European Review for Medical and Pharmacological Sciences 2014; 18: 2070-2075

Lunasin as a promising health-beneficial peptide

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Abstract. – Lunasin, a 43 amino acid polypeptide originally isolated from soybean, is known to produce multiple health benefits, including anti-hypertension, antioxidant activity, cancer prevention or therapy, anti-inflammation, hypocholesterolemic activity, anti-obesity and immunomodulation. These effects are believed to be due to its unique structure that includes a putative helical region, an Arg-Gly-Asp (RGD) motif and an Asp-rich carboxyl terminus. The focus of this article is to summarize the discovery, characterization and biological activities of lunasin, which will provide a reference for the future development and utilization of lunasin, and a basis for exploring the underlying mechanisms of these health-beneficial functions.

Key Words:
Lunasin, Anticancer, Anti-inflammation, Soybean peptide.

Discovery and Resources of Lunasin

There is a growing body of research suggesting that the consumption of soybean products can reduce the incidence of osteoporosis and some chronic diseases, especially cardiovascular diseases and cancers[1]. Consumption of soybean products is also associated with lower mortality rates from prostate, breast, colon, and endometrial cancers[2]. Therefore, the modification of nutritional habits (e.g., soybean consumption) is becoming increasingly interesting as a means of disease prevention and modulatory therapy. Previous studies have revealed that soybean contains a variety of phytochemicals including protease inhibitors, inositol hexaphosphate (phytic acid), b-sitosterol, saponins, and isoflavones[3].

Lunasin was first isolated in 1987 at Niigata University School of Medicine in Japan, during the screening of protease inhibitors from soybean seeds, and was subsequently identified as a promising anticancer candidate. It was initially characterized as a small polypeptide with poly-Asp (D) residues located at the carboxyl terminus, and was subsequently found in other beans, grains and herbal plants, including wheat, barley, rye, sunberry, wonderberry, bladder-cherry, jimson weed, *Solanum nigrum L.*, amaranth and triticale, at concentrations ranging from 0.013 to 70.5 mg protein lunasin/g of protein (Table I)[4]. Since then, lunasin has been the subject of several investigations, including a very important study on the cloning of the soybean lunasin gene and subsequent transfection into mammalian cells, at the California University at Berkeley (USA), which led to the discovery that the lunasin gene can disrupt mitosis and induce chromosome breakage, ultimately leading to cell apoptosis[5].

Characterization of Lunasin

Structural Characteristics

Lunasin is a unique peptide composed of 43 amino acid residues (sequence SKWQHQQD-S C R K Q K Q G V N L T P C E K H I M E K I Q-GRGDDDDDDDDD) (Figure 1) with a molecular weight of 5.5 kDa. This peptide contains 8 negatively-charged Asp (D) residues at the carboxyl terminus, thus providing a histone-binding property that allows it to act as a potent inhibitor of positively-charged H3 and H4 histone acetylation[6]. The poly-D chain is immediately preceded in the sequence by a cell adhesion motif Arg-Gly-Asp (RGD) that is responsible for the attachment of lunasin to the extracellular matrix, and a putative helical region (EKHIMEIQG) which is structurally homologous to a conserved region found in chromatin-binding proteins[7], suggesting that the helical region may target lunasin to core histones[8]. This putative helical region may function to enhance binding capability to the core histone. The RGD motif can also facilitate internal-

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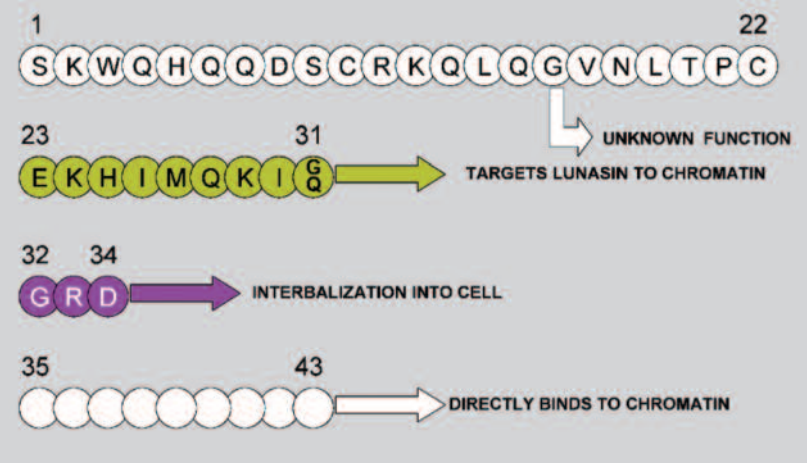

Figure 1. Amino acid sequences and functions of peptide fragments from lunasin. The function of the fragment spanning residues 1-22 remains to be defined; the fragment spanning residues 23-31 is responsible for chromatin-targeting; the Arg-Gly-Asp motif (amino acid residues 32-34) is responsible for peptide internalization into the cell nucleus; the polyaspartyl end (amino acid residues 35-43) is responsible for its direct binding to chromatin.

ization of lunasin into mammalian cells within several minutes, followed by localization in the nucleus within a few hours[8]. According to recent studies, the cell adhesion function of the RGD motif may be cell-line specific, with adhesion observed in C3H cells, but not in NIH3T3 cells. The RGD motif can induce cell apoptosis in different cell lines via a caspase-dependent mechanism[9]. In addition, lunasin may also be involved in cellular growth and proliferation, cellular function maintenance, and cell-cell signaling and interactions[10]. Based on our current knowledge, the major functions of lunasin appear to reside in the poly-D chain, RGD motif and putative helical region structures, while functions associated with the other amino acid residues or structures in lunasin remain undefined. Further research will be required to determine whether smaller peptides that include these functional domains from lunasin will be capable of similar health-promoting or therapeutic functions.

Physiological Characteristics

Since lunasin is a peptide, it is important to understand the degradation, absorption, translocation and distribution of lunasin in target tissues or organs subsequent to oral administration. Therefore, the bioavailability of lunasin has been explored in both *in vitro* and *in vivo* studies. Preliminary studies regarding bioavailability of lunasin in mice and rats supplied with lunasin-enriched soybean protein have revealed that 35% of ingested lunasin is distributed to the target tissues and organs in an intact and active form[11,12]. Similarly, a study on the bioavailability of lunasin in a sample from adult human males indicated that 4.5% of the ingested lunasin was distributed in the plasma in the intact or active form of the soybean protein[13]. The high capacity of lunasin to avoid degradation by gastrointestinal and serum proteinases or peptidases, and retain its bioactive form during the translocation process to blood and other organs, makes lunasin a promising can-

Table I. Lunasin content in soybean, grains and medicinal plants.

Plant (Latin name)	Plant (General name)	Lunasin content (mg/g protein)	Lunasin content (mg/g seed)
Triticum aestivum	Wheat		0.2-0.3
Hordeum vugare L.	Barley	5.9-8.7	0.01-0.02
Glycine max[5]	Soybean	4.4-70.5	0.5-8.1
Amaranthus hypochondriacus	Amaranth	9.5-12.1	
Physalis alkekengi var. francheti	Bladder-cherry	17.0	0.1
Solanum lyratum	Hyyodori-jogo	22.3	0.4
Solanum nigrum L.	Sunberry	36.4	1.8
Datura starmonium	Jimson weed	10.3	0.3

didate for the preventive treatment of cancers *in vivo*[14]. In addition, circular dichroism analysis reveals that lunasin exhibits high thermostability within the temperature range 25-100°C with no apparent change in either its secondary structure or its bioavailability[15].

Preparation of Lunasin

Current methods for the preparation of lunasin include isolation and purification from natural products, prokaryotic expression and chemical synthesis. Jeong et al[12] isolated lunasin from wheat seeds at different growth stages by extraction using MPBS buffer supplemented with fresh protease inhibitor and partial purification, and identified the product using mass peptide mapping. The isolated lunasin is evaluated by determing the bioactivity of the various purified fractions and intact protein. Vermont et al[26] applied a method that utilized the sequential application of anion-exchange chromatography, ultrafiltration, and reversed-phase chromatography[15].

The lunasin gene can be synthesized by overlapping extension polymerase chain reaction (PCR) and expression in *E. coli* BL21 (DE3) with the use of vector pET29a. The recombinant lunasin containing His-tag at the C-terminus can be expressed in soluble form so that it can be purified by immobilized metal affinity chromatography. The result of *in vitro* bioassays has revealed that the purified recombinant lunasin can inhibit histone acetylation. Recombinant lunasin also inhibits the release of pro-inflammatory cytokines (tumor necrosis factor-α [TNF-α] and interleukin-1β), and the production of nitric oxide (NO)[15]. Additionally, lunasin fused with a hexahistidine tag and tobacco etch virus protease site allows for the protease-mediated release of native lunasin, which can be more effectively analyzed using the tobacco etch virus[16]. Moreover, lunasin has also recently been synthesized using a solid phase peptide synthesis strategy.

Biological Activity of Lunasin

Anti-oxidant or Anti-free Radical Capacity

A previous investigation by Hernández-Ledesma et al[23] demonstrated that lunasin can reduce the production of reactive oxygen species (ROS) in lipolysaccharide (LPS)-induced macrophages in a dose-dependent manner, based on the determination of the inhibitory activity of linoleic acid oxidation and scavenging capacity of $ABTS^+$ free radicals. Lunasin appears to exhibit potent ABTS scavenging activity, with TEAC values of 1.41 and 1.90 μM, respectively[17].

Anti-inflammation

Lunasin also appears to inhibit the release of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in LPS-stimulated RAW264.7 cells, without impacting cell viability[17]. Its corresponding action mechanisms are strongly correlated with reduced inflammation in LPS-stimulated RAW 264.7 macrophages, induced by lunasin through inhibition of the nuclear factor kappa B (NF-kB) pathway[18]. Similarly, the treatment of RAW 264.7 macrophage with 100 μM lunasin apparently reduces the production of NO and prostaglandin E2 (PGE2), as well as reducing the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2).

Anticancer

Lunasin, as a novel chemopreventive peptide derived from soybean, also functions to suppress chemical carcinogens and viral oncogene-induced transformation of mammalian cells, and to inhibit carcinogen-induced skin tumorigenesis in mice. The addition of 125 nM lunasin can reduce transformation efficiency in dimethylbenzanthracene (DMBA)-treated C3H cells by 80%, while 3-methylcholantrene (MCA)-treated C3H cells treated with 10 nM lunasin for 24 h exhibited a 69% reduction in transformation efficiency. Purified lunasin at concentrations of 50, 100, 250 500 and 1000 nM can inhibit yGCN5 activity by 3, 13, 27, 48 and 65%, respectively[8,11]. Combinatorial application of anacardic acid and lunasin can result in the arrest of the cell cycle in S-phase, and apoptosis induction at a higher level, and these results are not observed with applications of each compound individually. Additionally, the combined application of both compounds produces synergistic inhibition of ERBB2, AKT1, JUN and RAF1 signaling genes, which are responsible for the growth of breast cancer cells and resistance to apoptosis when up-regulated. Moreover, lunasin inhibits cell proliferation in a dose-dependent manner[6,19,20]. Histological staining of samples collected from lunasin-treated tumors has revealed that destructive regions of tumors are replaced by apoptotic and necrotic cells, thus demonstrating that lunasin

treatment also results in a significant reduction of cell proliferation and obvious apoptosis induction in MDA-MB-231 tumors, although these effects are not observed in tumors from Bowman-Birk inhibitor (BBI)-treated mice. Together, these findings indicate that lunasin acts as a preventive agent for cancers *in vivo*, and that BBI prevents lunasin from digestion, thereby increasing its bioavailability. Pure lunasin isolated from Bowman-Birk inhibitor concentrate (BBIC) at 100 nM can reduce foci formation by 73%, which is identical to results observed using 100 nM synthetic lunasin, while pure BBI isolated from the BBIC at 100 nM decreases foci formation by 60%. Thus, lunasin is 18% more effective than BBI at equivalent concentrations[21].

Lunasin has been found to inhibit cell proliferation, arrest cell cycle in the S phase, and down-regulate both mRNA levels of CDK2, CDK4, CDC25A, caspase 8, Ets2, Myc, Erbb2, AKT1, PIK3R1, FOS and JUN signaling genes in MDA-MB-231 cells, and the expression of Bcl-2[7,14,22]. Research also suggests that lunasin can reduce protein levels of cyclin D1, cyclin D3, CDK4 and CDK6 in a dose-dependent manner in breast cancer cells, and down-regulate Bax and caspase-3 in HT-29 colon cancer cells[7,23]. In MCF-7 cells, lunasin may up-regulate the tumor suppressor phosphatase and tensin homolog deleted in chromosome ten (PTEN). Lunasin-induced cellular apoptosis is similar to apoptosis induced by genistein in that both are mediated by PTEN, although the lunasin mechanism is p53-independent. Additionally, both lunasin and genistein effect the promotion of E-cadherin and beta-catenin non-nuclear localization, although lunasin does not appear to have an impact on the oncogene Wnt1 in HC11 cells[24]. Similarly, lunasin can slow the proliferation of epidermal cells in mouse skin in both the absence and presence of 7,12-dimethylbenz(a)anthracene (DMBA). Lunasin applied topically at 250 mg/week suppresses skin papilloma formation in SENCAR (sensitivity to carcinogenesis) mice treated with DMBA and tetradecanoylphorbol-13-acetate by 70% when compared with the control[25]. Lunasin has also demonstrated the ability to significantly reduce keratinocyte proliferation in both normal and carcinogen-treated SENCAR mice[22]. When RWPE-2 cells are treated with 50 mM lunasin for 24 h, lunasin immunofluorescent signals can be visualized in both the cytoplasm and the nucleus. Synthesized lunasin internalizes in the cell through the RGD cell adhesion motif, co-localizes with hypoacetylated chromatin, binds preferentially to deacetylated histone H4 *in vitro* and inhibits the acetylation of histone H3 and H4 *in vivo* in the presence of histone deacetylase inhibitors[8]. Lunasin can also up-regulate the expression of genes directly or indirectly involved in chemoprevention in non-tumorigenic prostate epithelial RWPE-1 cells but not in tumorigenic RWPE-2 cells, and can enter both RWPE-1 and RWPE-2 cells in a dose-dependent manner and co-localize along the inner edge of the nuclear membrane, a region known to be the site of actively transcribed chromatin[26,27]. It can also bind tightly and specifically to H4 *in vitro* to mask the acetylation of H4K8 while enhancing the acetylation of H4K16, and can hyperacetylate H4K16 *in vivo* at the 5' end of the pro-apoptotic gene THBS1 in RWPE-1 cells, but not in RWPE-2 cells. It is hypothesized that H4K16 acetylation in chromatin destabilizes electrostatic interactions between adjacent nucleosomes, thus improving the accessibility of the basal transcription apparatus to the promoter[28-31].

Conclusions

Lunasin contains multiple functional sites, including a putative helical region, an RGD motif and a poly-D chain. The polyfunctional nature of lunasin makes it an attractive peptide for antioxidant and radical-scavenging, and both anti-inflammatory and anti-cancer functions. However, further investigation will be required to determine if shorter or smaller peptides containing these lunasin functional domains can exhibit similar health-promoting or therapeutic functions. Additional research will also be needed to determine the underlying mechanisms of lunasin, particularly with respect to other potential therapeutic applications, such as the ability to inhibit inflammation-related cancer progression or rheumatoid arthritis.

Acknowledgements

This work is financially supported by Chutian Scholar Program in Hubei Province and the Innovation Program from Wuhan Sports University by NC.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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