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Cancer-Preventive Peptide Lunasin from *Solanum nigrum* L. Inhibits Acetylation of Core Histones H3 and H4 and Phosphorylation of Retinoblastoma Protein (Rb)

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Lunasin, a unique 43 amino acid, 4.8 kDa cancer-chemopreventive peptide initially reported in soybean and now found in barley and wheat, has been shown to be cancer-chemopreventive in mammalian cells and in a skin cancer mouse model against oncogenes and chemical carcinogens. To identify bioactive components in traditional herbal medicines and in search for new sources of lunasin, we report here the properties of lunasin from *Solanum nigrum* L. (SNL), a plant indigenous to northeast Asia. Lunasin was screened in the crude extracts of five varieties of the medicinal plants of Solanaceae origin and seven other major herbal plants. An *in vitro* digestion stability assay for measuring bioavailability was carried out on SNL crude protein and autoclaved SNL using pepsin and pancreatin. A nonradioactive histone acetyltransferase (HAT) assay and HAT activity colorimetric assay were used to measure the inhibition of core histone acetylation. The inhibitory effect of lunasin on the phosphorylation of retinoblastoma protein (Rb) was determined by immunoblotting against phospho-Rb. Lunasin isolated from autoclaved SNL inhibited core histone H3 and H4 acetylation, the activities of the HATs, and the phosphorylation of the Rb protein. Lunasin in the crude protein and in the autoclaved crude protein was very stable to pepsin and pancreatin *in vitro* digestion, while the synthetic pure lunasin was digested at 2 min after the reaction. We conclude that lunasin is a bioactive and bioavailable component in SNL and that consumption of SNL may play an important role in cancer prevention.

KEYWORDS: Lunasin; core histone acetylation; histone acetyltransferases; retinoblastoma protein (Rb); phosphorylation; *Solanum nigrum* L. (SNL)

INTRODUCTION

In recent years, there has been a global trend toward the use of natural substances present in fruits, vegetables, oilseeds, and herbs as antioxidants and in functional foods (*1–3*). Several of these substances are believed to have potential value as cancer-chemopreventive or therapeutic agents. For instance, some vitamins and their derivatives have important biological roles related to cancer prevention and free-radical scavenging (*4*). *Solanum nigrum* L. (SNL) is a plant indigenous to northeast Asia that has been traditionally used in oriental medicine and is believed to have various biological activities (*5*).

Lunasin is a unique and novel 4.8 kDa cancer-preventive peptide from soy (*6*), barley (*7*), and wheat (*8*). It is 43 amino acids long and contains 8 aspartic acid residues in its carboxyl end preceded by a cell-adhesion motif Arg-Gly-Asp (RGD) (*9, 10*) 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 in a mouse model for skin cancer (*6, 7, 11–13*).

Histone acetylation and deacetylation have been associated with eukaryotic transcriptional regulatory mechanisms (*14*). Recent studies implicate alterations in chromatin structure by histone hyperacetylation/deacetylation as playing important roles in either the genesis or suppression of cancer. The role of histone hypoacetylation in cancer has been suggested in studies by Yang

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et al. (*15*), who demonstrated that the E1A oncoprotein stimulates proliferation by disrupting the growth-suppressive interactions of p300/CBP and p300/CBP-associated factor (p/CAF), both of which have been shown to have histone acetyltransferase (HAT) activity (*16*).

The 110 kDa retinoblastoma protein (Rb) is an important regulator of cell-cycle progression and differentiation (*17, 18*). Central to the role of the protein as a tumor suppressor is the ability of Rb to suppress inappropriate proliferation by arresting cells in the G1 phase of the cell cycle (*19, 20*). Inactivation of Rb can be achieved through multiple distinct mechanisms, including the direct loss of functional protein because of mutation, binding of oncoproteins of DNA tumor viruses, or overt phosphorylation of Rb, which inactivates its growth-suppressing function (*21–23*). It has been hypothesized that Rb must be phosphorylated and inactivated by cyclin-dependent kinase (CDK)/cyclin complexes in cells to allow for cell-cycle progression, and in tumor cells, this process is often deregulated (*21, 22*). Thus, amplification of the proto-oncogenes, CDK4 or cyclin D1, results in excessive phosphorylation and inactivation of Rb. Likewise, the loss of the tumor suppressor protein $p16^{ink4a}$, which normally serves to attenuate the activity of CDK4 and cyclin D1, results in deregulated phosphorylation and inactivation of Rb. It is thought that unphosphorylated (i.e., active) Rb prevents cellular proliferation by sequestering a host of factors and assembling complexes that inhibit cell-cycle progression (*23, 24*). Here, we tested whether lunasin is involved in Rb phosphorylation.

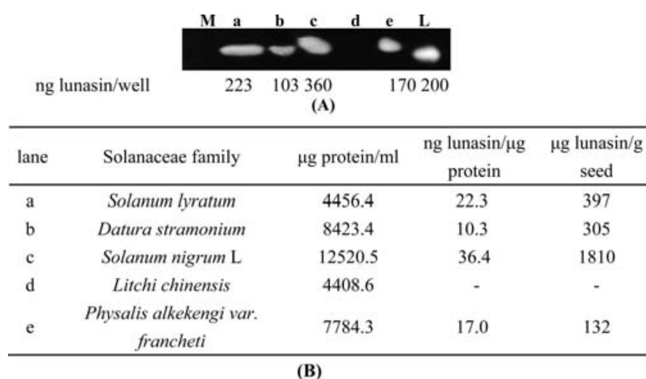
The affinity of lunasin for hypoacetylated chromatin suggests a role in chromatin modification, a process implicated in cell-cycle control, and in the role of tumor suppressors in carcinogenesis (*25*). We propose an epigenetic mechanism, whereby lunasin selectively kills cells that are being transformed or newly transformed by binding to deacetylated histones exposed by the inactivation of tumor suppressors that operate through histone acetylation−deacetylation (*12*). This leads to the inhibition of histone acetylation (*6*) and a disruption of the dynamics of histone acetylation−deacetylation. This is perceived as abnormal by the cell and eventually results in cell death.

To identify bioactive components in traditional herbal medicine and in the quest for other natural sources of lunasin, we report here the identification, purification, and biological properties of lunasin from SNL. We also account for the first time the ability of lunasin to inhibit Rb phosphorylation.

MATERIALS AND METHODS

Materials. *Reagents.* All electrophoresis chemicals were purchased from Bio-Rad Laboratories (Hercules, CA). Standard lunasin was synthesized by American Peptide Co. (Sunnyvale, CA). Polyclonal lunasin antibody was produced by Zymed, Inc. (Carlsbad, CA). The secondary antibody, goat antirabbit IgG-horseradish peroxidase (HRP) conjugated, for lunasin peptide was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa 488, the secondary antibody for immunostaining of lunasin was purchased from Invitrogen (Carlsbad, CA). Phospho-Rb (ser780) antibody was purchased from Cell Signaling Technology (Beverly, MA). p/CAF for core histone H4 acetylation was purchased from Upstate (Charlottesville, VA). Pepsin and pancreatin for the simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) digestion stability assay were purchased from Sigma (St. Louis, MO). Nonradioactive HAT assay kit and HAT activity colorimetric assay kit were purchased from Upstate (Charlottesville, VA) and BioVision (Mountain View, CA), respectively.

Seeds. Seeds of the herbal medical plants *Prunus armeniaca*, *Posralea corylifolia*, SNL, *Prunus persica*, *Litchi chinensis*, *Torreya nucifera*, *Ginkgo biloba*, *Thuja orientalis*, *Solanum lyratum*, *Datura stramonium*, and *Physalis alkekengi* var. *francheti* were obtained from the National Institute of Crop Science, Suwon, Gyunggido, South Korea.



lane	Solanaceae family	µg protein/ml	ng lunasin/µg protein	µg lunasin/g seed
a	*Solanum lyratum*	4456.4	22.3	397
b	*Datura stramonium*	8423.4	10.3	305
c	*Solanum nigrum* L	12520.5	36.4	1810
d	*Litchi chinensis*	4408.6	-	-
e	*Physalis alkekengi var. francheti*	7784.3	17.0	132

(B)

Figure 1. (**A**) Western blot of lunasin peptide from seeds of various medicinal plants. Lane M is the molecular-weight (MW) marker, and lane L is 200 ng of synthetic lunasin. Each well contains 10 μg of protein. (**B**) Contents of lunasin from various medicinal plants. Lunasin contents were quantified by Western blot using the software Un-SCAN-IT gel version 5.1 (Silk Scientific, Inc.).

Extraction and Western Blot of Lunasin Peptide. For the isolation of lunasin peptide from the various herbal medical plants, 1 g of seed flour was extracted with 4 mL of the distilled water by shaking for 24 h at 4 °C, and extracts were centrifuged at 15 000 rpm for 30 min 3 times. Each supernatant protein extract was used for subsequent experiments. Protein contents were determined using the Bradford assay (*26*). Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) of each extract was performed using 15% Tris-HCl ready gel (Bio-Rad, Hercules, CA), following the instructions of the manufacturer. Gels were transblotted onto polyvinylidene fluoride (PVDF) membranes for Western blot analysis. The membranes were 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 1:3000 dilution in Blotto B solution (3% nonfat milk and 1% Tween-20 in TBS) for 1 h. After washing, the membrane was incubated with an antirabbit secondary antibody at 1:3000 dilution in Blotto B solution for 1 h, washed again, and treated with the detection agent (Amersham Biosciences) and immediately developed in Polaroid film. Lunasin contents were quantified by Western blot using the software Un-SCAN-IT gel version 5.1 (Silk Scientific, Inc.).

Purification and Identification of Lunasin Peptide from SNL. A total of 10 g of SNL flour was extracted with 40 mL of the distilled water by shaking for 24 h at 4 °C, and extracts were centrifuged at 15 000 rpm for 30 min 3 times. The supernatants were dialyzed for 24 h at 4 °C against 1 L of distilled water using Spectra/Por 7 membrane [molecular-weight cut-off (MWCO) = 10 000], and the filtrate outside the bag was freeze-dried and redissolved in an appropriate volume of distilled water. The protein extracts were then purified by ion-exchange chromatography on Biogel resin AG1-X4, mesh size 100–200 (Bio-Rad Laboratories). The column was equilibrated with 0.1 M PBS buffer (pH 7.4) and about 100 mg of concentrated protein in PBS buffer was applied, and the column was washed with 150 mL of PBS buffer. The bound proteins were then eluted with 0.7 M NaCl in PBS buffer at 4 °C, dialyzed against distilled water, and freeze-dried. To further purify the extracts, 20 μL of filtrate was injected into high-performance liquid chromatography (HPLC) equipped with a C18 column (DELTA PAK, 15 μm, 300 A, 300 × 7.8 mm) equilibrated at ambient temperature and stabilized with the mobile phase (4:6 acetonitrile/water) at a flow rate of 2.5 mL/min for 12 min with the UV detector set at 295 nm. The SNL lunasin peak was identified by a comparison to the lunasin standard peak that appeared at a retention time of 4 min. The lunasin content of the sample peak was quantified by Western blot using the software Un-SCAN-IT gel version 5.1 (Silk Scientific, Inc.).

***In Vitro* Digestion with SGF and SIF of Lunasin in Crude Protein Extracted from SNL and Autoclaved SNL.** SGF and SIF consist of 3.2 mg/mL pepsin in 0.03 M NaCl at pH 1.2 and 10 mg/mL pancreatin in 0.05 M KH_2PO_4 at pH 7.5, respectively. Aliquots (200 μL) of SGF

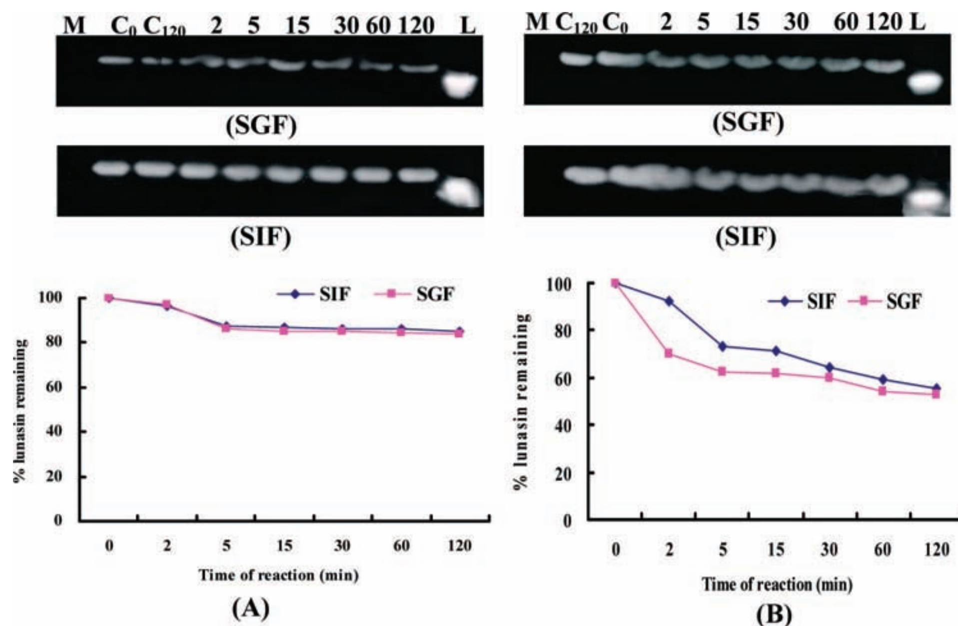


Figure 2. (**A** and **B**) Western blot of digests and plot of digestion time and percent of lunasin remaining after *in vitro* digestion with SGF and SIF of lunasin in SNL crude protein (**A**) and autoclaved SNL protein (**B**). The numbers above the blot indicate the time (in minutes) of the reaction with SGF and SIF. C_0 and C_{120} indicate the control not treated with SGF and SIF at 0 and 120 min. Lane L is synthetic lunasin (200 ng). Each graph represents the percent of lunasin remaining according to the reaction periods with the pepsin (SGF) and pancreatin (SIF). Lunasin contents were quantified by Western blot using the software Un-SCAN-IT gel version 5.1 (Silk Scientific, Inc.).

and aliquots (64 μL) of SIF were placed in 1.5 mL microcentrifuge tubes and incubated in a water bath at 37 °C, respectively. A total of 10 μL of crude and autoclaved protein from SNL (5 mg/ml) was added to each SGF and SIF vial to start the reaction, respectively. The ratio of pepsin and pancreatin to protein substrate was about 13:1 (w/w). At intervals of 0, 2, 5, 15, 30, 60, and 120 min, 50 μL of 1 N NaOH for SGF was added to each vial to stop the reaction. A total of 70 μL of 6× Laemmli buffer for SIF was added to each vial, and the reaction was immediately stopped by placing each vial in a boiling water bath for 10 min.

Inhibition of HAT Activities by Lunasin Purified from Autoclaved SNL. As HATs, yGCN5 uses acetyl-CoA to acetylate Lys-14 of H3 core histone protein, while p/CAF acetylates Lys-8 and Lys-16 of H4 histone to a lesser extent and generates acetylated histone and CoA (*26*−*28*). yGCN5 was prepared according to Jeong et al. (*29*). Inhibitions of HATs (yGCN5 for H3 and p/CAF for H4) activity by lunasin purified from autoclaved SNL were done using a HAT activity colorimetric assay kit (BioVision) according to the protocol of the manufacturer, with some modifications. A total of 8 μg of yGCN5/40 μL of distilled water or 500 ng of p/CAF/40 of μL distilled water was added to each well in a 96-well plate. Then, 65 μL of assay mix (50 μL of 2× HAT assay buffer, 5 μL of HAT substrate, 5 μL of NADH-generating enzyme) was added to each well, and the 96-well plates were incubated at 37 °C for 4 h. The plates were then read on a plate reader at a 440 nm wavelength.

Inhibition of Histone H3 and H4 Acetylation by Lunasin Purified from Autoclaved SNL. The inhibition assay of histone H3 and H4 acetylation was evaluated by the protocol as described in Jeong et al. (*29*), with some modification. A total of 8 μg of yGCN5 and 500 ng of p/CAF were used for acetylating H3 and H4, respectively. Lunasin (1000 nM) purified from autoclaved SNL was used in this assay.

Immunostaining of Lunasin in the Internalization Experiment. Immunostaining of lunasin in the internalization experiment was assessed by the protocol as described in ref *6*.

Western Blot of Phospho-Rb Protein in NIH 3T3 Cells Treated with Na−Butyrate. NIH 3T3 cells were plated in 6-well plates at a density of 5×10^3 cells per well and then incubated for 24 h. Lunasin (1 μM) purified from SNL and Na−butyrate (5 mM) were added and allowed to react for 24 h. Protein enriched for Rb was acid-extracted (standard protocols of Upstate Biotechnology) from the cells. A total of 25 μg of acid-extracted protein was separated using 15% Tris-HCl gel and transferred to a PVDF membrane. The membrane was blocked with blocking buffer (1× TBS and 0.1% Tween-20 with 5% w/v nonfat dry milk) for 1 h at room temperature and then washed 3 times for 5 min each with TBS/T buffer (1× TBS and 0.1% Tween-20). After the blocking, the membrane was incubated with primary antibody (1:1000) in the primary antibody dilution buffer [1× TBS and 0.1% Tween-20 with 5% bovine serum albumin (BSA)] with gentle agitation overnight at 4 °C. The membrane was then washed with TBS/T and incubated with a HRP-conjugated secondary antibody (1:2000) in the blocking buffer with gentle agitation for 1 h at room temperature. The membrane underwent additional washing, and the immunoreactive protein was visualized using the chemiluminescent reagent ECL (Santa Cruz Biotechnology, Santa Cruz, CA) according to the protocol of the manufacturer.

RESULTS

Lunasin Contents. In search for other natural sources of lunasin from the herbal medical plants, we report here for the first time the identification and biocharacterization of lunasin purified from SNL, a herbal medical plant used in northeast Asia. Lunasin was not detected in *P. armeniaca*, *P. corylifolia*, *P. persica*, *L. chinensis*, *T. nucifera*, *G. biloba*, and *T. orientalis*, while it was detected in the Solanaceae family. The presence of lunasin in the Solanaceae family and its absence in *L. chinensis* are shown in **Figure 1**. The contents of lunasin in *S. lyratum*, *D. stramonium*, SNL, and *P. alkekengi* var. *francheti* are 397, 305, 1810, and 132 μg of lunasin/g of seed, respectively. Lunasin is found only in the seeds and not in other tissues, such as the leaf, shoot, and root of SNL (not shown).

***In Vitro* Digestion of SNL Crude Protein with SGF and SIF.** Synthetic pure lunasin is completely digested by SGF and SIF after 2 min of reaction time (not shown), while lunasin in SNL crude protein is protected from both enzymes. After digestion for up to 120 min by SGF and SIF, lunasin remains at approximately 85% of the original (**Figure 2A**). Lunasin in autoclaved crude protein is also protected from both enzymes, remaining up to 56 and 53% of the original after 120 min of

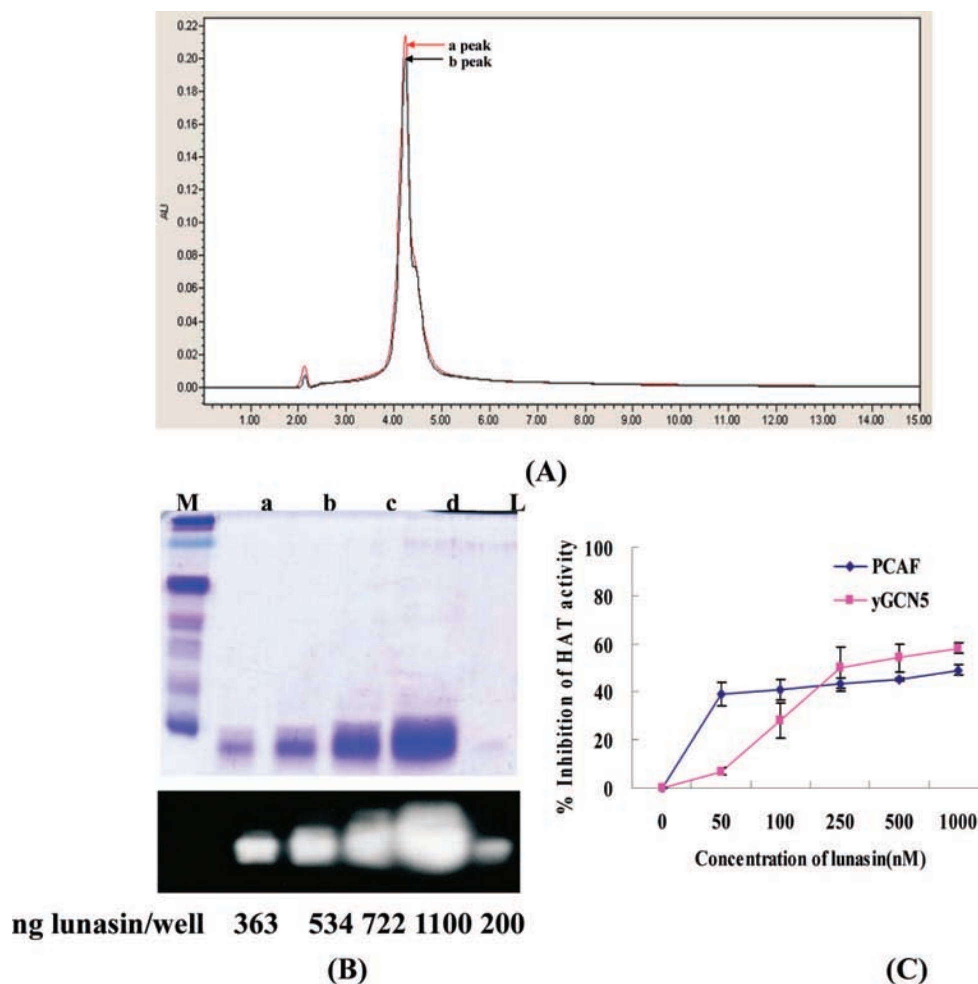


Figure 3. (**A**) Peaks of synthetic lunasin (a peak) and lunasin (b peak) purified from SNL by HPLC. (**B**) Commasie Blue staining and Western blot of lunasin purified from autoclaved SNL by HPLC. Lane M is the MW markers, and lanes a−d are loaded with 0.37, 0.54, 0.78, and 1.13 μg of protein per well. (**C**) Plot of the percent inhibition of HAT enzyme activities of yGCN5 for H3 and p/CAF for H4 core histones with increasing concentrations of lunasin. Lunasin contents were quantified by Western blot using the software Un-SCAN-IT gel version 5.1 (Silk Scientific, Inc.).

digestion by SIF and SGF, respectively (**Figure 2B**). This suggests that naturally present protease inhibitors in SNL seed protect lunasin from digestion by pancreatin and pepsin.

Inhibition of HAT Activities by Lunasin Purified from Autoclaved SNL. The lunasin purified by HPLC from autoclaved SNL was used in the inhibition assay of HAT activity to determine the effects of dose using the HAT activity colorimetric assay kit (**Figure 3C**). **Figure 3A** shows the peaks of synthetic lunasin and lunasin purified from autoclaved SNL with HPLC. The peak of purified lunasin was in accordance with that of synthetic lunasin. **Figure 3B** shows Coommasie Blue staining and Western blot of HPLC-purified lunasin according to different loadings per well. The purity of lunasin is calculated to be above 95% (protein/protein). The inhibitory effects of purified lunasin at increasing doses are shown in **Figure 3C** for yGCN5 and p/CAF, which acetylate histone H3 and H4, respectively. Purified lunasin inhibits yGCN5 activity by 7 and 58% at 50 and 1000 nM, respectively, while the p/CAF activity is inhibited by 39 and 49% at 50 and 1000 nM. yGCN5 activity is inhibited by increasing concentrations of lunasin until 250 nM, beyond which there is a leveling off, while p/CAF activity is inhibited at 50 nM lunasin and levels off. The greater sensitivity of p/CAF to inhibition at low lunasin concentrations is likely due to its specificity for acetylation of two Lys residues in histone H4 compared to that of yGCN5 that requires only one residue in histone H3.

Inhibition of Histone H3 and H4 Acetylation by Lunasin Purified from Autoclaved SNL. Parts **A** and **B** of **Figure 4** show the inhibitory effects of 1 μM lunasin purified from autoclaved SNL on histone H3 and H4 acetylation. In the absence of lunasin, yGCN5 produced 95 ng of acetylated H3, while p/CAF produced 9.1 ng of acetylated H4. In the presence of 1000 nM lunasin purified from autoclaved SNL, 21 ng of acetylated H3 and 2.5 ng of acetylated H4 are produced, which translate into the inhibition of H3 acetylation by 78% and H4 acetylation by 73%. An equal concentration of synthetic lunasin produced identical effects, demonstrating that there is no difference in the histone acetylation inhibitory effects of histone acetylation by natural and synthetic lunasin.

To demonstrate further the ability of lunasin purified from autoclaved SNL to inhibit histone acetylation, immunostaining with antiacetyl lysine in NIH 3T3 cells was carried out. **Figure 4C** shows the inhibition of histone acetylation by lunasin from autoclaved SNL in normal mouse fibroblast NIH 3T3 cells 24 h after treatment with Na−butyrate, a histone deacetylase (HDAC) inhibitor (5 mM), and purified lunasin (1 μM). Without the addition of Na−butyrate and purified lunasin (i.e., negative control), acetylated histones are not detected in the nuclei stained with DAPI, suggesting that the histones in these cells are predominantly nonacetylated under these conditions. When treated with Na−butyrate alone (i.e., positive control), acetylated histones are detected (white arrows), while the addition of both Na−butyrate

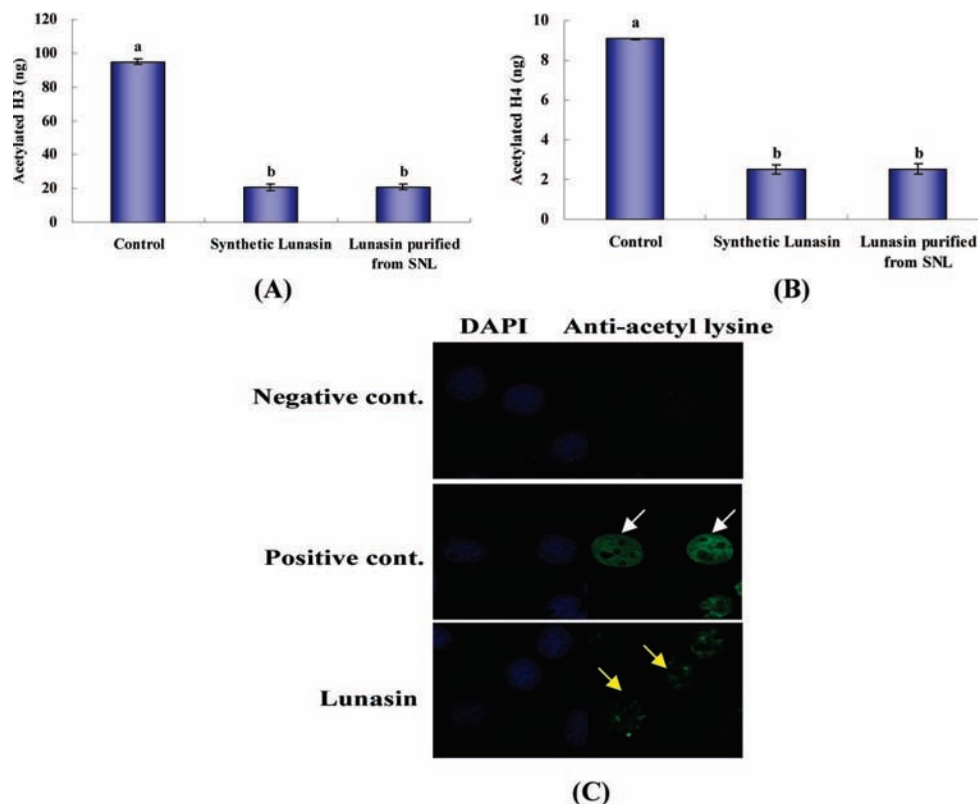


Figure 4. Inhibition of core histone H3 acetylation (**A**) and H4 acetylation (**B**) by synthetic lunasin and lunasin (1 μM) purified from autoclaved SNL. (**C**) Immunostaining with antiacetyl lysine to demonstrate the inhibition of histone acetylation by lunasin purified from autoclaved SNL 24 h after treatment with combinations of Na−butyrate (5 mM) and purified lunasin (1 μM). Nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI).

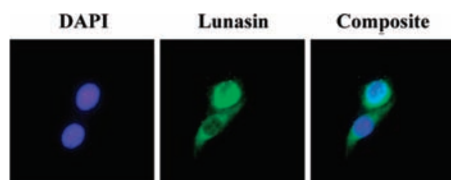
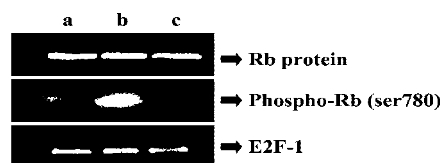


Figure 5. Internalization of lunasin purified from autoclaved SNL in mouse fibroblast NIH 3T3 cells 20 h after exogenous addition.

and 1 μM lunasin results in a significant reduction in the signal for acetylated histones (yellow arrows). Similar results have been observed with soy and barley lunasin (*6, 7*).

Internalization of Lunasin Purified from Autoclaved SNL in Normal Mouse Fibroblast NIH 3T3 Cells. A unique property of lunasin is its ability to internalize into mammalian cells upon exogenous addition and to localize mostly in the nucleus (*6, 12, 13*). The lunasin purified from autoclaved SNL (green fluorescent stain) internalizes into the mouse fibroblast NIH 3T3 cells and localizes in the nucleus (blue DAPI stain) within 20 h of administration (**Figure 5**). The bright green fluorescent stained lunasin gradually concentrates in the nucleus. Full penetration of lunasin into the nucleus is marked by the turning of the blue DAPI-stained nuclei into a faint green color.

Inhibition of the Phosphorylation of Rb by Lunasin from Autoclaved SNL. The phosphorylation of Ser780 in Rb is caused by cyclin D1, which results in the inactivation of Rb (*23*). Na−butyrate is known to strongly stimulate expression of cyclin D1 (*30, 31*). **Figure 6** shows that 1 μM lunasin from autoclaved SNL completely inhibits the phosphorylation (Ser780) of Rb, whereas the expression levels of Rb protein and E2F-1 are not affected (lane c). The addition of Na−butyrate alone leads to a higher level of phosphorylation compared to the control (lane b). This increase is completely nullified by the addition of lunasin.



lane a : NIH3T3

lane b : NIH3T3 + 5mM Na-butyrate

lane c : NIH3T3 + 5mM Na-butyrate + 1μM lunasin purified from autoclaved SNL

Figure 6. Inhibition of the phosphorylation of Rb at 46 h after treatment of NIH 3T3 cells with 1 μM lunasin purified from autoclaved SNL and 5 mM Na−butyrate. Lanes a−c were loaded with 25 μg of protein.

DISCUSSION

We isolated and characterized lunasin from soybean (*32*) and barley (*7*) previously. An identical peptide from soy was initially identified and isolated and was proposed to possess important biological functions because of its unique sequence, but none were demonstrated (*33*). In search for other natural sources of lunasin especially from medicinal plants, we report here the identification and bioassay of lunasin purified from SNL.

Establishment of the existence of lunasin in SNL is highly significant because it has been traditionally used in oriental medicines, is believed to have various biological activities (*5*), and suggests that lunasin might be a universal chemopreventive agent in plant seeds. Lunasin was proposed to have a role in arresting cell division to allow DNA endoreduplication without cell division during seed development (*13, 34*), implying the widespread presence of lunasin in angiosperm seeds, although our initial screening of a limited number of seeds suggests

otherwise (*32*). We have screened for lunasin in the crude extracts of five varieties of the medicinal plants of Solanaceae origin and seven other major herbal plants. Lunasin is present in all of the Solanaceae family, except *L. chinense*, and none were found in the other major herbal plants. Lunasin is lowest in *P. alkekengi* var. *francheti* (132 μg of lunasin/g of seed) and highest in SNL (1810 μg of lunasin/g of seed). The presence of lunasin in Solanaceae offers another source of this cancer-preventive peptide in a plant that is already used as a herbal medicine.

We have purified lunasin from SNL by membrane dialysis, ion-exchange column chromatography, and reverse-phase HPLC to yield lunasin at >95% purity. Therefore, these methods offer means to isolate fully functional lunasin from natural sources that can be used for chemoprevention in large-scale animal studies and ultimately for human subjects.

The inhibition of histone acetylation, HAT activities, and Rb phosphorylation has been confirmed with the lunasin extracted and purified from autoclaved crude protein from SNL. The inhibitory effect of lunasin on histone acetylation is relevant to the proposed epigenetic mechanism for the cancer-preventive property of lunasin and for the postulated role of lunasin in seed development (*6, 12, 13, 32, 34*).

The inhibitory effect of lunasin on Rb phosphorylation (Ser780) induced by cyclin D1 possibly may affect the cell-cycle control pathway, especially G1/S arrest, resulting in keeping the core histone deacetylated and inhibiting abnormal cell growth. Here, we suggest that lunasin binds to hypoacetylated histone and inhibits HAT activities, histone acetylation, and phosphorylation of Rb. In **Figures 4C** and **6**, when treated with only Na−butyrate, Rb phosphorylation and histone acetylation occur, while when treated with Na−butyrate and lunasin together, both processes are inhibited. This further suggests that lunasin inhibits the acetylation of the core histone by inhibiting Rb phosphorylation (Ser780) induced by cyclin D1.

Oral administration is an important feature of an ideal cancer-preventive agent. Because lunasin is a peptide, it is crucial to establish whether it survives digestion, is absorbed, remains intact, and hence, can be developed into a widely used cancer-preventive pill (*29*). Our findings on the *in vitro* digestibility of lunasin present in the crude SNL extract indicate that lunasin evidently ends up in the tissues intact and bioactive. It is very likely that protease inhibitors naturally present in SNL seeds protect lunasin from digestion.

The inhibition of core histone acetylation by lunasin from SNL suggests that SNL, a major herbal medicine plant, is another source of lunasin besides soybean, barley, and wheat. In addition, lunasin from SNL is very stable in *in vitro* digestibility and to heat. Thus, consumption of the herbal medicine using SNL may play a significant role in cancer prevention.

The internalization of lunasin from SNL into the nucleus observed by immunostaining is consistent with our previous data obtained in soybean and barley lunasin and further supports our postulate that lunasin exerts its anticancer activity by interacting with deacetylated histones in the nucleus. How lunasin crosses the nuclear membrane and localizes in the nucleus is another significant and interesting aspect of this peptide that needs to be investigated.

ABBREVIATIONS USED

SNL, *Solanum nigrum* L.; HAT, histone acetyltransferase; Rb, retinoblastoma protein; p/CAF, p300/CBP-associated factor; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; HDAC, histone deacetylase; CDK, cyclin-dependent kinase.

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