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# Identification and Bioavailability of a Chromatin-Binding Peptide (Lunasin) from Korean Soybean

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**Lunasin is a novel peptide with great potential as a nontoxic chemopreventive drug. This compound might account for part of the anticancer effects reported from studies with soybean (*Glycine max*). We studied its isolation, purification and biological assay, and observed that both its band from soybean and one from synthetic lunasin were <5 kD in their molecular weights. Among all the crop varieties tested, only the soybean produced a lunasin band on our western blot. Levels of this peptide ranged from 0.045 mg per gram of seed for the Hanbatkong cultivar to 0.156 mg per gram seed for 'Poolunkong'. The effect on colony formation by lunasin from different soybean extracts was significantly higher than for either the positive control or the synthetic lunasin. When lunasin was present in those natural extracts, histone acetylation decreased 100% compared with cells that were treated with Na-butyrate.**

*Keywords*: chemopreventive, histone acetylation, lunasin, soybean

Cancer is a leading cause of mortality worldwide and, therefore, a major research focus has been its chemoprevention (Singh and Lippman, 1998a, 1998b). This desirable and important facet of biomedical research helps to provide a practical approach to identifying potentially useful inhibitors of cancer development (Sporn and Shu, 2000).

The human diet contains a larger number of potential cancer chemopreventive substances (Vogel et al., 1968). Low-molecular-weight inhibitors of proteolytic enzymes are widely distributed among plant species (Vogel et al., 1968; Richardson, 1977). They tend to accumulate in storage structures (Chen and Mitchell, 1973), such as tubers and seeds (Richardson, 1977). Diets rich in products from the soybean (*Glycine max*) are associated with lower cancer mortality rates, particularly for colon, breast, and prostate cancers (Messina et al., 1994; Setchell and Cassidy, 1999). Those components believed to be capable of suppressing carcinogenesis include the Bowman-Birk protease (BBI), inositol hexaphosphate, β-sitosterol, and isoflavones (Messina and Barnes, 1991).

Lunasin was isolated from soybean seeds and sequenced 14 years ago. Although a number of biological functions were proposed for it, none has yet been proven (Odani et al., 1987). Lunasin is a subunit of one of the soluble proteins known as 2S albumins, which are expressed during cell expansion, i.e., the endoreduplication phase (Galvez and Lumen, 1999). It is a unique 43-amino acid soybean peptide that contains, at its carboxyl end, nine Asp (D) residues, an Arg-Gly-Asp (RGD) cell adhesion motif, and a predicted helix with structural homology to a conserved region of the chromatin-binding proteins (Odani et al., 1987). The RGD motif allows tumor cells to attach to an extracellular matrix (Ruoslahti and Pierschbacher, 1986).

Transfection of the lunasin gene into mammalian cells leads to mitotic arrest and cell death, which is characterized by cell lysis and chromosome fragmentation (Ruoslahti and Pierschbacher, 1986; Akiyama et al., 1995). With its chromatin-binding property, lunasin could be a chemopreventive agent. Using chemically synthesized lunasin, Galvez et al. (2001) havedemonstrated both in-vitro and in-vivo chemopreventive properties of this unique peptide. When added exogenously to mouse fibroblast cells (C3H 10 T1/2), lunasin suppresses foci formation of the cells induced by chemical carcinogens such as 7,12-dimethylbenz[α]anthracene (DMBA), and methylcholanthrene (MCA). Furthermore, in the first whole-animal model, Galvez et al. (2001) showed that lunasin, when applied topically to mouse skin, inhibited tumorigenesis.

Here, we report partial purification and bioavailability of biologically active lunasin, a chromatin-binding peptide found in soybean.

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## MATERIALS AND METHODS

### Materials

We obtained mature seeds of various agronomic crops from the Andong Seed Supply Institute, Andong, Kyungpook, Korea. These included *G. max* (cv. Gyenoonkong Taekwnagkong, Sangjupoolunkong, Andonggumjungkong, and Hanbatkong); *Pisum sativum* var. *arvense*; *Phaseolus angularis* and *Phaseolus radiatus*; *Oryza sativa* L. cv. Ilpum and *O. sativa* var. *glutinosa*; *Panicum miliaceum*, and *Panicum dichotomiflorum*. The sources for our experimental supplies were the following: all electrophoresis chemicals from Bio-Rad; the protease inhibitor cocktail from Sigma; synthetic lunasin from American Peptide (after being chemically synthesized); and the primary antibody Zymyl R1 from Alfredo F. Gavez (FilGen BioScience, Inc.). All the other chemicals were of analytical regent grade from Sigma, Bio-Rad, and the Santa Cruz Co.

### Isolation and Identification of Lunasin

#### Isolation of Crude Protein

After the seeds were individually cracked and ground to a flour, 30-g samples were extracted with 100 mL of phosphate-buffered saline (PBS; pH 7.4) that was supplemented with fresh protease inhibitor cocktail. 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 was obtained by centrifuging the dialyzed protein extract at 12,000*g* for 30 min, then re-extracted once with 10 mL of the extraction buffer. The supernatants from the two extractions were combined and used for further purification. Protein concentrations were determined by Bradford assay.

#### Ion Exchange Column Chromatography

The crude protein extracts were further purified by ion exchange chromatography on Biogel resin AG 1-X4, with a mesh size of 100 to 200. The 50 cm × 50 cm column (packed height, 40 cm) was equilibrated with 0.1 M PBS buffer (pH 7.0). Approximately 100 mg of concentrated protein in this PBS buffer was applied, and the column was washed with 150 mL of equilibration buffer. Elution was carried out with a 0.7-M NaCl solution at 4°C.

#### Gel Electrophoresis

SDS-PAGE for our seed extracts was performed using 15% Tris-HCl ready gel, following the manufacturers instructions. Samples were diluted in Laemmli buffer and boiled for 5 min prior to loading. The gels were then stained with Coomassie brilliant blue and transblotted to PVDF membranes for western blot analysis.

#### Western Blot Analysis

The PVDF membrane containing the transferred protein was blocked for non-specific binding for 1 h in Blotto A solution (5% nonfat milk and 1% Tween 20 in tris-buffered saline). It was then washed with fresh changes of the 1% TBS-T solution (1% Tween 20 in tris-buffered saline), and incubated with the primary antibody Zymyl R1, at a 1:5000 dilution, in Blotto B solution (3% nonfat milk and 1% TBS-T) for 1 h. After washing, the membrane was incubated for 1 h with an anti-rabbit secondary antibody (1:3000 dilution) in the Blotto B solution. Following another washing, the membrane was prepared for detection using an enhanced chemiluminescence (ECL) kit (Amersham). The intensities of the bands were quantified using the software Chemi-Imager 4400 v. 5.5 (Alpha Innotech Corp.).

### Bioassay of Lunasin from Soybean

#### Colony Assay

The 2-12 cells (Liu et al, 1998) were obtained from ATCC (Manassas, VA, USA). After being cultured at a concentration of $1 \times 10^4$ cells/mL in plastic, six-well plates, they were grown on 0.6% soft agar media in a humidified 5%-$CO_2$ atmosphere at 37°C. This Dulbeccos modified eagle media (DMEM) was supplemented with 10% fetal bovine serum (FBS) and streptomycin. Plating efficiencies were determined from three plates that were seeded with a cell density equal to that for all the plates used in the colony assay; these cultures were terminated after 14 d. The various treatment concentrations of lunasin (from 100 µM to 10 nM), purified by ion-exchange column chromatography, were calculated by densitometry of the western Blot, then compared with a standard curve for synthesized lunasin. Each calculated dose was treated with media and 20 µL/mL IPTG for 35 µL of the total volume. Wells that contained transformed cells manifested colonies of about 0.5 mm after 10 to 14 d. These colonies were counted under a 40X microscope.

#### In-Vivo Acetylation Assay

MCF-7 cells (ATCC), produced in DMEM + 10%

FBS, were released from confluency and allowed to grow for 24 h. Afterward, the lunasin was identified in the extracts ion-exchange column chromatography. Following standard protocols (Upstate Biotechnology; Anti-acetylated Histone H3), 100 μM of lunasin and 5 mM Na-butyrate were added to the media. Using approximately 0.8 mg from the cells of the acid-extracted proteins, 15% Tris-HCl gel electrophoresis was performed, and blotting was done on Hybond-ECL membranes (Amersham). Immunoblot analysis was conducted (ECL Western Blot, Amersham) using 1:1000-diluted primary antibodies against tetra-acetylated H3 and 1:2000-diluted HRP-labeled anti-rabbit IgG secondary antibody. After a detection agent was applied to the membranes, they were immediately exposed to film for 1 min. Densitometer readings were taken on an autoradiograph (using the Bio-Rad Molecular Imaging System GS525 and Molecular Analyst software) to measure the levels of acetylated H3.

## RESULTS AND DISCUSSION

### Identification of Lunasin in Extracts from Various Crop Species

Lunasin was identified from eight different agronomic crops, using SDS-PAGE (Fig. 1A) and western blots (Fig. 1B). Only the soybean had a lunasin band on the western blot. The SDS-PAGE showed that the molecular weights for the majority of the crop proteins ranged from 30 to 200 kD, with the rest being 4 to 14 kD. The band sizes for the naturally occurring lunasin as well as that for synthetic lunasin were <5 kD range, as was expected.

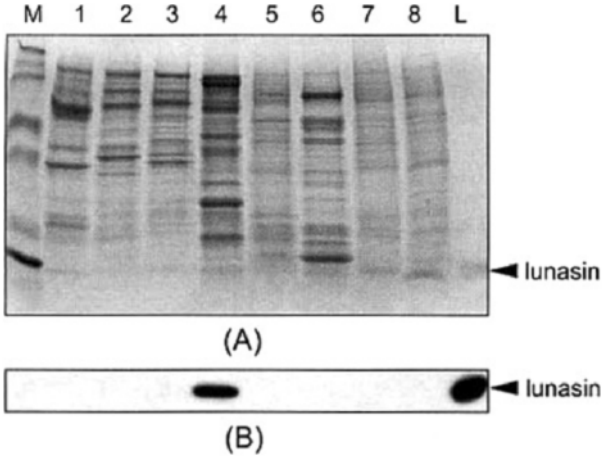


**Figure 1.** Identification of lunasin on SDS-PAGE (A) and western blot (B) of crude protein from various crops. Lane M, Marker; Lane 1, *P. sativum* var. *arvense*; Lane 2, *P. radiatus*; Lane 3, *P. angularis*; Line 4, *G. max* (cv. Hwangkumkong); Lane 5, *O. sativa* (cv. Ilpumbyeo); Lane 6, *O. sativa* var. *glutinosa* (cv. Sinsunchalbyo); Lane 7, *P. miliaceum*; Lane 8, *P. dichotomiflorum*; Lane L, Lunasin. Lanes 1 ~ 8 are 25 μg protein per well; Lane L is 250 ng of synthetic lunasin.

### Lunasin Content in Various Soybean Cultivars

Five soybean varieties were assayed for their lunasin contents, using SDS-PAGE (Fig. 2A) and western blots (Fig. 2B). All five showed bands on the latter. The levels of lunasin were determined by comparing individual quantities with a standard curve for a known concentration of synthetic lunasin. This method has a detection limit of approximately 15 ng lunasin, whereas the curve for signal intensity versus the actual amount of lunasin has a high correlation coefficient of 0.95. Concentrations ranged from 0.045 mg per gram seed for 'Andonggumjungkong' to 0.156 mg per gram seed for 'Sangjupoolunkong' (Table 1). In addition, 'Taekwangkong' had the highest lunasin content per g total protein.

### Purification of Lunasin from 'Taekwangkong' by Ion-Exchange Column Chromatography

To purify lunasin from the crude-protein extracts, we ran ion-exchange column chromatography with

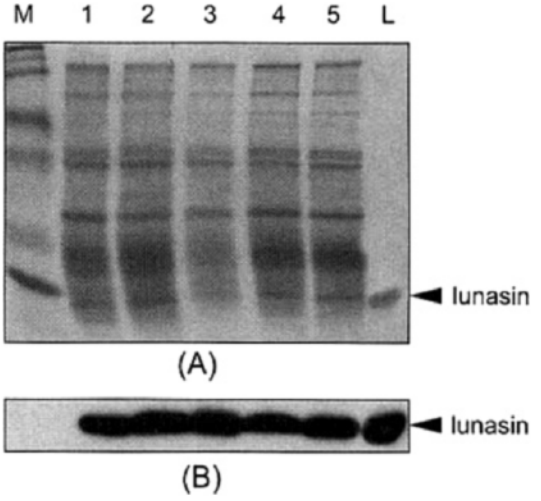


**Figure 2.** Identification of lunasin on SDS-PAGE (A) and western blot (B) of crude protein from several soybean cultivars Lane M, Marker; Lane 1, Gyenoonkong; Lane 2, Taekwangkong; Lane 3, Sangjupoolunkong; Line 4, Andonggumjungkong; Lane 5, Hanbatkong; Lane L, Lunasin. Lanes 1 ~ 5 are 25 μg protein per well; Lane L is 250 ng of synthetic lunasin.

**Table 1.** Lunasin content in crude protein from five soybean cultivars.

| Cultivar | g protein /mL | ng lunasin /μg protein | ng lunasin /mL | mg lunasin /g seed |
|---|---|---|---|---|
| Gyenoonkong | 16,997 | 3.75 | 63,738 | 0.085 |
| Taekwangkong | 12,303 | 5.33 | 65,574 | 0.132 |
| Sangjuoolunkong | 13,165 | 5.03 | 66,219 | 0.156 |
| Andonggumjungkong | 10,365 | 4.13 | 42,807 | 0.045 |
| Hanbatkong | 12,820 | 4.35 | 55,767 | 0.053 |

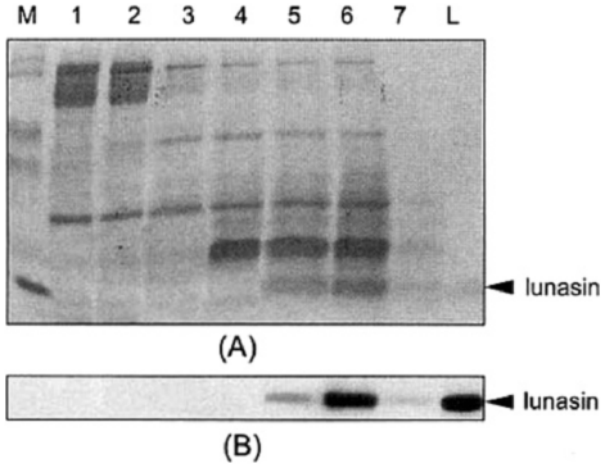


**Figure 3.** Identification of lunasin on SDS-PAGE (A) and western blot (B) of bound protein fraction eluted from ion-exchange column chromatography with 0.7 M NaCl phosphate buffer. Lane M, Marker; Lanes 1 through 7 are Fractions 1 to 7, respectively; Lane L, Lunasin. Lanes 1 ~ 7 are 10 μg protein per well; Lane L is 250 ng of synthetic lunasin.

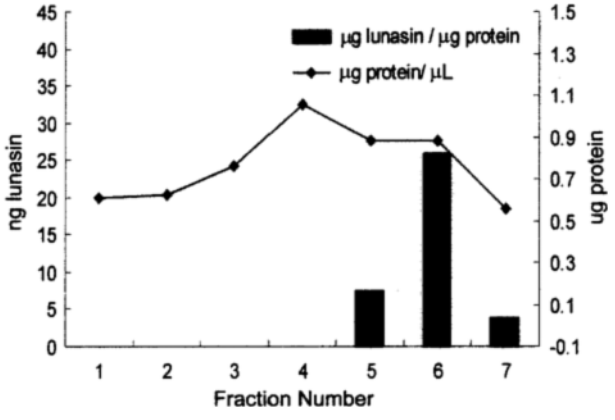


**Figure 4.** Amounts of lunasin on bound protein fractions eluted from ion-exchange column chromatography with 0.7 M NaCl phosphate buffer.

0.7 M NaCl. Most of the high-molecular-weight proteins were eluted at the 1st, 2nd, and 3rd fractions, while those with low molecular weights came off in the 4th, 5th, 6th, and 7th fractions (Fig. 3A). The latter three fractions, containing lunasin, were pooled (Fig. 3B).

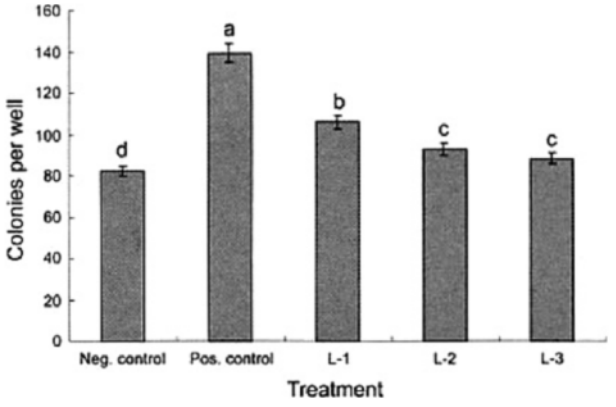


**Figure 5.** Colony formation of 2 to 12 cells in crude protein and purified lunasin. Neg. control, not treated with IPTG; Pos. control, treated with IPTG without lunasin; L-1, crude extract of soybean; L-2, purified fraction from ion-exchange column chromatography; L-3, synthetic lunasin. Combined means are compared using Duncan's multiple range test; treatment means flowed by the same letter are not significantly different.

When the 0.7-M NaCl phosphate buffer was used for eluting bound-protein fractions from 'Taekwangkong', lunasin was detected from the 5th, 6th, and 7th fractions (Fig. 4). In particular, Fraction 6 revealed the highest lunasin content per μg total protein.

Although the existence of lunasin had already been reported in soybeans, we refined the isolation technique by determining the proper concentration of NaCl phosphate buffer for use with ion-exchange column chromatography of soybean extracts. Furthermore, resolution on our western blots confirmed the enrichment of these fractions with respect to ~5-kD proteins such as lunasin.

## Suppression of Stably Ras-Transfected 2-12 Cells by Lunasin in Soybean Extracts

Colony assays are a simple and rapid quantitative tool for analyzing the oncogene-induced anchorage-independent growth of cells in culture. Our ion-

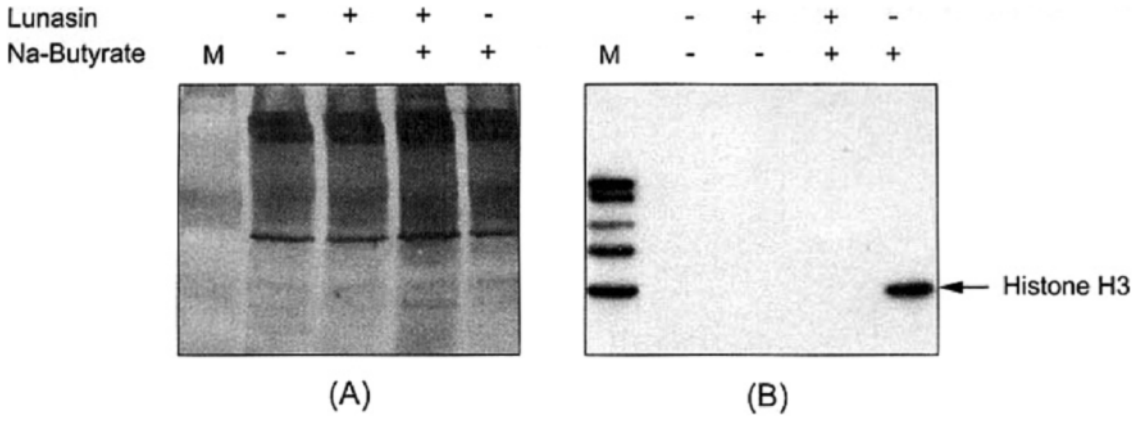


**Figure 6.** Lunasin inhibits in-vivo acetylation of histone H3 in the presence of Na-butyrate in MCF-7 cells. Silver stain (A) and immunoblot (B).

exchange column chromatography demonstrated that both the crude soybean lunasin and the purified lunasin were equally effective in inhibiting colony formation of stably ras-transfected 2-12 cells (Fig. 5). The number of colonies did not differ significantly among our five soybean cultivars, as shown by the Duncan's multiple range test.

These results suggest that the internalization of lunasin into cells could be the limiting step in its biological activities, which, however, remain to be established. A possible antimitotic effect in mammalian cells is consistent with the proposed biological role of lunasin as an effector molecule that inhibits mitosis, thereby allowing DNA endoreduplication and cell expansion to occur in the storage parenchyma cells during seed development. Lunasin, initially isolated from soybean cotyledons, is a novel and promising chemopreventive agent, with an epigenetic mechanism of action (Galvez et al., 2001).

### Acetylation of Histone H3 in-Vivo

To demonstrate the effect of lunasin on histone acetylation in-vivo, we treated MCF-7 cells with a histone deacetylase inhibitor, Na-butyrate. This was tested in the presence or absence of lunasin in the soybean extracts, using ion-exchange column chromatography. Histone acetylation and deacetylation are involved in chromatin remodeling, a process that has been associated with eukaryotic transcriptional regulatory mechanisms (Grunstein, 1997). Likewise, Na-butyrate is known to increase both histone acetylation and the level of those acetylated histones in cells (Candido et al., 1978). Silver staining and immunoblots revealed the activities of acid-extracted proteins in the presence of purified lunasin (Fig. 6). The level of tetra acetylated histone H3 was significantly reduced in Na-butyrate-treated cells when they were pretreated with 100 nM of purified lunasin. Histone acetylation by Na-butyrate increased in MCF-7 in the absence of lunasin. However, when lunasin was present, acetylation decreased 100% compared with cells treated with Na-butyrate.

The extent of the reduction in response to the immunopurified lunasin is comparable to that achieved using synthetic lunasin (Galvez et al., 2001). These results indicate that exogenous application of the lunasin peptide inhibits in-vivo acetylation of the N-terminal tails of histone H3 in mammalian cells. Therefore, we believe that lunasin has great potential as a nontoxic chemopreventive drug, and that its activity may account for part of the anticancer effects observed from soybean.

## ACKNOWLEDGMENTS

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