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Contents and Bioactivities of Lunasin, Bowman−Birk Inhibitor, and Isoflavones in Soybean Seed

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It has been previously demonstrated that lunasin is a novel and promising cancer preventive peptide from soybean. The Bowman−Birk protease inhibitor (BBI) and isoflavones are well-studied substances from soy. This study evaluated the levels and bioactivities of these three compounds as affected by stages of seed development and sprouting under light and dark conditions. BBI and lunasin appear at 7 and 6 weeks, respectively, after flowering and increase as the seed matures. Daidzein and genistein both decrease during seed maturation. During sprouting under light, BBI increases up to the 6th day and decreases thereafter, disappearing at the 9th day after soaking. Under dark conditions, BBI increases up to the 7th day after soaking and decreases thereafter, disappearing at the 10th day. Lunasin starts to decrease at 2 days after soaking and disappears completely at 7 days under light and dark conditions. Daidzein and genistein increase continuously during the 10 days of soaking, and both increase more in the dark than under light conditions. Protein extracts from early seed development (2−5 weeks after flowering) suppress cell viability to a greater degree than those from later stages (6−9 weeks). Inhibition of foci formation by protein extracts from later stages is greater than those from earlier stages. Lunasin and BBI suppress foci formation more than the isoflavones. Sprouting decreases lunasin and BBI contents but increases isoflavones. Protein extracts from early soaking times inhibit foci formation more and suppress cell viability less than those from later soaking times. Light and dark conditions have no influence on the bioactivities of protein extracts. These data are useful in the preparation of soy fractions enriched in lunasin, BBI, and isoflavones and in making dietary recommendations.

KEYWORDS: Lunasin; BBI; isoflavones; soybean; cancer preventive; sprouting; foci formation

INTRODUCTION

Consumption of soybean products is associated with overall low mortality rates caused by cancers such as prostate, breast, and colon (*1*, *2*). Southeast Asians have a 4−10-fold lower incidence of and death from breast and prostate cancers. Following emigration to the United States, the risk of these cancers rises rapidly in one generation to equal that of Americans (*2*, *3*). Differences in the diet are thought to account for a large part of this variation (*4*). The average intake of soy protein in Asia varies from 10 g/day in China to 30−50 g/day in Japan and Taiwan (*5*). In contrast, Americans eat no more than 1−3 g/day. Two-thirds of the reported studies in the epidemiological literature associated soy intake with reduction of cancer risk (*6*). More recent epidemiological studies, animal experiments, and in vitro studies show that soy products reduce cancer risks (reviewed in refs *7* and *8*). The two most studied bioactive substances in soy are the Bowman−Birk protease inhibitor (BBI or BBIC) and the isoflavones. BBI refers to the pure form, whereas BBIC refers to the BBI concentrate, a crude form.

BBI is a serine protease inhibitor (*9*, *10*) consisting of a single chain of 71 amino acid residues cross-linked by seven pairs of disulfide bonds (*11*, *12*) and a well-characterized ability to inhibit trypsin and chymotrypsin. BBIC has been shown to be cancer preventive in in vitro models of carcinogenesis as well as in a number of animal model systems (*13*). In preclinical studies, BBIC has been found to interfere effectively with the development of tumors induced by chemical carcinogens in the lung or gastrointestinal tract of mice (*14*−*16*), the esophagus of rats (*17*), and the oral cavity of hamsters (*18*) and with radiation-induced lymphosarcoma in mice (*19*).

Phytoestrogens, particularly soy isoflavones, are known to exhibit various health-beneficial effects (*20*−*25*) including relief of menopausal symptoms and preventive effects in the development of cardiovascular diseases and hormone-dependent cancers (*26*, *27*). Isoflavones are heterocyclic phenols with close similarity in structure to estrogens and a diphenolic character similar to that of lignans displaying both estrogenic and antiestrogenic activity, influencing sex hormone metabolism and

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their biological activities (*28*). They also may be important antioxidants (*29*).

Our laboratory has recently demonstrated the cancer preventive properties of a novel soy peptide that we called lunasin (*30*, *31*). Lunasin is a unique 43 amino acid soybean peptide that contains at its carboxyl end 9 Asp (D) residues, an Arg-Gly-Asp (RGD) cell adhesion motif, and a predicted helix with structural homology to a conserved region of chromatin-binding proteins. It has been shown to suppress carcinogenesis caused by chemical carcinogens and oncogenes in in vitro models and a mouse model for skin cancer (*30*−*32*).

Seed sprouts have long been used in human diets as health foods, and recent research shows that in addition to being a good source of basic nutrients, they also have important phytochemicals with disease preventive and health-promoting properties (*33*). Sprouting also removes antinutrients such as enzyme inhibitors in the seed that make sprouts safe for the diet (*34*).

Although we have studied the effects of stages of seed development on lunasin content and bioactivity (*35*, *36*), there are no such studies on comparable values for BBI and isoflavones. In this paper, we present the comparative contents and bioactivities of lunasin, BBI, and isoflavones as affected by seed maturation and sprouting.

MATERIALS AND METHODS

Materials. Soybean (*Glycine max* cv. Taekwangkong) was grown under usual greenhouse conditions at Andong National University, Andong, Korea. Flowers were tagged, and at the appropriate time, seeds were collected once a week for 8 weeks after flowering. Soybean seeds were sprouted and maintained in a growth chamber at 28 °C under dark or light conditions. The cotyledons were collected every 24 h from 2 to 7 days after soaking. All samples were stored in a −30 °C freezer until analyzed. Seeds and cotyledons were cracked and ground to a flour. Ten gram samples 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 12000*g* for 30 min, and the supernatant protein extract was used for subsequent experiments. Protein content was determined using the Bradford assay (*37*).

Chemicals. All electrophoresis chemicals were purchased from Bio-Rad. Synthetic lunasin (Synpep, Inc.) was used as a standard, and lunasin polyclonal antibody was made against the carboxyl half of lunasin (Zymed, Inc.). Monoclonal anti-BBI was generously provided to us by Dr. David Brandon of the USDA-WRRL (Albany, CA). Secondary antibody was purchased from Santa Cruz Biotechnology. BBI, the isoflavones daidzein and genistein, and dimethylbenzanthracene (DMBA) were purchased from Sigma. All HPLC solvents were purchased from J. T. Baker. All cell culture supplies and reagents were purchased from Gibco-BRL.

Gel Electrophoresis. SDS-PAGE of protein extracts was performed using 15% Tris-HCl ready gel as described by the Ready Gels Application Guide (Bio-Rad Laboratories). Laemmli buffer (Bio-Rad Laboratories) was used to dilute the sample, and the mixture was heated for 5 min at 95 °C prior to loading. Gels were stained with Coomassie brilliant blue and transblotted to PVDF membranes (Bio-Rad Laboratories) according to the Western blot procedure.

Western Blot. An immune-blot PVDF membrane was prepared for transfer by soaking in 100% methanol for 15 s. The SDS-PAGE gel was transblotted to the membrane for 90 min at 300 mA and 100 V. Upon the 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 anti-lunasin 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 anti-rabbit (for lunasin) or anti-mouse (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. Detection agent was applied to the membranes and immediately exposed to 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.)

HPLC Analysis of Isoflavones. Twenty microliters of filtered protein extract was injected into an HPLC equipped with a μBondapak C18 column after the system had been equilibrated at ambient temperature and stabilized using a UV detector (254 nm) with mobile phase (0.1% acetic acid in acetonitrile/0.1% acetic acid in water, 3:7 ratio for 15 min and 5:5 ratio over 15 min at a flow rate of 1 mL/min for 35 min). Isoflavones were identified by retention time and internal addition of standard, and their contents and recoveries were calculated by comparing peak areas with those of standards subjected to the same treatments.

Cell Growth Inhibition Assay. The cell growth inhibition effects of test samples were determined using the MTT assay on NIH3T3 cells (KCLB21658) obtained from the Korean Cell Line Bank (Cancer Research Institute, Seoul, Korea). Cells grew in DMEM supplemented with 10% FBS and 2% antibiotics (penicillin and streptomycin) and were maintained in a 37 °C incubator at 5% CO_2 level. Cells were cultured in a 96 well plate (5×10^4 cells/well). Cells were stabilized by incubation for 24 h at 37 °C, and 10 μL of test sample was added to each well. The plate was incubated at 37 °C. After 4 days, 50 μL of MTT solution (1 mg/mL PBS) was added to each well and the plate was incubated at 37 °C. After 4 h, the supernatant was aspirated, and 100 μL of DMSO was added and mixed thoroughly to dissolve the formazan crystals. The optical density was measured at 540 nm with a microplate reader (Asys Hitec, Expert96, Asys Co.).

Foci Formation Assay. NIH3T3 cells were used in the foci formation assay. The assay was carried out in a 12-well plate, and 1 mL of cell suspension (500 cells/mL) was added to each well. Cells were allowed to adhere for 20 h at 37 °C, and then the test samples were added. After 4 h, the chemical carcinogen (1.5 μg/mL of DMBA) was added to the medium. DMBA was dissolved with acetone. Acetone instead of DMBA was added to negative control. Cells were exposed to the carcinogen for 20 h and washed with 1 × PBS, and fresh medium was added. The mixture of medium and test samples was replaced once a week. After 5 weeks, each plate was washed with 0.9% NaCl, fixed with methanol, stained with Giemsa, and scored for transformed foci.

RESULTS AND DISCUSSION

Contents of BBI and Lunasin in Developing Soybean Seed. The protein patterns and those of lunasin and BBI at different stages of seed development are shown in **Figure 1**. The protein stain shows the biosynthetic patterns of various MW sizes including 80, 50, 35, 10, and 5 kDa during maturation. It is noteworthy that the bands increase significantly in weeks 5−7, peaking at week 7 and evidently decreasing thereafter, but the bands nonetheless remain prominent until week 9. The low MW proteins to which lunasin belongs show similar patterns. In a previous study, lunasin starts to appear at 5 weeks after flowering and remains in the mature seed (*35*). Although the variety is different, this confirms our earlier observation. The content of lunasin at the first appearance is 0.0109 μg of lunasin/g of seed and 0.1198 μg of lunasin/g of seed after maturation. BBI starts to appear at 7 weeks after flowering and increases as the seed matures, remaining in the mature seed. The content of BBI is calculated at 0.160 μg/g of seed at the first appearance and 3.56 μg/g of seed in the mature seed.

Seeds of legumes and other plants contain protease inhibitors, which block the digestion of proteins by inhibiting proteases in the animal digestive tract (*38*). The soybean-derived BBI is particularly effective in suppressing carcinogenesis (*15*). The

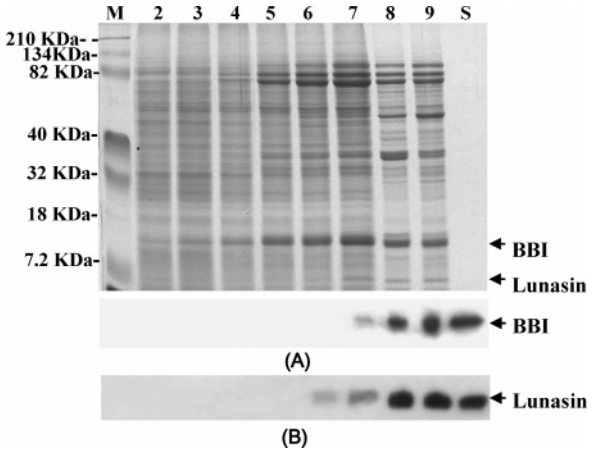

Figure 1. Lunasin and BBI contents in soybean seeds at different stages of development. M is the MW marker, numbers correspond to weeks after flowering, and S is the standard BBI (4 μg) or synthetic lunasin (200 ng). Each lane was loaded with 25 μg of protein. For BBI, primary and secondary antibody antibodies were diluted 1:4000. For lunasin, primary and secondary antibodies were diluted 1:5000.

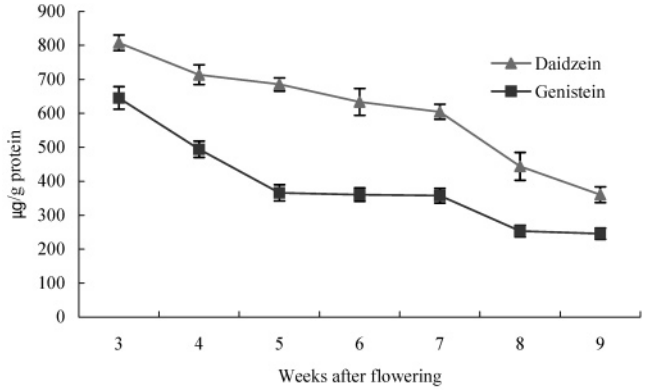

Figure 2. Isoflavone contents in protein extracts of developing soybean seed. Vertical lines on bars are ± SD, $n = 4$.

protein expression of BBI in the developing soybean seed is important in understanding the mutual biological relationship with lunasin in relation to their roles in seed development and their anticarcinogenic properties. It is likely that BBI and other protease inhibitors in soybean seed such as the Kunitz trypsin inhibitor (KTI) protect lunasin from digestion.

Contents of Isoflavones in Developing Soybean Seed. Phytoestrogens, particularly soybean isoflavones, have been studied in relation to hormone-related cancers. Isoflavones are diphenolic compounds and belong to a subclass of the more ubiquitous flavonoids. The primary isoflavones in soybean are genistein and daidzein (*39*), which occur as aglycon glucoside, acetylglucoside, or malonylglucoside (*40*).

The acid hydrolysis procedure used in this experiment leads to hydrolysis of glycosides, and therefore the data represent the total amount of isoflavones in soybean protein extracts, including glycosides and aglycons. The content of daidzein in total crude protein is higher than that of genistein throughout seed maturation. The genistein content at 2 weeks after flowering is 645.4 μg/g of protein and is 218.2 μg/g protein after maturation, whereas that of daidzein is 807.9 μg/g of seed protein at 2 weeks and 326.3 μg/g of seed protein after maturation. The complete biosynthetic pattern of the isoflavones is shown in **Figure 2**. Both isoflavones decrease during maturation, in contrast to lunasin and BBI. To our knowledge, this is the first documentation on the pattern of isoflavone biosynthesis during soybean seed development.

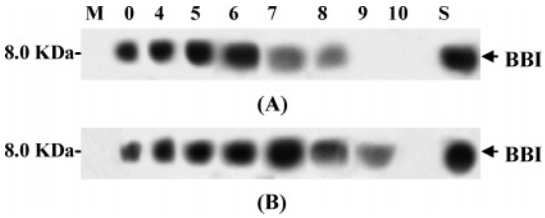

Figure 3. Contents of BBI as affected by sprouting under (**A**) light and (**B**) dark conditions. M refers to MW size, and numbers refer to days of soaking. S refers to BBI standard (4 μg). Each lane was loaded with 25 μg of protein. Primary antibody was diluted 1:3000 and secondary antibody 1:3000.

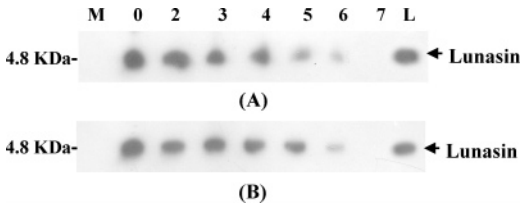

Figure 4. Contents of lunasin in soybean seed as affected by sprouting under (**A**) light and (**B**) dark conditions. M refers to MW marker, and numbers refer to days of soaking. L is lunasin standard (200 ng). Each lane was loaded with 25 μg of protein. Primary antibody and secondary antibody were diluted 1:5000.

Variation of BBI and Lunasin in Sprouting Soybean Seed. Soy and other seed sprouts, such as "kongnamul" in Korea, mung bean (*Phaseolus mungo*) sprouts in Southeast Asian countries, and soybean sprouts in the United States, are widely used in the human diet. Beans are typically sprouted by soaking under dark or light conditions.

Under light and dark conditions, crude protein patterns are similar (not shown). Under light, BBI increases up to 6 days after flowering, starts to decrease thereafter, and disappears completely at 9 days after soaking. In the dark, BBI continues to increase up to 7 days, decreases thereafter, and disappears at 10 days after soaking (**Figure 3**). The proteins were extracted only from the cotyledon because previous studies in our laboratory show the presence of lunasin mRNA only in this tissue (*41*). Under both light and dark conditions, lunasin continues to decrease after soaking and disappears completely at day 7 (**Figure 4**).

Variation of Isoflavones in Sprouting Soybean Seed. Both isoflavones were analyzed in cotyledon protein extracts and expressed in micrograms of isoflavone per gram of protein. **Figure 5** shows the pattern of isoflavone contents in sprouting soybean seeds under light and dark conditions. Under light, both isoflavones remain constant, start to increase slightly at day 5 up to day 7, and level off. Relative to 0 day, the increase in both isoflavones is ~30%. Under dark conditions, both isoflavones remain constant up to day 5 of soaking, increase dramatically at days 6 and 7, and increase slowly thereafter. Relative to day 0, the increase of the isoflavones at day 10 is ~230%. Daidzein content increases to 426.5 μg/g of protein (light condition) and 1072.2 μg/g of protein (dark condition), whereas genistein content increases to 521.6 μg/g of protein (light condition) and 1210.5 μg/g of protein (dark condition) at day 10 after soaking. It is clear that soaking under dark conditions leads to a much higher content (~3-fold) of the isoflavones. This is the first set of data documenting the patterns of isoflavone contents sprouted under light and dark conditions.

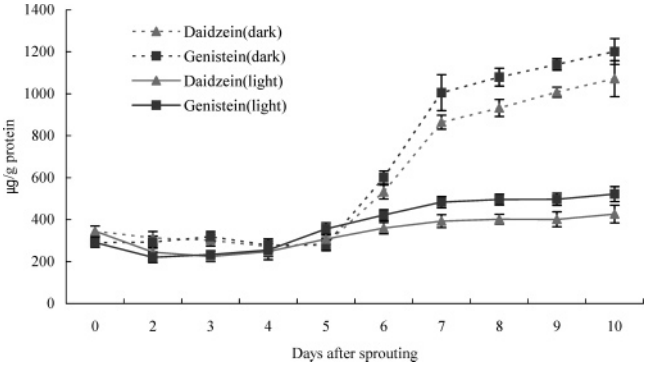

Figure 5. Contents of isoflavones in sprouting soybean seeds. Numbers refer to days after sprouting. Vertical lines on bars are ± SD, $n = 4$.

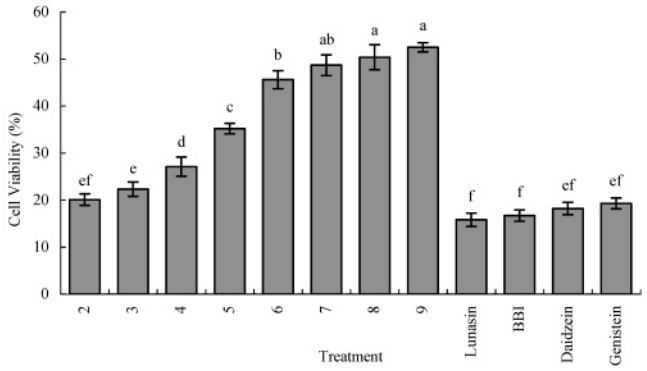

Figure 6. Effects of protein extracts obtained from various stages of soybean seed development and of standard lunasin, BBI, and isoflavones on cell viability as measured by the MTT assay of NIH 3T3 cells. The percentage of viable cells was normalized in relation to untreated cells. Numbers correspond to weeks after flowering, and each sample contained 35 μg of protein/mL. The standards used were lunasin, 480 ng/mL (~100 nM); BBI, 4 μg/mL (~400 nM); daidzein, 12 μg/mL; and genistein, 8 μg/mL. Vertical lines on bars are ± SD, $n = 4$. Bars with different letter designations are statistically significant from one another.

Effect of Protein Extracts on Cell Proliferation. Lunasin has been found to have no effects on cell proliferation rate of normal and cancerous mammalian cells (*32*). In contrast, typical chemopreventive agents are known to slow cell proliferation of cancer cells but not normal cells. Therefore, there is interest in determining the comparative effects of lunasin, BBI, and isoflavones on cell proliferation of NIH3T3 cells. The effects of protein extracts on seed viability as measured by the MTT assay are shown in **Figures 6** and **7**. Proteins were extracted from the seeds at various stages of seed development (**Figure 6**) and at various days after soaking (**Figure 7**). The lunasin, BBI, and isoflavone standards have no effect on cell viability. Interestingly, the protein extracts collected at weeks 2−9 after flowering promote cell viability of NIH3T3 cells with the effect increasing with later stages of seed development (**Figure 6**). On the contrary, the protein extracts from the sprouting seeds collected at days 0−10 after soaking have the opposite effects of decreasing cell viability, the negative effects becoming more pronounced with longer days of soaking (**Figure 7**). The contrasting effects on cell viability of proteins biosynthesized during seed maturation and that of proteins formed during sprouting are a novel finding.

Effects of Protein Extracts on Inhibition of Foci Formation in NIH3T3 Cells Induced by DMBA. Lunasin and BBI have been shown in our laboratory to inhibit foci formation in mammalian cells induced by chemical carcinogens and oncogenes (*30*, *32*). However, the effects of seed development and sprouting on the cancer preventive properties of these two substances as well as isoflavones have not been determined. The protein extracts from the seeds at various stages of development and from different days of soaking were tested for their ability to inhibit foci formation in NIH3T3 cells induced by DMBA (**Figure 8**). The lunasin, BBI, daidzen, and genistein standards all inhibit foci formation, lunasin and BBI being more effective than the isoflavones. On a molar basis, lunasin is 4-fold more effective than BBI.

This is the first time that the isoflavones have been tested in a foci formation assay. The protein extracts all inhibit foci formation with increasing efficacy as the seed matures. The inhibitory effect of the protein extracts from week 9 is 100% relative to the values of the positive control (no lunasin) and negative control (no DMBA). This particular sample contains 480 ng of lunasin/g of protein. In decreasing order of efficacy, week 9 is followed by weeks 7−8, week 6, and week 2. Although these can be explained by increasing concentrations of lunasin and BBI during seed maturation, it is not true for the

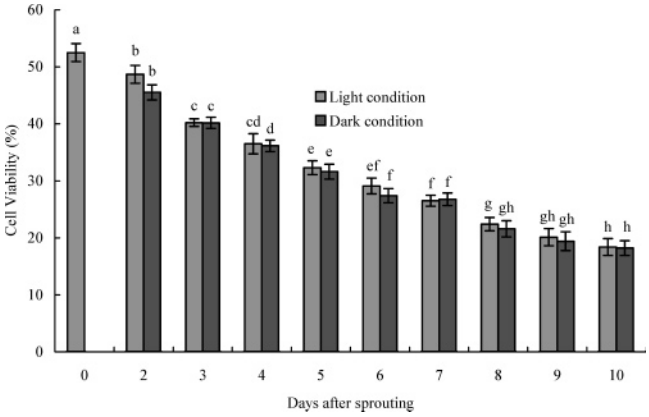

Figure 7. Effects of protein extracts from seeds sprouted under light and dark conditions on cell viability as determined by the MTT assay of NIH 3T3 cells. Numbers correspond to days after sprouting; the samples contained 35 μg of protein/mL. Vertical lines on bars are ± SD, $n = 4$. Bars with different letter designations are statistically significant from one another.

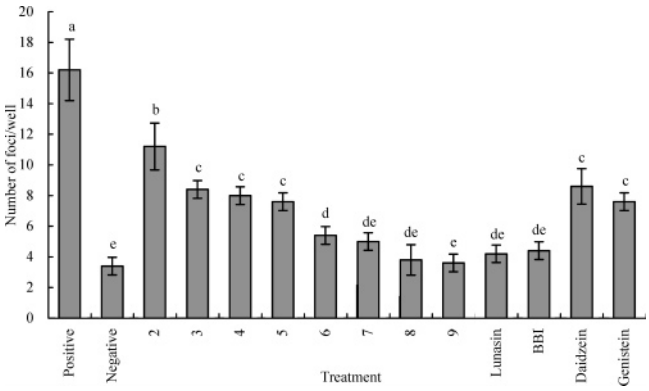

Figure 8. Effects of protein extracts from different stages of seed development and of standard lunasin, BBI, and isoflavones on inhibition of foci formation in NIH 3T3 cells induced by DMBA. Numbers refer to weeks after flowering, and each sample contains 35 μg of protein/mL. The standards used were lunasin, 480 ng/mL (~100 nM); BBI, 4 μg/mL (~400 nM); daidzein, 12 μg/mL; and genistein, 8 μg/mL. Positive refers to cells treated with DMBA and no lunasin, and negative refers to cells not treated with DMBA. Vertical lines on bars are ± SD, $n = 4$. Bars with different letter designations are statistically significant from one another.

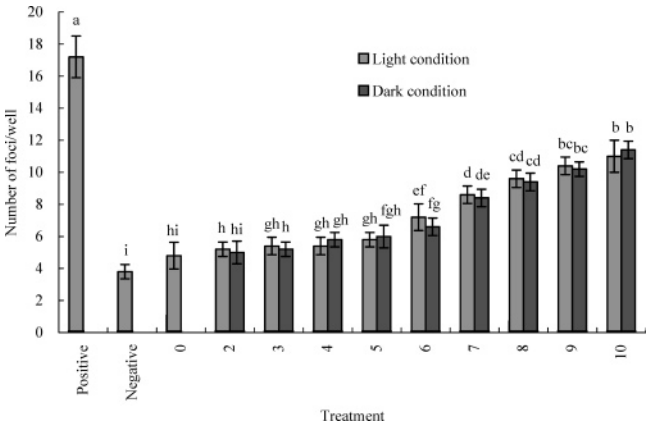

Figure 9. Effects of protein extracts from soybean seeds at various days of sprouting on inhibition of foci formation in NIH 3T3 cells induced by DMBA. Numbers refer to days after sprouting, and each sample contains 35 μg of protein/mL. Positive refers to cells treated with DMBA and no lunasin, and negative refers to cells not treated with DMBA. Vertical lines on bars are ± SD, $n = 4$. Bars with different letter designations are statistically significant from one another.

isoflavones, which decrease with seed maturation (**Figures 1** and **2**).

Figure 9 shows the effect of protein extracts from the sprouted seeds at various days after soaking under light and dark conditions. All samples exhibit inhibition of foci formation, the efficacy decreasing with soaking time. Greatest inhibition is observed at 0 days of soaking followed by a continuing decline with time of soaking. The light or dark conditions do not have any effect on the inhibition of foci formation. Again, the contrasting effects of proteins biosynthesized during seed development and that formed during sprouting are clear. This pattern is similar to the effects on cell viability and is a new finding.

In summary, the data presented here document for the first time the comparative contents and bioactivities of the three major cancer preventive substances in soybean as affected by seed maturation and sprouting under light and dark conditions. Lunasin and BBI contents increase as the seed matures, whereas isoflavones decrease. Sprouting leads to a continuing decrease of lunasin and BBI with soaking time, whereas the opposite is true for the isoflavones. The protein extracts from the developing seeds have clearly opposite effects on cell viability and inhibition of foci formation compared with those from sprouting seeds. Light and dark conditions affect the contents of BBI and isoflavones but not lunasin and have no influence on cell viability and inhibition of foci formation. These data are useful in the preparations of soy fractions enriched in these three substances and in recommendations of dietary intakes.

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