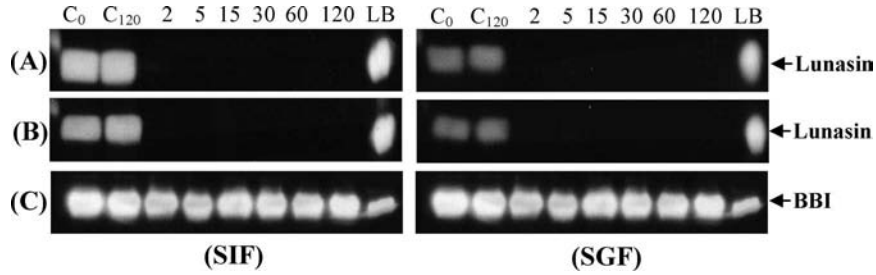

Figure 3. In vitro digestibility of synthetic lunasin, purified soy lunasin, and BBI using SIF and SGF. C0: Control (**A**, no pancreatin; **B**, no pepsin) 0 min. C120: Control 120 min. (**A**) Purified soy lunasin. (**B**) Synthetic lunasin. (**C**) BBI. The numbers correspond to times (min) of digestion.

resin AG 1-X4 (*13*). The various fractions were eluted with different concentrations of NaCl (0, 0.1, 0.5, 0.7, and 1 M) in phosphate-buffered saline (0.1 M PBS, pH 7.0). The column fraction collected at 0.7 M NaCl was purified further by concentrating with YM-10 and YM-3 Microcon centrifugal filters (Millipore Corp). Protein content was determined using the Bradford assay (*19*).

Chemicals. All digestion reagents were purchased from Sigma and electrophoresis chemicals from Bio-Rad Laboratories. Synthetic lunasin (Synpep, Inc.) and BBI (Sigma) was used as a standard. A lunasin polyclonal antibody against the carboxyl epitope (CEKHIMEKIQGRGD-DDDD) was custom-produced (Zymed, Inc., called Zymed R1) and provided by Filgen Biosciences, Inc. Monoclonal anti-BBI was purchased from Agdia Inc. Secondary antibody was purchased from Santa Cruz Biotechnology.

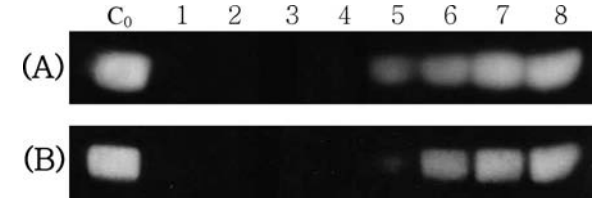

Figure 4. Effects of lunasin/BBI ratios in digestion mixtures on digestibility using (**A**) SIF and (**B**) SGF. C0: Control (no pancreatin or pepsin). 1: lunasin/BBI (1:1). 2: lunasin/BBI (1:2). 3: lunasin/BBI (1:5). 4: lunasin/BBI (1:10). 5: lunasin/BBI (1:20). 6: lunasin/BBI (1:30). 7: lunasin/BBI (1:40). 8: lunasin/BBI (1:50).

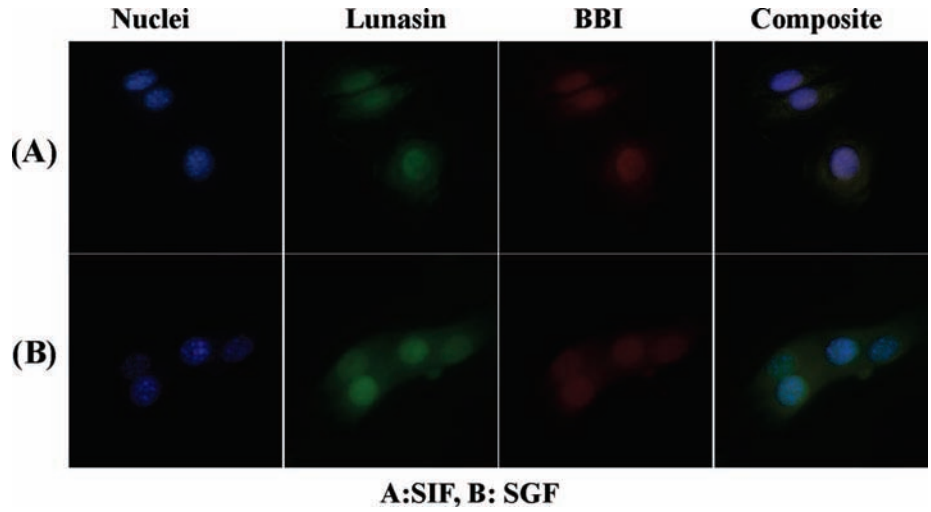

Figure 5. Internalization of *in vitro* digested lunasin and BBI in NIH3T3 cells 18 h after exposure.

SIF Digestion Stability Assay. SIF was prepared as described in the United States Pharmacopoeia (*20*) and consists of 10 mg/mL of pancreatin in 0.05 M KH_2PO_4, pH 7.5. Aliquots (64 μL) of SIF were placed in 1.5-mL microcentrifuge tubes and incubated at 37 °C for 10 min in a water bath. Soybean protein sample was prepared as 10 mg protein/mL in 0.05 M KH_2PO_4, pH 7.5. Samples containing different ratios of lunasin/BBI were prepared according to the content of lunasin (150 μg lunasin/mL) by standardizing with the soybean protein sample. The test samples (10 μL) were added to each of the microcentrifuge tubes to start the reaction. The ratio of pancreatin to soybean protein was about 6.4:1 (w/w) (*20*). At intervals of 0, 0.5, 5, 15, 60, and 120 min, 20 μL of 5 × Laemmli buffer was added to each tube, and the reaction was immediately stopped by placing the tube in a boiling water bath for 10 min The samples (30 μL) were loaded in SDS-PAGE according to the procedure described below.

SGF Digestion Stability Assay. SGF was prepared as described in the United States Pharmacopoeia (*20*) and consists of 3.2 mg/mL pepsin in 0.03 M NaCl at pH 1.2. Aliquots (200 μL) of SGF were placed in 1.5 mL microcentrifuge tubes and incubated in a water bath at 37 °C. Ten microliters of the test protein (10 mg/mL in 0.03 M NaCl) was added to each of the SGF vials to start the digestion reaction. The ratio of pepsin to soybean protein was about 6.4:1 (w/w). At intervals of 0, 0.5, 5, 15, 60, and 120 min, 75 μL of 1 N NaOH was added to each vial to stop the reaction. Next, 70 μL of 5 × Laemmli buffer was added to the sample before it was heated for 10 min in a boiling water bath. The samples (50 μL) were loaded in SDS-PAGE as described below.

Gel Electrophoresis. SDS-PAGE of protein samples was performed using 15% Tris-HCl gel as described by the Ready Gels Application Guide (Bio-Rad Laboratories). Gels were stained with Coomassie brilliant blue and transblotted to PVDF membranes (Bio-Rad Laboratories) according to the Western blot procedure described below.

Western Blot. An immune-blot PVDF membrane was prepared for transfer by soaking in 100% methanol for 15 s. The proteins on SDS-PAGE gel were transblotted to the membrane for 90 min at 300 mA, 100 V. Upon completion of transfer, the nonspecific binding sites were blocked by immersing the membrane for 1 h in 5% nonfat milk dissolved in Tris-buffered saline 1% Tween 20 (TBS-1T). The membrane was washed with fresh changes of the TBS-1T at room temperature, incubated in either antilunasin or anti-BBI as the primary antibody with 3% nonfat milk in TBS-1T for 1 h, and then washed with fresh changes of the TBS-1T at room temperature. The membrane was then incubated using antirabbit (for lunasin, 1:5000) or antimouse (for BBI) streptavidin HRP as the secondary antibody with 3% nonfat milk in TBS-1T for 1 h and subsequently washed with fresh changes of the TBS-1T at room temperature. The primary antibodies against lunasin and BBI were diluted 1:5000 and 1:3000, and secondary antibodies were diluted 1:5000 and 1:3000, respectively. The ECL Western blotting detection agent (RPN2106, Amersham, Inc.) was applied to the membranes and immediately exposed to Polaroid film.

Quantification of Lunasin and BBI. Lunasin and BBI contents of the samples were calculated by comparing the band intensities with those of known standards run under the same conditions. The intensities of the bands were quantified using the software *Un-SCAN-IT gel* version 5.1 (Silk Scientific, Inc.).

Cell Immunostaining Assay. The digested samples were purified to remove the digestive enzyme and buffer reagents by centrifugal filter devices using YM-3 and YM-10 (Millipore). The concentrations of lunasin and BBI were calculated by Western blot and image analysis program (*Un-SCAN-IT gel* version 5.1, Silk Scientific, Inc.). The ratios of lunasin/BBI from purified protein were 1:15 and 1:20 for SIF and SGF digestions, respectively. The identification of the treated samples was based on green fluorescent stain for lunasin, red fluorescent stain for BBI, and blue fluorescent stain (DAPI) for the nucleus (see below).

Sterilized glass coverslips were placed in 6-well plates that were then plated with NIH 3T3 cells (30 000 cells per well). Cells were stabilized by incubation for 24 h at 37 °C, and *in vitro* digested lunasin and BBI were added to each well to a final concentration of 10 uM. The plates were incubated at 37 °C. After 18 h, 2% formaldehyde was added to fix the cells. PBS/FBS (PBS with 10% fetal bovine serum) was then added to block nonspecific binding. The primary antibodies against lunasin and BBI were diluted 1:250 with 0.1% saponin/PBS/FBS solution. The secondary antibodies Alexa-Fluor 488 goat antirabbit IgG and Alexa-Fluor 647 goat antimouse IgG (Invitrogen Corporation) were used against lunasin and BBI, respectively. Once the secondary antibody incubation was done, coverslips were then washed and inverted onto a drop of antifade mounting medium (Sigma). Mounted coverslips were viewed under a fluorescence microscope using a 60× oil immersion objective. The excitation wavelength for DAPI was 359 nm, 494 nm for lunasin, and 610 nm for BBI.

RESULTS AND DISCUSSION

***In Vitro* Digestibility of Soy Lunasin and BBI.** Soy lunasin and BBI contents after incubation at various times with SIF are shown in **Figure 1A**,**B**. The Coomassie blue staining in **Figure 1A** shows a marked decrease in the 40 and 82 KDa protein bands starting at 15 min digestion with SIF. Generally, low amounts of lunasin and BBI are not clearly seen in Coomassie blue stained gel, but were clearly detected by Western blot. The Western blot panel shows a slight decrease in the amounts of lunasin and BBI with the progress in digestion times, and the bands for both peptides are still prominent even at 120 min. The quantities of both peptides are quantified as percent of the original in **Figure 1B**. The contents of lunasin and BBI in soy crude protein were 15.5 ng and 405 ng per microgram protein, respectively, a ratio of 1:26 (w/w). The lunasin content decreases slightly with increasing incubation

times, reaching a value of approximately 78% of the original at 120 min after the SIF digestion. The digestion rate of BBI is relatively faster, with the amount dropping down to about 80% of the original at 2 min incubation and eventually to about 60% of the original at 120 min.

The digestibility of soy lunasin and BBI by SGF are shown in **Figure 2A**,**B**. In general, the digestibility of the two peptides is more pronounced compared with that of SIF. Protein staining in the upper panel of **Figure 2A** shows marked decreases with increasing incubation times of protein bands corresponding to 20, 40, and 82 KDa. The Western blot data show that soy lunasin and BBI bands are still evident after 120 min of SGF digestion, but the amounts are less compared with that of SIF digestion. Lunasin and BBI contents decrease dramatically to about 30% and 19% of the original, respectively, at 120 min after SGF digestion (**Figure 2B**).

These data suggest that a significant degree of digestion of soy lunasin and BBI occurs in the stomach and to a lesser degree in the small intestines when soy protein is consumed orally. Further, the data show that BBI is digested faster than lunasin, indicating that BBI, in the process of protecting lunasin, becomes more vulnerable to digestion. The remaining undigested lunasin and BBI evidently end up in the tissues intact and bioactive (*17*, *18*).

***In Vitro* Digestibility of Synthetic Lunasin, Purified Soy Lunasin, and BBI.** Synthetic lunasin (>98% pure), lunasin purified from soy protein (estimated by Western blot to be about 90% pure), and BBI (purchased from Sigma) were subjected to *in vitro* digestions by SIF and SGF. **Figure 3** shows the stability of pure lunasin and BBI to *in vitro* digestion by SIF and SGF. While the pure BBI is stable even up to 120 min digestion with SIF and SGF, the purified lunasin from soybean and synthetic lunasin are easily digested after 2 min in both *in vitro* digestions.

Effects on Digestibility of Lunasin/BBI Ratios in Digestion Mixtures. It is clear from the data gathered so far that lunasin in soy protein is resistant to *in vitro* digestion by both SIF and SGF, but synthetic and purified soy lunasin are not. This suggests that there is a sufficient amount of BBI and other protease inhibitors such as the Kunitz Trypsin inhibitor (KTI) to protect lunasin in soy protein (*21*). Lunasin and BBI show similar patterns of biosynthesis and degradations in developing soybean seeds (*22*), indicating that the ratio of the two peptides in the mature seed is sufficient for protection of lunasin by BBI. However, in *in vitro* digestibility experiments, the ratio of lunasin/BBI would be critical. We therefore proceeded to determine the effects of this parameter on the digestibilities of the two peptides, and the results are shown in **Figure 4**.

Figure 4 shows that lunasin is easily digested when the ratio of lunasin/BBI is 1:10 or less. Protection against digestion begins at a ratio of 1:20 and continues to increase up to the maximum of 1:50 that was tested. We analyzed the soy protein sample we are using and found a lunasin/BBI ratio of 1:26 (see previous section). Although we have focused our attention on BBI, the protective effects of other soy protease inhibitors such as KTI cannot be excluded here, because they were not measured. It is likely that the combined protection provided by these soy natural protease inhibitors against digestion plays a major role in making lunasin bioavailable in soy protein (*22*). In the development of any nutraceutical or dietary supplement, this information has to be taken into consideration.

Internalization of Lunasin and BBI in NIH3T3 Cells after *In Vitro* Digestion. Our results so far show that lunasin and BBI are protected from digestion by naturally occurring soy protease inhibitors such as BBI. An interesting biological property of lunasin is its ability to internalize into the cell and localize in the nucleus (*12*). We therefore proceeded to determine if lunasin and BBI that have gone through *in vitro* digestion retain this ability as a measure of their bioactivities.

Figure 5 shows the internalization of lunasin and BBI after being subjected to *in vitro* digestion by SIF and SGF. The lunasin/BBI ratios used in the digestion are 1:15 for SIF and 1:20 for SGF, and the NIH3T3 cells were exposed to digested soy protein containing the equivalent of 1 μM lunasin and fixed after 18 h of exposure. The images clearly demonstrate that lunasin and BBI subjected to *in vitro*digestion retain the ability to internalize into the cells and localize in the nucleus. This is in confirmation of previous results in our laboratory where synthetic lunasin internalizes into the cells and localize in the nucleus, evidently binding to hypoacetylated chromatin (*9*, *12*).

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Received for review July 13, 2007. Revised manuscript received October 24, 2007. Accepted October 25, 2007. This work was partly supported by the Korea Research Foundation grant (KRF-2005-214-C00101) (to P.J.H.) and by the U.S. Department of Defense CDMRP and the American Association for Cancer Research (to B.O.dL.).

JF072107C