Korean J. Plant Res. 22(3) : 203~208 (2009)

Effect of Lunasin Extracted from Millet (*Panicum miliaceum*) on the Activity of Histone Acetyltransferases, yGCN5 and p/CAF

Jae Ho Park[1], Jin Boo Jeong[1], Jeong Rak Lee[1] , Ben O. de Lumen[2], Hyung Jin Jeong[1]*

[1]School of Bioresources Sciences, Andong National University, Andong, 760-749, Korea
[2]Div, of Nutritional Science and Toxicology, University of California, Berkeley, CA 94720-3104

Abstract - Lunasin is a unique 43-amino acid peptide which has shown a chemopreventive in mammalian cells and in a skin cancer mouse model. In search for new sources of lunasin and the role of cereals in cancer prevention, we report here the properties of lunasin purified from millet. Stability of millet lunasin was measured by *in vitro* digestibility assay using pepsin and pancreatin. Inhibition of HAT (histone acetyltransferase) and nuclear localization in mammalian cells were used to measure lunasin bioactivity as the cancer chemopreventive agent. Lunasin present in millet crude protein was stable to pepsin and pancreatin in *in vitro* digestion and inhibited the activities of HATs. When added exogenously, lunasin purified from millet internalized in the nuclei of mouse fibroblast cells. On the base of this result, we conclude that lunasin in millet is bioactive and consumption of millet may play an important role on cancer prevention in millet-consuming populations.

Key words - Cancer chemopreventive peptide, Histone acetyltransferases, Histone H3, Histone H4

Introduction

Cancer is one of the leading causes of death in the world (La *et al*., 2001). Cancer development is a long-term process that appears to proceed by step-by-step carcinogenesis events that ultimately spread from one area of the body to other parts of the body during the late metastasis stage (Chen and Tony Kone, 2005). Histone acetylation and deacetylation have been associated with eukaryotic transcriptional regulatory mechanisms (Shogren-Knaak *et al*., 2006). Recent studies implicate alterations in chromatin structure by histone hyper-acetylation/deacetylation as playing important roles in either the genesis or suppression of cancer. The role of histone hypo-acetylation in cancer has been suggested in studies by Yang *et al* (1996), who demonstrated that the E1A oncoprotein stimulates proliferation by disrupting the growth-suppressive interactions of p300/CBP and p/CAF, both of which have been shown to have HAT activity (Sonia and Richard, 1999). Recent studies implicate the alteration in chromatin structure by histone deacetylation as playing an important role in the suppression of cancer (Archer and Hodin, 1999). Evidence has been presented for the role of histone hyperacetylation in carcinogenesis (Alland *et al*., 1999; Yasui *et al*., 2003).

Lunasin is a unique and novel 4.8 kDa cancer-preventive seed peptide reported in soy, barley, wheat , Solanum family and amaranth. 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 (Galvez *et al*., 2001; Jeong *et al*., 2002; Jeong *et al*., 2007a; Jeong *et al*., 2007b; Silva- Sánchez *et al*., 2008). Lunasin added exogenously localizes in the nuclei of mammalian cells and inhibits core H3 and H4 histone acetylation (de Lumen, 2005; Galvez *et al*., 2001; 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, 2005; Lam *et al*., 2003).

Millet have been mostly used for foods in Africa, Asia, and South America, and contributed significantly to protein nutrition in these areas. Five millets are common; *Setaria italica*, *Pennisetum glaucum*, *Eleusine coracan*, *Echinocloa frumentacea* and *Pannicum miliaceum* (Lorenz, 1983). *Panicum miliaceum* is known as common millet in Korea.

*Corresponding author. E-mail : jhj@andong.ac.kr

In this report, we report here the bioavailability and bioactivity of lunasin from millet in the quest for other readily available natural sources of lunasin and to expand the information on the role of cereals in cancer prevention.

Materials and Methods

Isolation, purification and identification of lunasin from millet

To determine levels of lunasin during seed development, millet (*Panicum miliaceum*) seeds obtained from Andong, Gyeongbuk, Korea were grown in a greenhouse and seeds were collected every week after flowering. To isolate lunasin, seeds were ground to flour and 80 g of the flour was extracted with 200 ml distilled water, supplemented with fresh protease inhibitor cocktail (Sigma, St.Louis,MO) at a concentration of 0.1% v/v (Sigma) by shaking for 48 h at 4℃. The extract was centrifuged at 15,000 rpm for 30 min and the supernatants were collected. Protein contents were determined using Bradford assay (Bradford, 1976). Standard lunasin was synthesized by American Peptide Co. (Sunnyvale, CA). Anti-lunasin polyclonalanty antibody was produced by Zymed Inc. (South San Francisco, CA). The lunasin secondary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). SDS-PAGE of extracts was run in 15% Tris-HCl ready gel (Bio-Rad, Hercules, CA) following the manufacturer's instructions. The gel was transblotted on to PVDF membranes for western blot analysis. After the transblotting, the membrane was blocked for non-specific binding for 90 min in Blotto A (5% non-fat milk and 1% Tween 20 in Tris-buffered saline) and then washed with 1% TBS-T solution (1% Tween 20 in TBS). After washing, the membrane was incubated with the lunasin primary antibody R1 at 1:2000 dilution in Blotto B solution (3% non-fat milk and 1% Tween 20 in TBS) for 1h and then the membrane was re-incubated with an anti-rabbit secondary antibody at 1:2000 dilution in Blotto B solution for 1 h, washed again and treated with the detection agent (Amersham Biosciences) and immediately developed in Polaroid film. For the purification of lunasin from millet, millet was extracted with water (1:10, g:ml) for 1 h, and centrifuged at 15,000 rpm for 1 h. The supernatants were dialyzed for 24 h at 4℃ against 2 L of distilled water using Spectra/Por 7 membrane (MWCO: 10,000) and the distilled water outside the bag was freeze-dried and re-dissolved in an appropriate volume of distilled water. To purify lunasin from the extracts, 20 μl filtrate was injected into HPLC C_{18} column (DELTA PAK, 15 μl, 300A, 300×7.8 mn) equilibrated at ambient temperature and stabilized with the mobile phase (acetonitrile:water, 4:6) at a flow rate of 2.5 ml/min for 15 min with the UV detector set at 295 nm. The rye lunasin peak was identified by comparison with the lunasin standard peak that appeared at a retention time of 4 min. Lunasin content of the sample peak was quantified by western blot using the software UN-SCAN-IT gel Version 5.1 (Silk Scientific, Inc.).

Inhibition of HAT Activities by lunasin purified from millet

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 (Kim *et al*., 2000; Tanner *et al*., 2000). yGCN5 was prepared according to Jeong *et al* (2007). Inhibition of HATs (yGCN5 for H3 and p/CAF for H4) activity by varying concentrations of lunasin purified from millet was done using a HAT activity colorimetric assay kit (Bio Vision) according to the protocol of the manufacturer, with some modifications. 8 μg yGCN5/40 μl distilled water or 500 ng p/CAF/40 μl distilled water was added to each well in a 96-well plate. And then, 65 μl Assay mix (50 μl of 2×HAT Assay buffer, 5 μl HAT substrate, 5 μl NADH generating enzyme) was added to each well, and then the 96-well plates were incubated at 37℃ for 4 hours. The plates were then read on a plate reader at 440 nm wavelength.

***In vitro* digestion with SGF and SIF of lunasin in crude protein extracted from millet**

SGF and SIF consist of 3.2 mg /ml pepsin in 0.03M NaCl at pH 1.2 and 10 mg/ml pancreatin in 0.05M KH_2PO_4 at pH 7.5, respectively. Aliquots (200 μl) of SGF and aliquots (64 μl) of SIF were placed in 1.5ml microcentrifuge tubes and incubated in a water bath at 37℃, respectively. Ten microliter of crude protein from millet (5 mg/ml) was added to each SGF and SIF vial to start the reaction, respectively. The ratio of pepsin for SGF and pancreatin for SIF to protein substrate was about 13:1 (w/w). At intervals of 0, 2, 5, 15, 30, 60 and 120min, 50 μl of 1N NaOH for SGF was added to each vial to stop the reaction. 70 μl of 6×Laemmli buffer for SIF was added to each vial, and the reaction was immediately stopped by placing each vial in

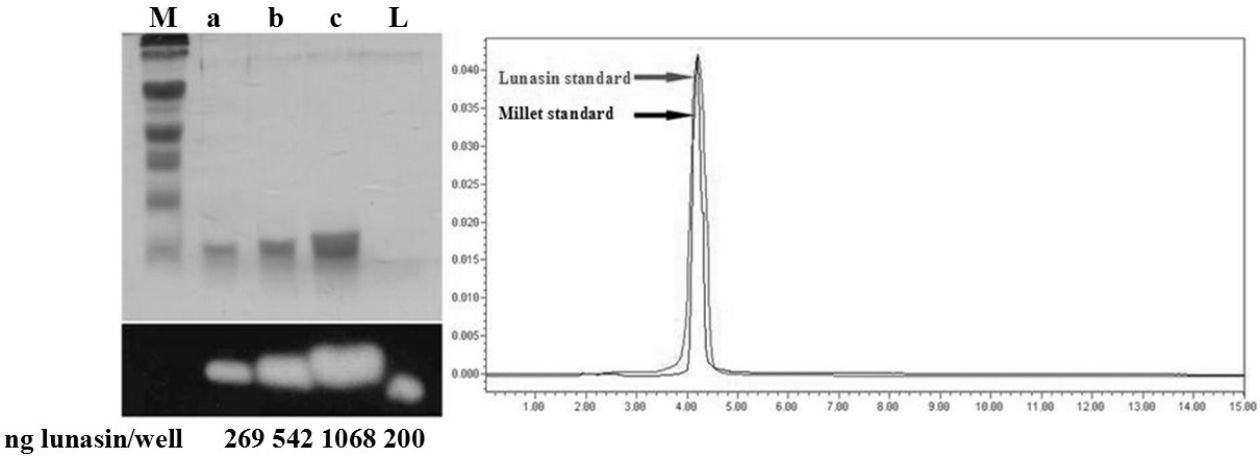


Fig. 1. (A): Commasie blue staining and western blot of lunasin purified from millet by HPLC. Lanes are M (MW markers); a-c are loaded with 0.28, 0.56, 1.12 μg protein per well. Lunasin contents were quantified by western blot using the software Un-SCAN-IT gel Version 5.1 (Silk Scientific, Inc.). (B): The peaks of synthetic lunasin (a peak) and lunasin (b peak) purified from millet by HPLC.

boiling water bath for 10min.

Internalization of lunasin purified from millet in the nucleus of NIH3T3 cells

Immunostaining of purified millet lunasin internalized into the mouse fibroblast cell line NIH 3T3 within 20h after administration was performed as described by Lam *et al* (2003) with some modifications. Sterilized glass cover slips were placed in 6-well plates and seeded with 40,000 NIH3T3 cells per well. To each well, 10 μM of purified millet lunasin was added. After the lunasin treatment for 20 hours, the cells were fixed for an hour with 2% formaldehyde. It was washed with PBS for 5 min 3 times. PBS with 10% fetal bovine serum (PBS/FBS) was then added to block nonspecific binding for an hour. Anti-lunasin polyclonal antibody (Zymed Laboratories, San Francisco, CA) was diluted in 0.1% saponin/PBS/FBS solution at 1:100 dilution for an hour, and then washed with PBS for 5 min 3 times. A goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted at 1:250 was added and incubated in the dark for an hour. Fluorescence was read at 520 nm with an excitation wavelength of 494 nm. DAPI stain was used to localize the cell nucleus during the secondary antibody incubation. Antifade mounting medium (Sigma, St. Louis, MO) was added after incubation and mounted cover slips were washed and inverted onto a drop of antifade mounting medium and viewed under a fluorescence microscope using a 60X oil immersion objective. The excitation wavelength for DAPI was 359 nm and emission wavelength was at 461 nm.

Results and Discussion

Purification and Identification of Lunasin

Lunasin for the further experiments was purified by HPLC and subsequently identified and determined for purity using western blot. Figure 1A shows commassie blue staining and western blot of HPLC-purified lunasin using different protein loadings per well. Figure 1B shows the HPLC peaks of lunasin purified from millet and synthetic lunasin. The two peaks coincided exactly with each other. The purity of rye lunasin is calculated to be above 95% (protein/protein). In our previous studies, we have purified lunasin by ion-exchange chromatography after dialysis or membrane filtration (Jeong *et al*., 2002, 2007a, 2007b). Although, those methods were efficacious for purification of natural lunasin, the process was more complicate and lowered the purity of lunasin than HPLC method. Therefore, HPLC methods offer the 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.

Inhibition of HAT activities by lunasin purified from millet

HATs function enzymatically by transferring an acetyl group from acetyl-coenzyme A (acetyl-CoA) to the ε-amino group

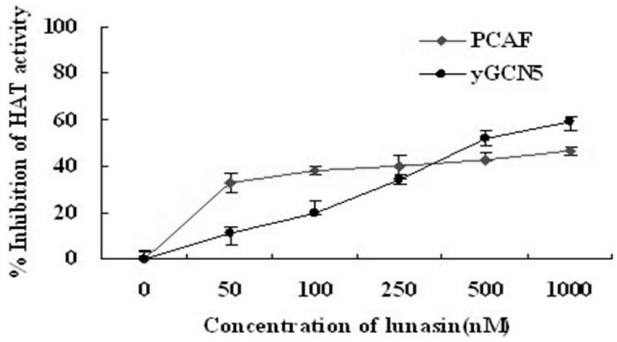


Fig. 2. Inhibition of HAT activities of yGCN5 and p/CAF with increasing concentrations of lunasin purified from millet.

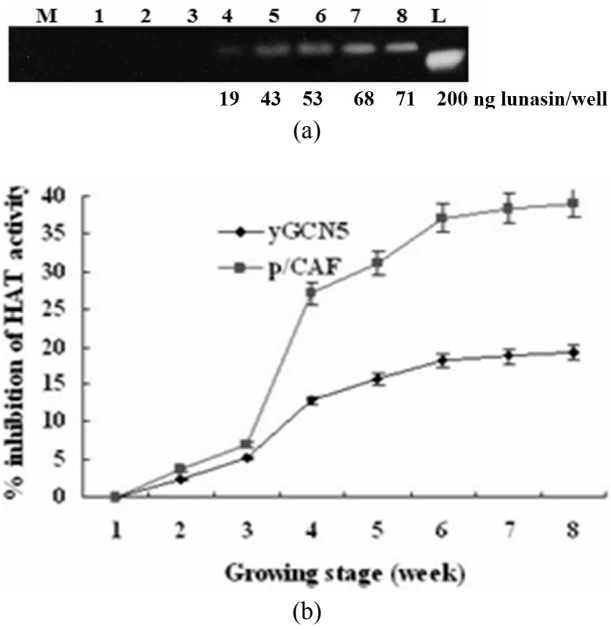


Fig. 3. (A) Western blot of lunasin in millet seeds at various stages of development (A) and (B) inhibitory property on HATs (GCN5 and p/CAF) of lunasin extracted from millet seeds at different stages of seed development. In Western blot, each well was loaded with 12.5 μg protein. The numbers mean the weeks after flowering. In HAT activity, 25 μg protein was used.

of certain lysine side chains within a histone's basic N-terminal tail region (Loidl, 1994). 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 (Kim *et al*., 2000; Tanner *et al*., 2000).

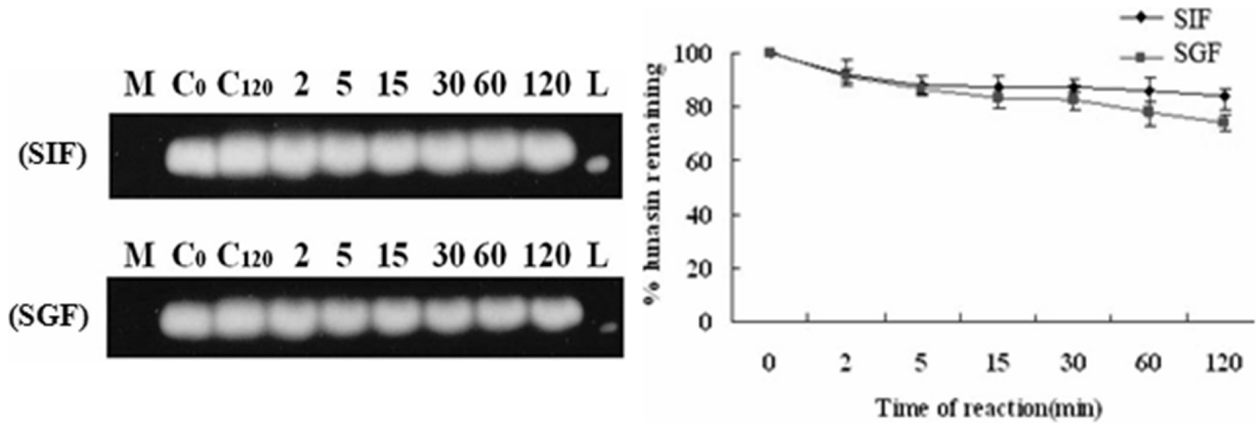
Purified lunasin from millet was used in the inhibition assay of HAT activity to determine the effects of dose using the HAT activity colorimetric assay Kit (Fig. 2). The inhibitory effects of purified lunasin at increasing doses are shown in Fig. 2 for yGCN5 and p/CAF which acetylate histone H3 and H4, respectively. Purified lunasin inhibits yGCN5 activity by 7% and 53% at 50 nM and 1000 nM, respectively, while the p/CAF activity is inhibited by 35% and 43% at 50 nM 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 concentration is likely due to its specificity for acetylation of two Lys residues in histone H4 compared with that of yGCN5 that requires only one residue in histone H3.

Expression of lunasin and inhibition of HAT activity in developing rye seeds

In a previous study, soybean lunasin starts to appear at 5 weeks after flowering and remains in the mature seed and their bioactivities depends on lunasin content (Park, 2005). In this study we determine levels of lunasin and inhibitory property on HATs of lunasin extracted from millet seeds at different stages of seed development. Lunasin was detectable at 4 weeks after flowering and thereafter at a level of about 1.3, 3.5, 4.2, 5.4 and 6.4 ng lunasin/ μg protein from 4 weeks to 8 weeks (Fig. 3A). Although lunasin was detectable only at 4 weeks after flowering, the protein extracts from 2 week and thereafter inhibit the activities of yGCN5 and p/CAF thereafter (Fig. 3B). Lunasin from 8 week seeds inhibited the activities of yGCN5 and p/CAF by 15.6% and 36.2%, respectively. The extents of HAT inhibition correlate with the lunasin contents at each week of seed development.

***In vitro* digestion of rye crude protein with SIF and SGF**

Lunasin in millet crude protein is protected from both pancreatin (SIF) and pepsin (SGF) (Fig. 4A). After digestion for up to 120 min by SIF and SGF, lunasin remains up to 85% and 79% of the original, respectively (Fig. 4B). According to Park (2007), the purified lunasin from soybean and synthetic lunasin are easily digested after 2 min in SIF and SGF digestions, but lunasin from soy protein containing BBI is comparatively stable after both *in vitro* digestions. Seeds of legumes and other plants contain protease inhibitors, which block the digestion of proteins by inhibiting proteases in the animal digestive tract (Heldt,



(a) (b)

Fig. 4. (A) Western blot of digests and (B) plot of digestion time and percent of lunasin remaining after *in vitro* digestion with SIF and SGF of lunasin in millet crude protein. The numbers above the blot indicate the time in minutes of the reaction with SIF and SGF. C_0 and C_{120} indicate the control not treated with SIF and SGF at 0 and 120 min. Lane L is synthetic lunasin (200 ng). The numbers mean the reaction time (min). Each graph represents the percent of lunasin remaining according to the reaction periods with the pancreatin (SIF) and pepsin (SGF).

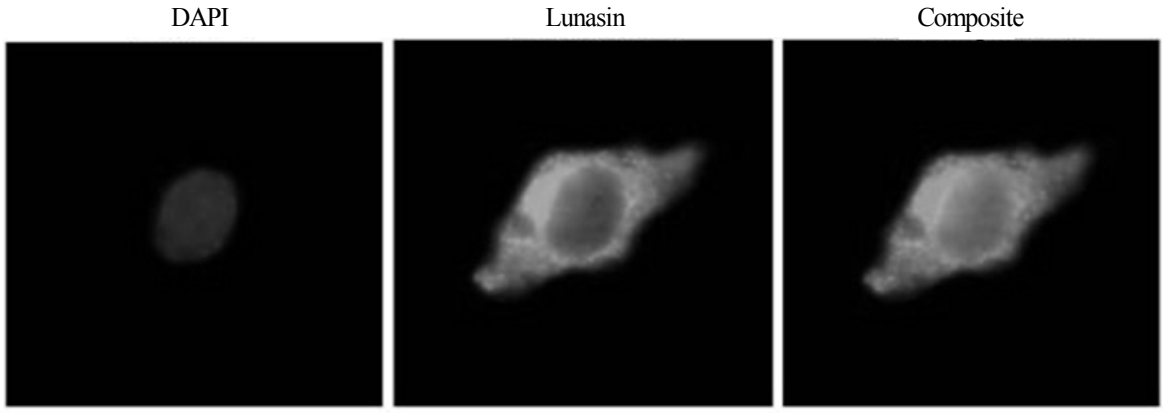


Fig. 5. Internalization of the lunasin purified from rye (green fluorescent stained) into the nuclei (blue-DAPI stain) of mouse fibroblast NIH 3T3 cell line.

1997). Therefore, this result suggests that naturally present protease inhibitors in millet seed protect lunasin from digestion by pancreatin and pepsin.

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

A unique property of lunasin is its ability to internalize into mammalian cells upon exogenous addition and to localize mostly in the nucleus (de Lumen, 2005; Galvez *et al*., 2001; Lam *et al*., 2003). The lunasin purified from millet (green fluorescent stain) internalizes into the mouse fibroblast NIH 3T3 cells and localizes in the nucleus (blue DAPI stain) within 20 hours of administration (Fig. 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. This results is consistent with our previous data obtained in soybean, wheat and Solanum nigrum 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.

Acknowledgements

This work was partly supported by the Korea Research Foundation Grant (MOEHRD) (KRF-2006-353-F00006).

Literature Cited

Alland, L., R. Muhle, H. Hou, J. Potes, L. Cjin, N. Schreiber-Agus and R.A. DePinho. 1999. Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. Nature 387: 49-55.

Archer, S.Y. and R.A. Hodin. 1999. Histone acetylation and cancer. Curr. Opin. Genet. Dev. 9: 171-174.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248.

Chen, C. and A.N. Tony Kone. 2005. Dietary cancer-chemopreventive compounds: from signaling and gene expression to pharmacological effects. Trends Pharmacol. Sci. 26: 318-326.

de Lumen, B.O. 2005. Lunasin: A cancer preventive soy peptide. Nutr. Rev. 63: 16-21.

Galvez, A.F., N. Chen, J. Macasieb and B.O. de Lumen. 2001. Chemopreventive property of a soybean peptide (lunasin) that binds to deacetylated histones and inhibits acetylation. Cancer Res. 61: 7473-7478.

Heldt, H. W. *Plant Biochemistry and Molecular Biology*; Oxford University Press: Oxford, U.K., 1997; pp 310-315.

Jeong, H.J., Y. Lam and B.O. de Lumen. 2002. Barley lunasin suppresses ras-induced colony formation and histone acetylation in mammalian cells. J. Agric. Food. Chem. 50: 5903-5908.

Jeong, H.J., J.B. Jeong, D.S. Kim, J.H. Park, J.B. Lee, D.H. Kweon, G.Y. Chung, E.W. Seo, E.W. and B.O. de Lumen. 2007a. The cancer preventive peptide lunasin from wheat inhibits core histone acetylation. Cancer Lett. 255: 42-48.

Jeong, J.B., H.J. Jeong, J.H. Park, S.H. Lee, J.R. Lee, H.K. Lee, G.Y. Chung, J.D. Choi, B.O. de Lumen. 2007b. Cancer preventive peptide lunasin from *Solanum nigrum L.* inhibits acetylation of core histones H3 and H4 and phosphorylation of retinoblastoma protein (Rb). J. Agric. Food Chem. 26: 10707-13.

Kim, Y., K.G. Tanner and L.M. Denu, 2000. A continuous, non-radioactive assay for histone acetyltransferases. Anal. Biochem. 280: 308-314.

Loidl, P. 1994. Histone acetylation: facts and questions. Chromosoma 103: 441-449.

Lorenz K. 1983. Tannins and phytate content in proso millets (*Panicum miliaceum*). Cereal Chemistry, 60, 424- 426

Lam, Y., A.F. Galvez, and B.O. de Lumen. 2003. Lunasin suppresses E1Amediated transformation of mammalian cells but does not inhibit growth of immortalized and established cancer cell line. Nutr. Cancer. 47: 88-94.

La, V., A. Altieri, A. Tavani. 2001. Vegetables, fruit, antioxidants and cancer: a review of Italian studies. Eur. J. Nutr. 40: 261-267.

Park J. H., H.J. Jeong and B.O. de Lumen. 2005. Contents and bioactivities of lunasin, bowman-birk inhibitor, and isoflavones in soybean seed. J. Agric. Food. Chem. 53: 7686-7690.

Park J. H., H.J. Jeong and B.O. de Lumen. 2005. *In vitro* digestibility of the cancer-preventive soy peptide lunasin and BBI. J. Agric. Food. Chem. 53: 7686-7690.

Shogren-Knaak M, H. Ishii, J.M. Sun, M.J. Pazin, J.R. Davie and C.L. Peterson. 2006. Histone H4-K16 acetylation controls chromatin structure and protein interactions. Science 311: 844-847.

Silva-Sánchez, C., A.P. Barba de la Rosa, M.F. León-Galván, B.O. de Lumen, A. de León-Rodríguez and E.G. de Mejía. 2008. Bioactive Peptides in Amaranth (*Amaranthus hypochondriacus*) Seed. J. Agric. Food Chem. 56(4):1233-40.

Sonia, Y.A. and A.H. Richard. 1999. Histone acetylation and cancer. Curr. Opin. Genet. Dev. 9: 171-174.

Tanner, K.G., M.R. Langer, Y. Kim, J.M. Denu. 2000. Kinetic mechanism of the histone acetyltransferase GCN5 from yeast, J. Biol. Chem. 29 (275): 22048-22055.

Yang, X.J., V.V. Ogryzko, J. Nishikawa, B.H. Howard and Y. Nakatani. 1996. A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. Nature 382: 319-324.

Yasui, W., N. Oue, S. Ono, Y. Mitani, R. Ito and H. Nakayama. 2003. Histone acetylation and gastrointestinal carcinogenesis. Ann. N.Y. Acad. Sci. 983: 220-231.

(Received 29 October 2008 ; Accepted 16 March 2009)