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Characterization of Lunasin Isolated from Soybean

HYUNG J. JEONG,† JAE H. PARK,† YI LAM,‡ AND BEN O. DE LUMEN*,§

School of Bioresources, Andong National University, Kyungpook, Korea Bio-Rad Laboratories, Hercules, California 94547, and Division of Nutritional Sciences & Toxicology, University of California, Berkeley, California 94720

Lunasin is a novel and promising chemopreventive peptide from soybean. We have shown previously that lunasin suppresses transformation of mammalian cells caused by chemical carcinogens and inhibits skin carcinogenesis in mice when applied topically. Although the *lunasin* gene was cloned from soybean, all experiments carried out so far in our lab have used synthetic lunasin and therefore there is no detailed description of natural lunasin isolated from soybean. We report here the first characterization of soybean lunasin that includes definitive identification by mass peptide mapping, partial purification, and measurement of bioactivities of the various purified fractions and protein expression in the developing seed. The identity of lunasin in the seed extracts was established by Western blot analysis and mass spectrometric peptide mapping. All lunasin fractions partially purified by anion exchange and immunoaffinity column chromatography suppress colony formation induced by the *ras*-oncogene and inhibit core H3-histone acetylation. During seed development, lunasin peptide appears 5 weeks after flowering and persists in the mature seed. Western blot analysis of different soybean varieties and commercially available soy proteins shows the presence of the peptide in varying amounts. These results demonstrate the feasibility of producing large quantities of natural lunasin from soybean for animal and human studies.

KEYWORDS: soybean lunasin; purification; suppression of colony formation; inhibition of core histone acetylation; protein expression

INTRODUCTION

Epidemiological evidence suggesting that diets rich in soybean are associated with lower cancer mortality rates, particularly colon, breast, and prostate cancers (*1*, *2*), has led to studies on soy components believed to be responsible in preventing carcinogenesis. The most extensively studied are the Bowman−Birk protease inhibitor (BBI) and the isoflavones (*3*). BBI, now in human clinical trials, suppresses carcinogenesis in laboratory animals and in in vitro transformation systems (*4*).

While searching for methionine-rich proteins from soybean, we isolated and cloned a cDNA for a posttranslationally processed 2S albumin (Gm2S-1) from midmaturation soybean seed (*5*, *6*). Gm2S-1 codes for a signal peptide, a small subunit (we termed lunasin), a linker peptide, and a large subunit methionine-rich protein. Lunasin, is a unique 43-amino acid peptide with (a) a carboxyl end of nine aspartic acids (D)**9** residues, (b) a cell adhesion motif −RGD− just before the poly-D, and (c) a predicted, conserved, helical region with structural homology to chromatin binding proteins (*7*). Transfection of mammalian cells with the *lunasin* gene leads to mitotic arrest and cell death characterized by cell lysis and chromosome fragmentation (*7*). The antimitotic effect of lunasin is attributed to binding of its poly-aspartyl carboxyl end to regions of hypoacetylated chromatin, like that found in centromeres. The affinity of the lunasin peptide for hypoacetylated chromatin suggests that lunasin may be involved in chromatin modification, a process implicated in cell-cycle control and suppression of carcinogenesis by tumor suppressors (*8*, *9*). The −RGD− cell adhesion motif (*9*, *10*) in lunasin and its chromatin-binding property suggest that it could be a chemopreventive agent. We recently demonstrated in vitro and in vivo the chemopreventive properties of this peptide against chemical carcinogens (*11*).

Although the *lunasin* gene was isolated from soybean, all the experiments carried out in our lab have used synthetic lunasin. The high cost of synthesizing lunasin makes it impractical to use synthetic lunasin for animal experiments and human studies. Therefore, there is a need to isolate, characterize, and demonstrate the biological activity of lunasin from soybean.

Knowledge of lunasin peptide expression in the developing seed will contribute to understanding its native role in the seed and is relevant because immature seeds (i.e. green seeds) are a major part of the human diet. With the isolation and sequencing of lunasin from soybean seeds 16 years ago, a few interesting biological roles were proposed, but none has been proven (*12*). The antimitotic effect of lunasin when the gene is transfected led us to propose that lunasin could be an effector molecule that arrests cell division to initiate cell expansion during the

* Corresponding author. Phone: 510-642-8144. Fax: 510-642-0535. E-mail: nitto@nature.berkeley.edu.
† Andong National University.
‡ Bio-Rad Laboratories.
§ University of California.

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second stage of angiosperm seed development, when massive synthesis of storage proteins, carbohydrates, and lipids occurs in the endosperm and cotyledon (*13*). This role, however, remains to be established.

We report here the first characterization of soybean lunasin that includes protein expression in the developing seed, definitive identification by mass peptide mapping, partial purification, and measurement of bioactivities of the various purified fractions, using colony formation and histone acetylation inhibition, and analyses in different soy bean varieties and soy protein fractions.

MATERIALS AND METHODS

Seeds. Mature seeds of soybean of different cultivars were obtained from the Andong Seed Supply Institute, Andong, Kyungpook, Korea. To determine protein expression during seed development, the cultivar Hwangkumkong was grown in a greenhouse, the flowers were tagged carefully, and the pods were harvested at various weeks after flowering. Kidney bean, mung bean, and pinto bean that were used for the initial screening of lunasin in other seeds were purchased from a local grocery store at Berkeley, CA. Commercially available soy proteins were obtained from SPI Group, Inc (San Leandro, CA).

Reagents. All electrophoresis chemicals were purchased from Bio-Rad Labs (Hercules, CA). Protease inhibitor cocktail was purchased from Sigma (St Louis, MO). Standard lunasin was custom synthesized by American Peptide Co (Sunnyvale, CA). Antibodies other than lunasin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate Biotechnology (Lake Placid, NY). Polyclonal lunasin antibody was produced by Zymed, Inc, (South San Francisco, CA).

Isolation and Purification of Lunasin. *Isolation of Total Protein.* Soybean seeds of unknown variety were purchased from a local grocery store at Berkeley. The seeds were cracked individually and ground to flour and 30 g was extracted with 100 mL of phosphate-buffered saline (PBS, pH 7.4, 0.1 M) supplemented with fresh protease inhibitor cocktail (Sigma). For the commercially available soy proteins, samples were extracted directly with the extraction buffer. The extraction mixture was shaken for 48 h at 4 °C, and the protein extract was dialyzed for 24 h at 4 °C in distilled water. A protein pellet obtained by centrifugation of the dialyzed protein extract at 12000*g* for 30 min was re-extracted once with 10 mL of extraction buffer. The supernatants from the two extractions were combined and used for further purification or analyzed directly for lunasin contents.

Ion Exchange Column Chromatography. The protein extract was further purified by ion exchange chromatography on Biogel resin AG 1-X4, mesh size 100−200 (Bio-Rad Labs). The column (5.0 × 50 cm, packed height of 40 cm) was equilibrated with PBS buffer (pH 7.4, 0.1M) and about 100 mg of concentrated protein in PBS buffer was applied and the column washed with 150 mL of equilibration buffer. The bound proteins were then eluted with 0.7 M NaCl in PBS buffer at 4 °C, at a flow rate of 30 mL/h, and 24-mL fractions were collected. Our previous work on barley lunasin purification showed that lunasin elutes with 0.7−0.9 M NaCl (*14*). The column fractions were concentrated to a volume of 150 μL using a YM3 Microcon centrifugal filter (Millipore Corp., Bedford, MA). Each fraction was analyzed for protein concentration by Bradford assay (Bio-Rad Laboratories) and ran on SDS−PAGE and Western blot. Lunasin purified by the ion exchange column was analyzed by MALDI (matrix-assisted laser desorption ionization) peptide mass mapping for confirmation of identity.

Immunoaffinity Column Chromatography. A rabbit polyclonal antibody (called R1) against the carboxyl end of lunasin was custom produced (Zymed Inc., South San Francisco, CA). The coupling of the antibody to Affi-Gel Hz gel (Bio-Rad Laboratories) and subsequent sample application and elution were according to manufacturer's instructions. Briefly, the coupling involved periodate oxidation of vicinal hydroxyls of the carbohydrate moiety in the Fc region of the antibody to form aldehyde groups for specific coupling to the agarose matrix in Affi Gel Hz gel. The immunoaffinity gel was contained in a 1.0 cm × 10 cm Econo-Pac chromatography column. After applying the concentrated sample from ion exchange chromatography, unbound proteins were washed with 2 bed volumes of 0.5 M NaCl and 0.1 M NaCl in the application buffer. Bound fractions were eluted with 20 mL of 0.2 M glycine-HCl (pH 2.5), and 1.0-mL fractions were collected for protein assays, SDS−PAGE, Western blot analysis, and bioassays.

Identification of Lunasin. *Gel Electrophoresis.* SDS−PAGE of seed extracts and column fractions was performed using 15% Tris-HCl Ready Gel (Bio-Rad Laboratories) following manufacturer's instructions. Samples were diluted in Laemmli buffer (Bio-Rad Laboratories) and boiled for 5 min prior to loading. A goat anti-rabbit Western-compatible molecular weight standard (Santa Cruz Biotechnology) was used. Gels were stained with Coomassie Blue and transblotted to PVDF membranes for Western blot analysis.

Western Blot. The PVDF membrane with the transferred protein was blocked for nonspecific binding for 1 h in Blotto A (5% nonfat milk and 1% Tween 20 in Tris-buffered saline), washed with fresh changes of the 1% TBS-T solution (1% Tween 20 in Tris-buffered saline), and incubated with the lunasin primary antibody R1 at 1:4000 dilution in Blotto B solution (3% nonfat milk and 1% Tween20 in TBS) for 1 h. After washing, the membrane was incubated with an anti-rabbit secondary antibody at 1:3000 dilution in Blotto B solution for 1 h. After washing, the membrane was prepared for detection using a chemiluminescence kit (Santa Cruz Biotechnology) following manufacturer's instructions. The intensities of the bands were quantified using the software ChemImager 4400 v. 5.5 (Alpha Innotech Corp.). Lunasin in samples was quantified by comparison with a standard curve established using known concentrations of synthetic lunasin. The method has a detection limit of approximately 15 ng lunasin, and the curve of signal intensity versus the amount of lunasin has a high correlation coefficient of 0.95 (*14*).

Identification of Lunasin by MALDI (Matrix-Assisted Laser Desorption Ionization) Peptide Mass Mapping. Lunasin purified by ion exchange column was analyzed by MALDI peptide mass mapping for confirmation of identity. The band corresponding to the molecular weight of lunasin, based on comparison with chemically synthesized lunasin, was excised from SDS−PAGE gel and submitted for MALDI analysis (*15*) to the Protein Structure Laboratory at University of California, Davis. The identity of lunasin was established by comparison of the peptide mass map of the putative peptide obtained from in-gel tryptic digest with predicted masses of lunasin cleaved by trypsin (*16*).

Bioassay of Lunasin. Chemically synthesized lunasin has been shown in our lab to suppress anchorage-independent growth of mouse fibroblast cells in soft agar (colony assay) induced by *ras*-oncogene transfection and to inhibit histone acetylation in mammalian cells in the presence of sodium butyrate, a histone deacetylase inhibitor (*11*, *17*).

Colony Assay. Stably *ras*-transfected cells (Dr. Peter Stambrook, Cincinnati Medical School) were used for the colony formation assay in 6-well plates. The cell line was established by introduction of a *Ha-ras* oncogene (valine mutation at codon 12) into NIH 3T3 cells (*18*, *19*). An SV40 promoter drives the *Ha-ras* oncogene cloned from the human T24 bladder carcinoma cell line with the *Escherichia coli lac* repressor-binding operator. Transfectants of one cell line designated as 2-12, after isopropyl β-D-thiogalactoside (IPTG, from Sigma) induction, show high expression level of *Ha-ras* oncogene and strong characteristics of transformation, including anchorage independence. The inducibility of 2-12 cells makes this cell line a simple yet convenient assay to determine the effect of lunasin on anchorage independence of transformed cells. 2-12 cells were routinely grown and maintained in a 37 °C incubator at 5% CO_2 level. Normal growth medium was DMEM supplemented with 10% calf serum (CS). For each well, 1 mL of 0.6% base layer agar was prepared by diluting 120 μL of 5% agar in 880 μL of growth medium. The bottom agar layer was set at room temperature for 15 min. For each well, 1 mL of 0.33% upper agar layer was then prepared by combining 550 μL of 0.6% agar, 330 μL of growth medium, 20 μL of 1 M IPTG, and 100 μL of 2-12 cells (40 000 cells/mL). When lunasin was used for treatment, the volume in the solution displaced the same volume of medium. Once the upper agar solution was transferred to the wells, cells were incubated for 14 days. Colonies were counted under a microscope at a magnifica-

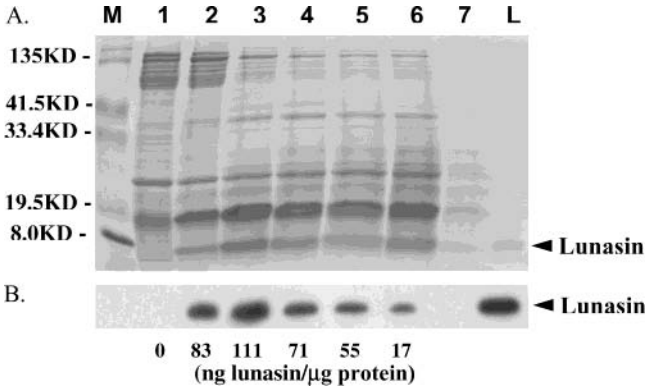


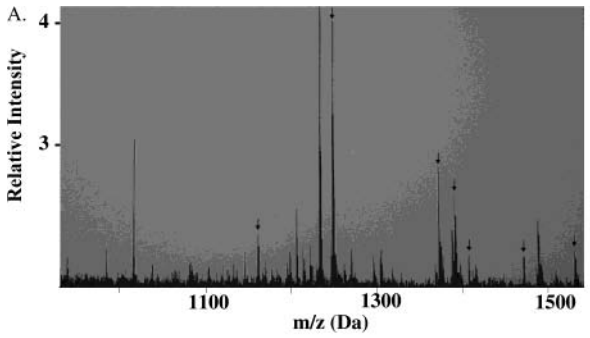
Figure 1. (**A**). Coomassie Blue stain and (**B**) Western blot of various fractions eluted with 0.7 M NaCl from Biogel resin AG 1-X4 and ran on 15% Tris-HCl gels. About 100 mg of concentrated total protein extract in PBS buffer was applied and the column washed with 150 mL of equilibration buffer. The bound proteins were then eluted with 0.7 M NaCl in PBS buffer at 4 °C, at flow rate of 30 mL/h. Twenty-four-milliliter fractions were collected and concentrated down to 150 μL, and 3.3 μL containing 25 μg proteins was applied to each well. Lanes 1–7 represent the 24-mL fractions sequentially eluted with 0.7 M NaCl. Lane M is prestained MW marker and lane L is 200 ng of synthetic lunasin standard.

tion of 40×. The treatment concentrations varied from 1 nM to 10 μM for lunasin purified from ion-exchange and immunoaffinity column chromatography.

Core Histone Acetylation Assay. Human breast cancer cells MCF-7 (ATCC) were grown in DMEM + 10% FBS in a 37 °C incubator at 5% CO_2 level. After release from confluency, the cells were treated with various combinations of 10 μM soybean lunasin from immunoaffinity column chromatography and 5 mM sodium butyrate, a histone deacetylase inhibitor and grown for 24 h. Using standard protocols (Upstate Biotechnology), proteins enriched for histones were acid-extracted from the cells. Approximately 0.8 mg of acid-extracted proteins was run on 16.5% Tris-Tricine gel (Bio-Rad), blotted onto Hybond-ECL membranes (Amersham), and silver-stained for proteins (Bio-Rad). Immunoblot analysis was conducted using primary antibodies against tetraacetylated H3 histone (H3-Ac) (Upstate Biotechnology), diluted 1:1000, and HRP-labeled anti-rabbit IgG secondary antibody, diluted 1:2000. Densitometer readings were taken on autoradiograph using the Bio-Rad Molecular Imaging System GS525 and Molecular Analyst software, to measure levels of acetylated H3.

RESULTS

Purification of Lunasin by Anion Exchange Chromatography. The total protein extracts from the seeds were purified by anion exchange chromatography to obtain samples for MALDI identification and for bioassay. Since our previous work on barley lunasin showed that lunasin elutes with 0.7−0.9 M NaCl (14), 0.7 M NaCl was used to elute lunasin from the column. **Figure 1** shows the Coomassie Blue staining and Western blot of the different fractions eluted by 0.7 M NaCl in PBS buffer after washing with 150 mL of equilibration buffer. Each lane represents 3.3 μL containing 25 μg of protein out of 150 μL concentrated from 24 mL of collected fraction. Lunasin started eluting in the second 24-mL fraction (lane 2), maximizes in the third fraction (lane 3), and diminishes thereafter, until none is detected in the seventh fraction (lane 7). It is evident that the high MW proteins predominate in the early fractions (lanes 1 and 2), while low MW proteins that include lunasin were eluted in subsequent fractions (lanes 3−7). All the fractions containing lunasin were pooled and subsequently used for the MALDI identification of the lunasin band and further purification by immunoaffinity column.



Monoisotopic masses	Sequence
1187.501	QLQGVNLTPCEK
1244.458	WQHQQDSC*R
1369.621	qLQGVNLTPC*EK
1386.645	QLQGVNLTPC*EK
1402.628	SKWQHQQDSCR
1459.650	SKWQHQQDSC*R
1514.771	KQLQGVNLTPC*EK

C*: Cys was modified to carboxy amidomethyl-Cys

q : Gln is cyclized to Pyroglutamic acid

Figure 2. (**A**). MALDI peptide mass map of putative lunasin band cut out from SDS−PAGE of the ion-exchange-purified fraction of soybean extract eluted with 0.7 M NaCl. The masses of seven peptide fragments marked with arrows match with those of predicted sequences deduced from tryptic cleavage within 30 ppm, unambiguously identifying lunasin as the major peptide in the band. (**B**) The masses of the seven fragments and their predicted sequences. The tryptic peptides were used for calibration of the spectrum. The proteins were analyzed using the automated analysis at ProtanaA/S, Odense, Denmark.

MALDI Analysis of In-Gel Tryptic Digests of Lunasin. Figure 2A shows the mass spectrum of the tryptic digest of the putative lunasin band cut out from the SDS−PAGE of the pooled fractions from the anion exchange column chromatography. The average absolute mass accuracy is 30 ppm over the *m*/*z* range from 1000 to 1500 (*15*), and seven peptide peaks match within the expected tryptic masses for the protein sequence retrieved by automated database searching. All Lys sites would not be cleaved completely because they are neighboring with Glu or Arg, or are close to N-terminal residues, which would be cleaved only partially. The monoisotopic masses of the seven peptide fragments and the corresponding predicted sequences are shown in **Figure 2B**. These data establish unambiguously that lunasin is the major peptide in the putative lunasin band.

Soybean Lunasin Fractions Suppress Colony Formation in Stably *ras*-Transfected 2-12 Cells. The colony assay provides a simple and rapid quantitative tool for analysis of the oncogene-induced anchorage-independent growth in transformed cells. In all the experiments, the negative control received no IPTG during the assay and the positive control was induced by IPTG but received no lunasin treatment. **Figure 3A** shows that relative to the positive control, total protein soy extract (Lunasin 1) suppresses colony formation by approximately 30%, both partially purified lunasin from an ion exchange column (Lunasin 2) and lunasin from an immunoaffinity column (Lunasin 3) by approximately 43% compared with 15% for the synthetic lunasin (Lunasin 4). **Figure 3B** shows the dosage effect of immunopurified lunasin on suppression of colony formation, which is linear with $R^2 = 0.92$ when plotted in a semilog manner. A 40%

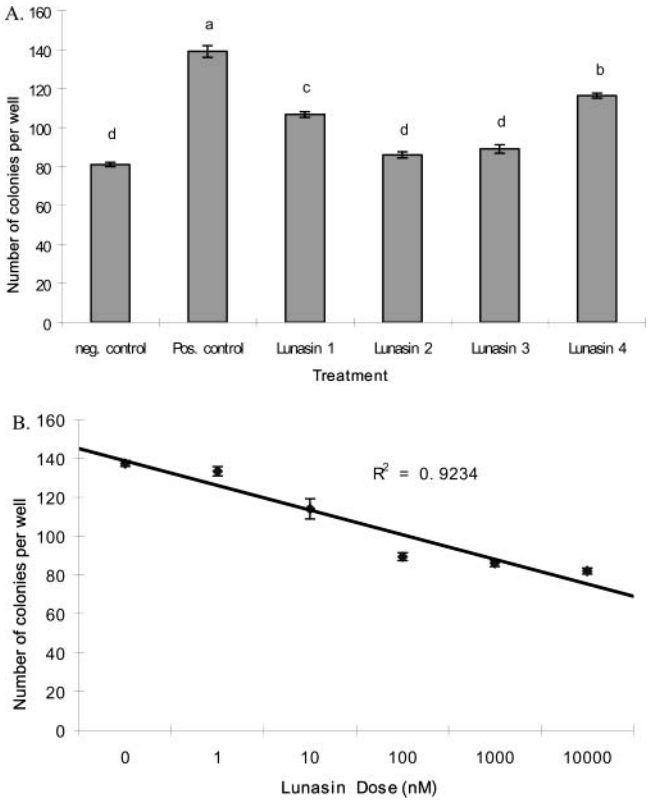


Figure 3. Soybean lunasin fractions inhibit colony formation in IPTG-induced *ras*-oncogene stably transfected 2-12 cells. (**A**) At a concentration of 10 μM, soybean lunasin fractions at different stages of purification are effective in inhibiting colony formation. Negative control is not treated with IPTG, while the positive control is treated with IPTG without lunasin. Lunasin 1 is the total protein extract of soybean seed, lunasin 2 is the concentrated pooled fractions from ion-exchange column chromatography, lunasin 3 is the concentrated pooled fractions from immunoaffinity column chromatography, and lunasin 4 is synthetic lunasin. Each treatment represents the average of triplicate experiments. Treatment means (±standard error of the means) with different letters are significantly different from each other as determined by one-way ANOVA followed by Duncan's multiple range test. (**B**) Dosage effect of immunopurifed lunasin soybean fraction on suppression of colony formation. Each dose treatment mean (±standard error of the means) is the average of triplicate experiments.

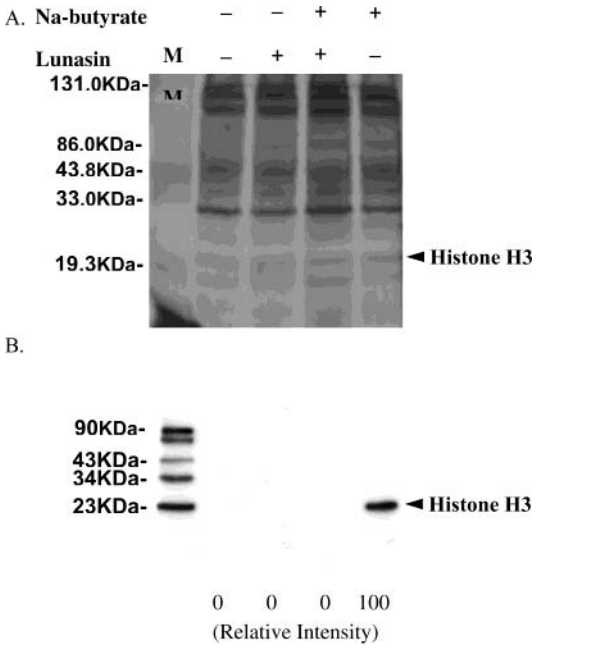


Figure 4. Soybean lunasin inhibits acetylation of histone H3. (**A**) Silver stain and (**B**) Western blot of acid-extracted proteins from human breast cancer MCF-7 cells are shown. After being released from confluency, the cells were treated with different combinations of 10 μM soybean lunasin purified by immunoaffinity column chromatography and 5 mM of the histone deacetylase inhibitor sodium butyrate and grown for 24 h. The acid-exracted proteins enriched for histones from the different treatment combinations were run on SDS–PAGE, blotted on to PVDF membranes, silver-stained, and probed with the antibody against tetracetylated histone H3. H3-Ac was visualized using an HRP-conjugated anti-rabbit secondary antibody. Silver-stain of the acid extracted proteins shows approximately equal loading of proteins for each well that contains 15 μg of protein each. Lane M shows a prestained MW marker. Relative intensity corresponds to densitometry readings normalized against that of controls untreated with sodium butyrate.

reduction is seen even at a concentration of 100 nm. However, increasing the concentration of lunasin from 100 nM to 10 μM does not produce any further statistically significant reduction in colony formation, suggesting that the internalization of lunasin into the cells might be the limiting step in the biological activities of lunasin. Overall, these results indicate that lunasin isolated and purified from soybean at different stages of purification are biologically active. This is the first piece of evidence indicating that lunasin isolated from its initial natural source can be an effective chemopreventive agent against oncogene-induced transformation in cells.

Inhibition of H3-Histone Acetylation by Immunopurified Soybean Lunasin Fraction. Histone acetylation and deacetylation are involved in chromatin remodeling, which has been associated with eukaryotic transcriptional regulatory mechanisms (*20*). Transfection of the lunasin gene into mammalian cells leads to mitotic arrest and cell lysis, resulting in lunasin bound to the chromatin (*7*). Subsequently, we showed that exogenous addition of synthetic lunasin peptide to mammalian cells inhibits histone acetylation in the presence of sodium butyrate, a histone deacetylase inhibitor (*11*). Thus, inhibition of histone acetylation has proven to be an excellent and simple bioassay for lunasin. MCF-7 cells were treated with Na-butyrate, in the presence and absence of soy lunasin fractions from immunoaffinity column chromatography. Sodium butyrate is known to increase histone acetylation and the level of acetylated histones in the cell (*21*). **Figure 4** shows the silver stain of SDS−PAGE (**A**) and Western blot (**B**) of acid-extracted proteins. In the absence of lunasin, sodium butyrate increases significantly the level of H3-Ac to a relative intensity of 100 (lane + -, Western blot). With the addition of 10 μM immunopurified lunasin fraction, this increase is completely neutralized, as shown by the absence of the H3-Ac band in the Western blot (lane + +, Western blot). The extent of reduction in acetylation achieved using immunopurified lunasin is comparable to that achieved with synthetic lunasin (*11*).

Analysis of Lunasin in Soybean Cultivars and Other Legumes. Five soybean cultivars and three beans from the Phaseolus family, kidney, mung, and pinto beans, were analyzed for lunasin to extend the analysis on soybean and to screen initially the presence of lunasin in other seeds (**Figure 5A,B**). Interestingly, lunasin was present only in the five soybean cultivars and not in any of the *Phaseolus* beans. Our lab has reported the presence of lunasin in barley, the first cereal crop demonstrated to have lunasin, and an extensive characterization has been carried out (*14*).

Lunasin Contents of Commercially Available Soy Proteins. Table 1 shows the lunasin contents of four commercially

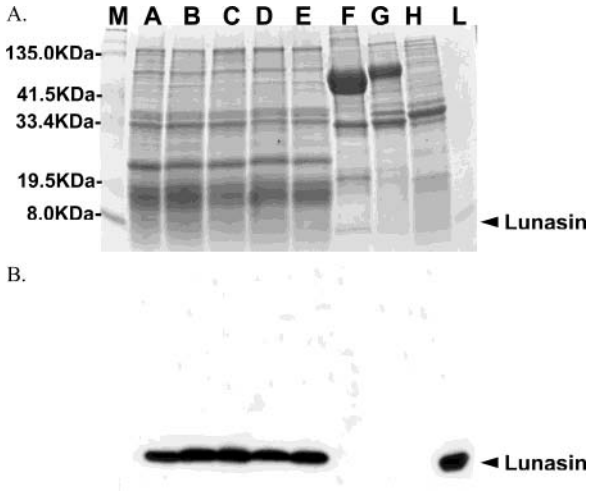


Figure 5. (**A**) Coomassie Blue stain and (**B**) Western blot of protein extracts from five varieties of soybean and three beans of the *Phaseolus* family. Lanes A–E contain protein extracts of five Korean soybean cultivars, blackbean, bluebean, goldbean, hanbatbean, and miceeye bean, and lanes F–H contain protein extracts from pinto bean, red kidney bean, and mung bean, respectively. Lane M is the MW marker, and lane L is 200 ng of synthetic lunasin. Each well contains 25 μg of proteins.

Table 1. Lunasin Contents of Some Commercially Available Soy Proteins[a]

soy protein	mg of lunasin/ g of protein	% lunasin in product
defatted soy flour	5.48 ± 0.17	0.054 ± 0.0017
soy concentrate (alcohol washed)	8.72 ± 0.19	0.065 ± 0.0015
soy isolate	6.92 ± 0.16	0.059 ± 0.0014
soy concentrate (water washed)	16.52 ± 0.23	0.091 ± 0.0013

[a] The soy proteins were extracted with physiological phosphate buffer (PBS, 0.1 M, pH 7.4) as described in Materials and Methods. The extracts were run on SDS–PAGE, using 25 μg of proteins per well and blotted onto membranes for Western analysis. The concentration of lunasin in each sample was calculated by comparing the densitometry readings of the lunasin band with the signals from a standard curve of standard synthetic lunasin ran under similar conditions. Each value represents three independent experiments.

available soy protein preparations, expressed in milligrams of lunasin/gram of protein and in percent lunasin in the product. All these proteins are used in the formulation of soy food products. Defatted soy flour containing typically 50% protein is the starting material for producing soy concentrate (70% protein) and soy isolate (90% protein). Defatted soy flour has the lowest amount of lunasin, while the water-washed soy concentrate has the highest. Soy isolate and the alcohol-washed soy concentrate have intermediate lunasin values. The lunasin content of the alcohol-washed soy concentrate is approximately half that of the water-washed soy concentrate, likely due to lunasin being washed away by the alcohol. The alcohol-washed soy concentrate has also the least amount of isoflavones (SPI, Inc.), which are intensely studied as chemopreventive agents. The soy isolate has a lower lunasin content expressed in milligrams of lunasin/gram of protein than the two types of soy concentrates, possibly because of selective removal of lunasin during the isoelectric precipitation step used to make isolate from concentrate. The varying amounts of lunasin in these soy proteins reflect the effects of the preparation methods on lunasin contents. Overall, the presence of lunasin in these soy proteins, which are used to make soy food products, indicate the widespread presence of lunasin in the end products consumed by the public. However, the concentration and bioactivity of lunasin in soy food products remain to be established.

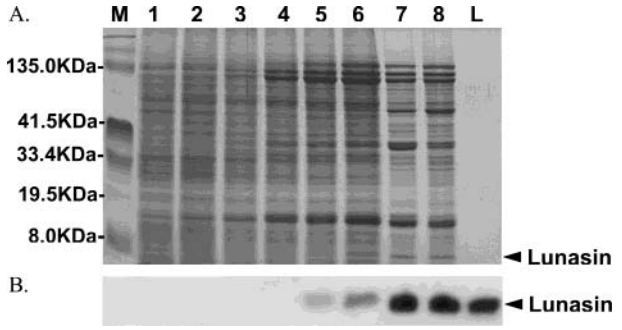


Figure 6. (**A**) Coomassie Blue stain and (**B**) Western blot of total protein extracts from soybean seeds collected at different stages of seed development. Soybean (cv Hwangkumkong) was grown in a greenhouse, the flowers were tagged carefully, and the pods were harvested at various weeks after flowering, which are indicated above the wells. Protein extracts were run on 15% Tris-HCl gels and each well contains 25 μg of proteins. Lane M is the prestained MW marker and lane L is 200 ng of synthetic lunasin.

Lunasin Expression in Developing Soybean Seed. The protein expression of lunasin in the developing soybean seed is not only important in understanding the native role of lunasin in the seed but also because of the widespread use of immature seeds in the human diet, such as "green soy" or Japanese "edamame". Lunasin peptide starts to appear at 5 weeks after flowering and remains in the mature seed (**Figure 6**). The presence of lunasin in mature soybean seeds is confirmed by the analysis of several soy varieties and soy proteins as shown above. In a separate study, we showed that lunasin mRNA appears at 3 weeks after flowering and is found only in the cotyledon and not in other tissues such as the leaf, stem, root, and pod (*22*).

DISCUSSION

Lunasin is a novel and promising chemopreventive agent that has been shown to suppress transformation of mammalian cells induced by chemical carcinogens and inhibits skin tumorigenesis in mice (*11*). In all of these experiments, synthetic lunasin was used and therefore there is no information on the efficacy of natural lunasin from soybean from which the *lunasin* gene was originally cloned in our lab (*6*, *7*). The high cost of producing synthetic lunasin makes it important to gather information on the efficacy of natural lunasin isolated from soybean as an abundant and relatively inexpensive source for animal and human studies.

Partially purified lunasin fractions suppress colony formation induced by the *ras*-oncogene in MCF-7 cells stably transfected with an inducible form of the oncogene. Colony formation is a measure of anchorage-independent cell growth, one of the characteristics of transformed cells. These lunasin fractions also inhibit histone acetylation when added in the presence of sodium butyrate, a deacetylase inhibitor that is known to increase the level of acetylated histones in the cell (*21*). The bioactivities of these fractions, which are impure, suggest that there are no components in soybean that antagonize lunasin's efficacy and there might even be compounds that enhance its efficacy.

The ability of natural soy lunasin to inhibit histone acetylation is an intriguing property in the light of the role played in carcinogenesis of reversible, covalent histone modifications, such as acetylation, phosphorylation, methylation, and their possible connection to DNA methylation (*23*). Preclinical and

clinical studies provide interesting evidence that part of the anticancer properties attributed to several bioactive food components may relate to DNA methylation patterns (*24*). Our data clearly showed that the global increase of H3-Ac in MCF-7 cells due to sodium butyrate is completely neutralized by the addition of soy lunasin. In a previous study, we showed that synthetic lunasin reduces H3 histone acetylation 50-fold in mouse fibroblast C3H cells and 3-fold in human breast cancer MCF-7 cells (*11*). Sodium butyrate, a well-studied anticancer agent that has shown inconsistent results in vivo (*25*, *26*), might affect carcinogenic pathways different from that of lunasin, because of its opposite effect on histone acetylation.

The varying levels of lunasin in four different soy proteins clearly demonstrate the effects of processing on lunasin levels. Their widespread use in the formulation of soy food products suggests the presence of lunasin in food products available to consumers. We established that synthetic lunasin in aqueous solution survives boiling for 5 min, as shown by detection on Western blot (unpublished observation). Analysis of lunasin levels in food products and establishing their bioactivities by in vitro assays, such as used here, and by animal studies remain to be determined.

The absence of an immunoreactive band in three commonly eaten beans (pinto, kidney, and mung) belonging to the *Phaseolus* family is unexpected, considering that lunasin may play a crucial role in seed development. The ability of lunasin to arrest cell division in mammalian cells when the *lunasin* gene is transfected and the exclusive presence of lunasin in the seed suggest that it might be an effector molecule that triggers mitotic arrest to initiate the second major stage of seed development when the parenchyma cells in the cotyledon stops dividing and enlarge (*27*, *28*). This is accompanied by endoreduplication of DNA without cell division and the massive biosynthesis of carbohydrates, lipids, and proteins that make seeds a very important part of the human diet. A more comprehensive screening of seeds taking into account the possible presence of lunasin homologues, the use of other extraction procedures, and the stage of seed development is needed. We reported recently the presence and characterization of lunasin in barley, the first report of its presence in a major cereal crop (*14*).

The presence of lunasin in the developing seed is relevant from the chemopreventive perspective because of the widespread use of green immature seeds in the human diet.

In summary, this is the first report on the bioactivities of lunasin fractions isolated from soybean and on the ability of natural soy lunasin to suppress transformation of mammalian cells by an oncogene. Our data support the concept that lunasin and possibly its homologues might be universal chemopreventive agents that contribute to the known anticancer properties of plant foods. The isolation of bioactive lunasin from soybean lays the foundation for the large-scale preparation of lunasin-enriched soy and other seeds for use in animal and human studies.

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LITERATURE CITED

(1) Greenwald, P.; Clifford, C. K.; Milner, J. A. *Eur. J. Cancer* **2001**, *37*, 948−965.
(2) Messina, M. J.; Persky, V.; Setchell, K. D. R. *Nutr. Cancer* **1994**, *21*, 113−131.
(3) Kennedy, A. R. *J. Nutr.* **1995**, *125*, 733S−743S.
(4) Kennedy, A. R. *Am. J. Clin. Nutr.* **1998**, *68*, 1406S−1412S.
(5) Revilleza, M. J.; Galvez, A. F.; Krenz, D. C.; de Lumen, B. O. *J. Agric. Food Chem.* **1996**, *44*, 2930−2935.
(6) Galvez, A. F.; Revilleza, M. J.; de Lumen, B. O. Plant Gene Register #PGR97-103. *Plant Physiol* **1997**, *114*, 1567,
(7) Galvez, A. F.; de Lumen, B. O. *Nature Biotech* **1999**, *17*, 495−500.
(8) DePinho, R. A. *Nature* **1998**, *391*, 533−536.
(9) Ruoslahti, E.; Pierschbacher, M. D. *Cell* **1986**, *44*, 517−518.
(10) Akiyama, S. K.; Olden, K.; Yamada, K. M. *Cancer Metastasis Rev.* **1995**, *14*, 173−189.
(11) Galvez, A. F.; Chen, N.; Macasieb, J.; de Lumen, B. O. *Cancer Res.* **2001**, *61*, 7473−7478.
(12) Odani, S.; Koide, T.; Ono, T. *J. Biol. Chem.* **1987**, *262*, 10502−10505.
(13) Spencer, D.; Higgins, T. J. V. Molecular aspects of seed protein biosynthesis. In *Commentaries in Plant Science;* Smith, H., Ed.; Pergamon Press: New York, 1981; Vol. 2, pp 175−189.
(14) Jeong, H. J.; Lam, Y.; de Lumen, B. O. *J. Agric. Food Chem.* **2002**, *50*, 5903−5908.
(15) Andersen, J. S.; Mann, M. *FEBS Lett.* **2000**, *480*, 25−31.
(16) Shevchenko, A.; Jensen, O. N.; Podtelejnikov, A. V.; Sagliocco, F.; Wilm, M.; Vorm, O.; Mortensen, P.; Boucherie, H.; Mann, M. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 14440−14445.
(17) Lam, Y.; de Lumen, B. O. Unpublished observation.
(18) Liu, H. S.; Scrable, H.; Villaret, D. B. Lieberman, M. A.; Stambrook, P. J. *Cancer Res.* **1992**, *52*, 983−989.
(19) Liu, H. S.; Chen, C. Y.; Lee, C. H.; Chou, Y. I. *Br. J. Cancer* **1998**, *77*, 1777−17786.
(20) Grunstein, M. *Nature* **1997**, *389*, 349−352.
(21) Candido, E. P.; Reeves, R.; Davie, J. R. *Cell* **1978**, *14*, 105−113.
(22) de Lumen, B. O.; Galvez, A. F.; Revilleza, J. M.; Krenz, D. C. In *Chemicals via Higher Plant Bioengineering, Advances in Experimental Medicine and Biology;* Shahidi, et al., Eds.; Kluwer Academic: New York, 1999; Vol. 464, pp 117−126.
(23) Cervoni, N.; Detich, N.; Seo, S. B.; Chakravarti, D.; Szyf, M. *J. Biol. Chem.* **2002**, *277*, 25026−25031.
(24) Anonymous. Executive Summary, Diet, DNA methylation processes and health workshop. NCI Division of Cancer Prevention, Aug 6−8, 2001, Bethesda, MD.
(25) Caderni, G.; Luceri, C.; De Filippo, C.; Salvadori, M.; Giannini, A.; Tessitore, L.; Dolara P. *Carcinogenesis* **2001**, *22*, 525−527.
(26) Freeman, H. J. *Gastroenterology* **1986**, *91*, 596−602.
(27) Schweizer, L.; Yerk-Davis, G. L.; Phillips, R. L.; Srienc, F.; Jones, R. J. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7070−7074.
(28) Spencer D.; Higgins, T. J. V. *Molecular aspects of seed protein biosynthesis. in Commentaries in Plant Science:* Smith, H., Ed.; Pergamon Press: New York, 1981; Vol. 2, pp 175−189.

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