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The Anticancer Properties of Lunasin Peptide from Aged Callus Induced by the Soybean Tissue Culture

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Abstract - Lunasin is small subunit peptide of coded from Gm2S-1 gene in soybean. It has been previously demonstrated that lunasin is a novel and promising cancer preventive peptide. Lunasin peptide is found only in the seed and not other tissues. And lunasin peptide starts to appear at 5 weeks after flowering and remains in the mature seed. We report here firstly lunasin peptide identified from soybean callus induced by the tissue culture and demonstrate its anticancer properties. The lunasin was identified and purified from soybean callus aged for 6 months. The callus lunasin (1μM) inhibited the acetylation of histone H3 and H4 by 58.8% and 56.5%, respectively. And it fully inhibited foci formation compared to the values of the positive control (no lunasin) and negative control (no MCA). Purified lunasin was able to internalize into the cell and localized in the nucleus.

Key words - Lunasin, Gm2S-1, Embryo culture, Histone acetylation

Introduction

Lunasin, a 43-amino acid peptide naturally present in soy protein, has been found to suppress transformation of mammalian cells induced by carcinogens and viral oncogenes E1A and RAS (de Lumen, 2005; Galvez and de Lumen, 1997; Galvez *et al.*, 2001; Jeong *et al.*, 2003; Lam *et al.*, 2003). In the first animal model, lunasin, applied topically, reduces skin tumor formation in mice (Galvez *et al.*, 2001). Lunasin is found in the 2S soybean albumin fraction. It contains a carboxyl end of nine aspartic acids residues, preceded by a cell adhesion motif (RGD), and a predicted helical region that has structural homology to chromatin binding proteins (de Lumen, 2005; Galvez and de Lumen, 1997; Galvez *et al.*, 2001).

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 (de Lumen *et al.*, 1999). In our separate study, we showed that lunasin peptide was found only in the seed and not other tissues. And lunasin peptide starts to appear at 5 weeks after flowering and remains in the mature seed (Lam *et al.*, 2003).

Lunasin has been shown to inhibit core histone acetylation, by binding to non-acetylated H3 and H4 histones (Galvez *et al.*, 2001), an epigenetic mechanism believed to be responsible for the anti-carcinogenic property of this chromatin-binding peptide. Histone acetylation and deacetylation have been associated with eukaryotic transcriptional regulatory mechanisms (Shogren-Knaak *et al.*, 2006). The affinity of lunasin for hypoacetylated chromatin suggests its role in chromatin modification, a process implicated in cell cycle control and in the role of tumor suppressors of carcinogenesis (Yasui *et al.*, 2003). Since lunasin inhibits core H3 and H4 histone acetylation in mammalian cells (Lam *et al.*, 2003), we have postulated an epigenetic mechanism whereby lunasin selectively kills cells that are being transformed or newly transformed by disrupting the dynamics of histone acetylation-deacetylation triggered by the inactivation of tumor suppressors that operate through histone acetylation-deacetylation (de Lumen, 2003).

Although we have studied the cancer chemopreventive properties of synthetic or natural lunasin from soybean seed, there has not been a report on lunasin properties from soybean callus. In this report, we tried producing a lunasin in soybean callus and demonstrated its cancer chemopreventive properties by inhibitory effect of histone acetylation and foci formation in chemical induced mammalian cells.

Materials and Methods

Callus induction by soybean tissue culture

Soybean callus was induced by the tissue culture of the matured

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soybean seeds (*Glycine max cv. Taekwangkong*) on a medium composed of Murashige and Skoog (MS) inorganic elements (Murashige and Skoog, 1962). MS medium was adjusted with 1 N NaOH or HCl to pH 5.7, gelled with 0.8% agar before autoclaving for 15 min at 1.2kg/㎠. Callus samples were collected after 2 weeks, 3 months and 6 months without the sub-culture, respectively.

Protein extraction and purification

Ten grams of soybean callus were extracted with 50 mL of phosphate-buffered saline (0.1M PBS, pH 7.0) supplemented with fresh protease inhibitor cocktail (Sigma). The mixture was centrifuged at 12,000 × g for 30 min and the supernatant protein extract was used for subsequent experiments. Callus protein was purified further by concentrating with YM-10 and YM-3 Microcon centrifugal filters (Millipore Corp). Protein content was determined using the Bradford assay (Bradford, 1976).

Chemicals

All digestion reagents were purchased from Sigma and all electrophoresis chemicals were purchased from Bio-Rad Laboratories. Synthetic lunasin (Synpep, Inc) was used as standards. A lunasin polyclonal antibody against the carboxyl epitope (CEKHIMEKIQGRGDDDDD) was custom produced (Zymed, Inc., called Zymed R1) and provided by Filgen Biosciences, Inc. Secondary antibody was purchased from Santa Cruz Biotechnology.

Gel electrophoresis

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

Western blot

An immune-blot PVDF membrane was prepared for transfer by soaking in 100% methanol for 15 sec. The proteins on SDS-PAGE gel were transblotted to the membrane for 90 min. at 300 mA, 100 V. Upon completion of transfer, the non-specific binding sites were blocked by immersing the membrane for 1 hour in 5% non-fat 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 as the primary antibody with 3% nonfat-milk in TBS-1T for 1 hour and then washed with fresh changes of the TBS-1T at room temperature. The membrane was then incubated using anti-rabbit streptavidin HRP as the secondary antibody with 3% nonfat-milk in TBS-1T for 1 h and subsequently washed with fresh changes of the TBS-1T at room temperature. The primary antibody of lunasin was diluted at a ratio of 1:5,000 and secondary antibody was diluted at a ratio of 1:5,000. The ECL western blotting detection agent (RPN2106, Amershim holding Inc) was applied to the membranes and immediately exposed to Polaroid film.

Preparation of yGCN5

The catalytic domain of yGCN5 was recombinantly expressed as described by Tanner *et. al.* (Tanner *et al.*, 2000) and purified from bacteria according to Kim et al. (Kim *et al.*, 2000) with some modifications. BL21 cell was used to express yGCN5 and the enzyme was extracted with distilled water and dialyzed with Spectra/Por Membrane (MWCO: 50,000) (Spectrum Laboratories Inc.) at 4℃ for 24 h and subjected to Ni-NTA Chelating Agrose CL-60 (Peptron, Inc.) ion-exchange chromatography eluted with 400mM imidazole gradient. Purified yGCN5 (assessed by SDS-PAGE) was pooled and stored at -20℃ until use. Protein concentrations were determined by Bradford method.

Spectrophotometric determination of yGCN5 activity

The reaction mixtures of yGCN5 HAT assay contained 0.2mM NAD, 0.2mM thiamine pyrophosphate (TPP), 5mM MgCl2, 1mM DTT, 2.4mM a-ketoglutarate, 63mM acetyl-CoA, 60mM histone H3 peptide, 0.07 units of a-ketoglutarate dehydrogenase (1 unit of dehydrogenases is defined by the manufacturer to be the conversion of 1.0 mmol of b-NAD to b-NADH per minute at pH 7.4 at 30℃ in the presence of saturating levels of coenzyme A), 350nM yGCN5, 100mM sodium acetate, 50mM Bis-Tris, and 50mM Tris (TBA buffer), pH 7.5, in a total volume of 300mL, unless otherwise noted. A tenfold stock solution containing NAD, TPP, $MgCl_2$, DTT, and a ten-fold stock solution of α-ketoglutarate in 20mM Hepes (pH 7.5) were prepared fresh daily. All assay except yGCN5 were incubated at 25℃ for 5 min and the reaction was initiated by the addition of yGCN5. The rates were analyzed continuously for 5 min by measuring NADH production at 340nm ($\in \frac{NA}{340} = 6230\ M^{-1}\ cm^{-1}$).

Core histone acetylation assay

The histone acetyl transferase (HAT) yGCN5 uses acetyl-CoA to

acetylate Lys-14 of H3 core histone protein while p300/CBP associated factor (PCAF) acetylates Lys-8 and Lys-16 of H4 histone to a lesser extent and generates acetylated histone and CoA (Kim *et al.*, 2000). Non-radioactive HAT assays were done using a HAT assay kit (Upstate Biotechnology, Inc.) according to the manufacturer' s protocol with some modifications. PCAF for HAT assay of histone H4 peptide was purchased from Upstate Biotechnology. Purified yGCN5 for H3 or PCAF for H4 was mixed with 100μM acetyl-CoA and 1×HAT assay buffer and incubated in ELISA assay plate (streptavidin-coated strip plate) precoated with 100 ng histone H3 or H4 and purified lunasin for 30 min. After washing with TBS, HAT assay was initiated by adding 50μl HAT reaction cocktail (10μl 5×HAT assay buffer, 10μl of 500μM acetyl-CoA, 1.5μg yGCN5 for H3 or 100ng PCAF for H4) and acetylated histones were detected using an anti-acetyl-lysine rabbit polyclonal antibody followed by the horseradish peroxidase-based colorimetric assay. The strip plate was read on a plate reader at wavelengths of 450 and 570nm. Acetylated histone H3 or H4 peptides in the samples were determined by comparison with standard curves.

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 20h at 37℃, and then the test samples were added. After 4h, the chemical carcinogen (1.5μg/mL of MCA) was added to the medium. MCA was dissolved with acetone. Acetone instead of MCA was added to negative control. Cells were exposed to the carcinogen for 20h 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.

Cell immunostaining assay

Sterilized glass cover slips were placed in 6-well plates that were then plated with NIH 3T3 cells (30,000 cells per well). Cells were stabilized by incubation for 24 hour at 37℃, and callus lunasin was added to each well to a final concentration of 10μM. The plates were incubated at 37℃. After 18h, 2% formaldehyde was added to fix the cells. PBS/FBS (PBS with 10% fetal bovine serum) was then added to block non-specific binding. The primary antibody against lunasin was diluted at a ratio of 1:250 with 0.1% saponin in PBS/FBS solution. The secondary antibody, Alexa-Flour 488 goat anti-rabbit IgG, was used against lunasin. Once the secondary antibody incubation was done, cover slips were then washed and inverted onto a drop of antifade mounting medium (Sigma). Mounted cover slips were viewed under a fluorescence microscope using a 60×oil immersion objective. The excitation wavelengths for DAPI were 359nm, and 494nm for lunasin.

Results and Discussion

Lunasin induced in aged soybean callus

Lunasin is small subunit peptide of coded from Gm2S-1 gene in soybean. The Gm2S-1 transcript is detected from the beginning of 3 weeks after flowering and persists up to 7 weeks after flowering (late maturation) but is completely gone in the mature seed (Galvez *et al.*, 1997). Lunasin peptide was known to presence in only plant seeds. Therefore, the isolation of natural lunasin was depended on plant seeds of the soybean, barley and wheat etc. Fig. 1 shows the coomassie blue stained gel and western blot of lunasin peptide from aged soybean callus. The weak band of lunasin peptide was appeared at 6 months after sub-culture, but lunasin peptide from the callus was not found in 2 weeks and 3 months after sub-culture. The lunasin content of the callus aged for 6 months was 2.05ng lunasin per μg protein. This is the first data on presence of lunasin peptide from the callus. Although we didn' t apply any biological

Fig. 1. Coomassie blue stain (A) and western blot (B) of protein extracts from soybean callus.
M: Size Marker, 1: 2 weeks after sub-culture, 2: 3 months after sub-culture, 3: 6 months after sub-culture, 4: synthetic lunsin (200 ng). Each lane was loaded with 20μg of protein.

treatment to the callus, this data provide the possibility of mass production of lunasin peptide from the callus treated by specific agents as plant hormones related with plant aging and biological stress.

Inhibition of histone acetyltransferase (HAT) by purified lunasin from callus

The acetylation of histones by HATs has an important role in transcriptional regulation by remodeling chromatin structure (Luo and Dean, 1999). yGCN5 uses acetyl-CoA to acetylate Lys-14 of H3 histone protein, and generates the products, acetylatedhistone and CoA. In the spectrophotometric coupled assay, the CoA produced by yGCN5 is concomitantly used as a substrate for either a-ketoglutarate dehydrogenase. Because of their ability to reduce NAD to NADH while oxidizing substrate (Hunter and Ferreira, 1995). The production of NADH is simply followed spectrophotometrically at 340nm.

Exogenous addition of synthetic lunasin and purified lunasin peptide from soybean to mammalian cells inhibits histone acetylation in the presence of sodium butyrate, a histone deacetylase inhibitor (Jeong *et al.*, 2007).

The lunasin purified by Microcon centrifugal filters from the soybean callus was used in the spectrophotometric determination of yGCN5 activity. The inhibitory effects of NADH production of purified lunasin at different doses on yGCN5 acetylating Lys-14 of H3 histone are shown in Fig. 2. The dose effect of callus lunasin on

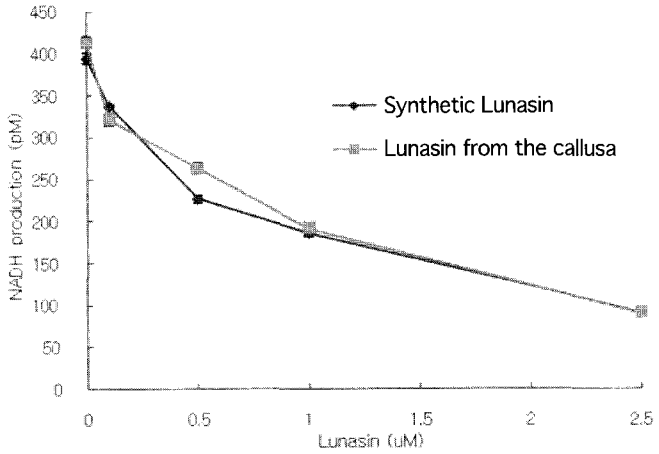

Fig. 2. Inhibition of yGCN5 histone acetyltransferase activity using α-ketoglutrate dehydrogenase-coupled reaction.
Vertical lines on bar are ± SD, n = 3.

inhibition of NADH production was indistinguishable from that of synthetic lunasin.

Fig. 3 shows the inhibitory effects of synthetic and purified lunasin (1μM) from the callus on histone H3 and H4 acetylation, respectively. In the control for histone H3 and H4 acetylation treated alone with yGCN5 and PCAF, acetylated Histone H3 and H4 were produced by 95ng and 9.2ng, while there were produced by 39.1ng and 4ng in the case of the treatment of 1μM lunasin, respectively. In other words, compared to the control, 1μM purified lunasin inhibited the acetylation of histone H3 and H4 by 58.8% and 56.5%, respectively. These inhibitory effects were similar to those by synthetic lunasin.

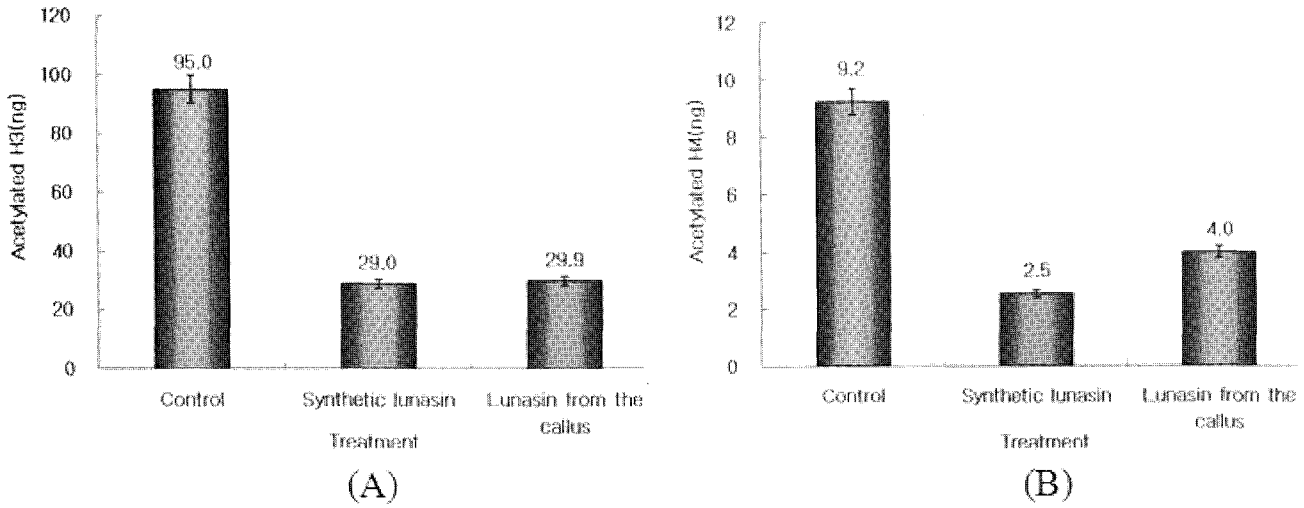

Fig. 3. Amount of acetylated core histone H3 (A) and H4 (B) measured after carrying out the HAT assay in the presence of synthetic and callus lunasins.
Vertical lines on bar are ± SD, n = 3. yGCN5 and PCAF were used for the HAT assay for H3 and H4 peptide, respectively.

Inhibition of foci formation of callus lunasin peptide in NIH3T3 cells fnduced by MCA

Lunasin inhibits foci formation in mammalian cells induced by chemical carcinogen and oncogenes (Galvez *et al.*, 2001; Jeong *et al.*, 2003). The purified lunasin from the callus was tested for their cancer prevention using foci formation assay in NIH3T3 cells induced by MCA (Fig. 4). 1μM Synthetic lunasin and purified lunasin were used per well. Callus lunasin fully inhibited foci formation relative to the values of the positive control (no lunasin) and negative control (no MCA).

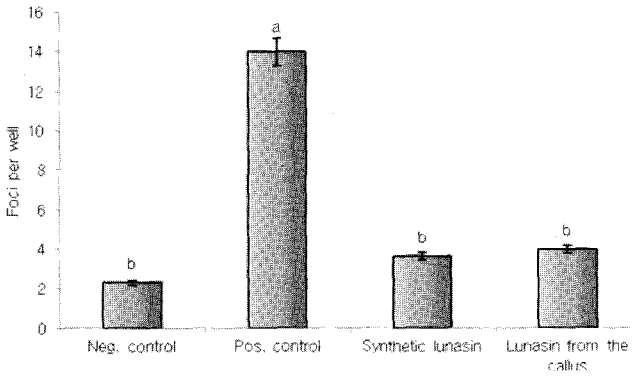

Fig. 4. Inhibitory effect of synthetic and callus lunasin against foci formation on NIH3T3 cells induced by MCA.
Vertical lines on bar are ±SD, n = 3. Bar with different letters are statistically significant from one another.

Internalization of lunasin purified from callus in normal mouse fibroblast NIH3T3 cells

An interesting biological property of lunasin is its ability to internalize into the cell and localize in the nucleus (Lam *et al.*, 2003). In our separated study, we demonstrated that natural lunasin from the soybean, barley, wheat and rye was localized in the nucleus of mammalian cells by exogenous addition. The lunasin purified from callus (green fluorescent stain) internalizes into the mouse fibroblast NIH 3T3 cells and localizes in the nucleus (blue DAPI stain) within 18 hours of administration (Fig. 5). NIH3T3 cells were exposed to purified lunasin containing the equivalent of 1 μM lunasin. The bright green fluorescent stained-lunasin gradually was concentrated 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. This result exerts its anticancer activity by interacting with deacetylated histones in the nucleus.

Acknowledgements

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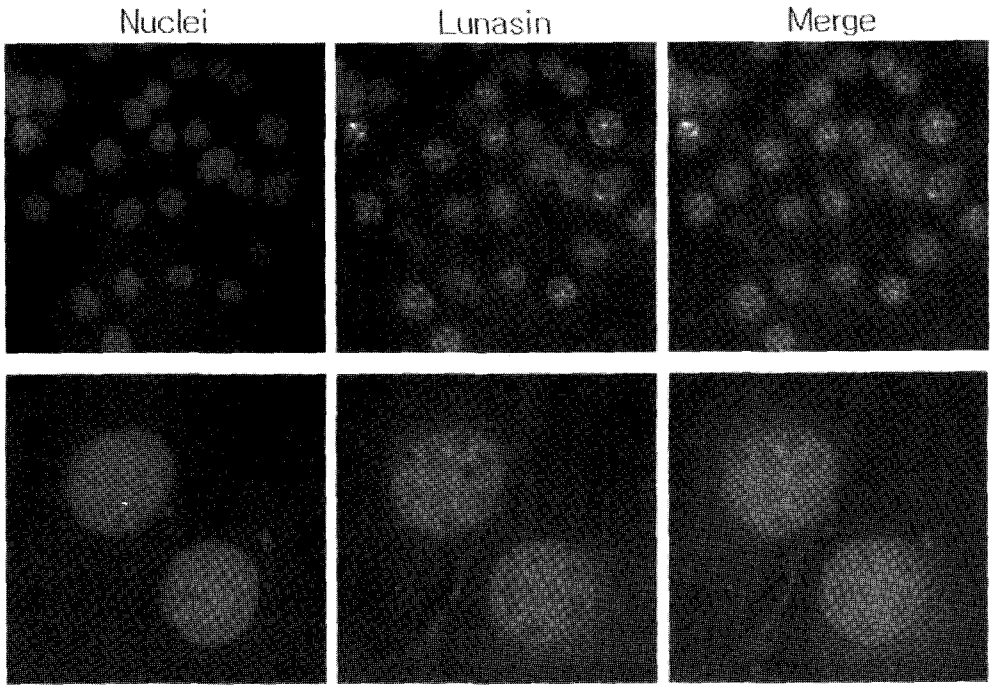

Fig. 5. Internalization of lunasin from the callus in NIH3T3 cells 18 hrs after exposure.

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