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(54) Titre : ALIMENTS FONCTIONNELS A BASE DE PROTEINE DE SOJA
(54) Title: SOYBEAN PROTEIN NUTRACEUTICALS

(57) **Abrégé/Abstract:**

The invention provides methods and compositions for delivering effective amounts of lunasin as a nutraceutical. The general formulation comprises a composition comprising an active unit dosage of a lunasin polypeptide and a pharmaceutically acceptable excipient. The formulations may be delivered or administered by oral ingestion, by topically contacting skin using well known techniques for dermal delivery, by introducing into a retained physiological fluids. The invention also provides methods for making the subject formulations by purifying lunasin polypeptides to the requisite purity, and combining said lunasin polypeptide with a pharmaceutically acceptable excipient in an orally active unit dosage. The lunasin source material may be soybeans, a recombinant lunasin polypeptide expression system, a synthetically produced lunasin, or extract or fraction thereof.



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(21) International Application Number: PCT/US00/11657 (22) International Filing Date: 30 April 2000 (30.04.00) (30) Priority Data: 09/303,814 30 April 1999 (30.04.99) US (71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 1111 Franklin Street, 12th Floor, Oakland, CA 94607-5200 (US). (72) Inventors: DE LUMEN, Benito, O.; 231 Morgan Hall, University of California, Berkeley, Berkeley, CA 94720-3104 (US). GALVES, Alfredo, F.; 609 Madison Street, Albany, CA 94706 (US). (74) Agent: OSMAN, Richard, Aron; Science & Technology Law Group, 75 Denise Drive, Hillsborough, CA 94010 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: SOYBEAN PROTEIN NUTRACEUTICALS (57) Abstract The invention provides methods and compositions for delivering effective amounts of lunasin as a nutraceutical. The general formulation comprises a composition comprising an active unit dosage of a lunasin polypeptide and a pharmaceutically acceptable excipient. The formulations may be delivered or administered by oral ingestion, by topically contacting skin using well known techniques for dermal delivery, by introducing into a retained physiological fluids. The invention also provides methods for making the subject formulations by purifying lunasin polypeptides to the requisite purity, and combining said lunasin polypeptide with a pharmaceutically acceptable excipient in an orally active unit dosage. The lunasin source material may be soybeans, a recombinant lunasin polypeptide expression system, a synthetically produced lunasin, or extract or fraction thereof.		

Soybean Protein Nutraceuticals

INTRODUCTION

Field of the Invention

The field of the invention is soybean proteins having chemopreventive effects.

5 Background

The concept that dietary factors play an important role in the etiology of different kinds of cancer is well supported by epidemiological data (World Cancer Fund, 1997). For instance, there is much evidence to suggest that diets containing large amount of soybean products are associated with overall low cancer mortality rates, particularly for cancers of the colon, breast and prostate, which has given impetus towards identifying specific compounds in soybean that could be responsible for its cancer preventive effects (Messina and Barnes, 1991; Kennedy, 1995).

A soybean-derived protease inhibitor, Bowman-Birk inhibitor (BBI) has been shown to be a particularly effective chemopreventive agent (Kennedy, 1998). BBI has been characterized as a protein of 8 kD of defined sequence and structure (reviewed by Birk, 1985), however most studies demonstrating the efficacy of BBI have used BBIC, a soybean extract enriched in BBI. BBIC is highly effective in suppressing carcinogenesis (1) induced by several different carcinogens, (2) in three different animal model systems (rats, mice and hamsters) and in *in vitro* transformation systems (Kennedy et al, 1993a), (3) in several tissues/organs (colon, liver, lung, esophagus and oral epithelium, (4) when administered to animals in several different routes (ip, iv, topical, dietary), (5) involving different kinds of tumors (squamous cell carcinomas, adenocarcinomas, angiosarcomas, etc, (6) in different cell types (epithelial cells in liver, colon, lung, esophagus and cheek pouch as well as connective tissue cells (fibroblasts both in vitro as well as in liver) which give rise to angiosarcomas. Thus, the chemopreventive ability of BBIC has been demonstrated in a variety of different carcinogenesis assay systems, achieving Investigational New Drug status from the FDA in 1992 (IND no. 34671) and now in human clinical trials.

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WO 00/66625

PCT/US00/11657

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WO 00/66625

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SUMMARY OF THE INVENTION

- The invention provides methods and compositions for delivering effective amounts of lunasin as a nutraceutical. The general formulation comprises a composition comprising an active unit dosage of a lunasin polypeptide and a pharmaceutically acceptable excipient, said composition comprising at least 50% by polypeptide weight said lunasin polypeptide and less than 10% by polypeptide weight Bowman-Birk Inhibitor polypeptide. In other embodiments, the composition comprises at least 70%, preferably at least 90%, more preferably at least
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- 4 -

98%, most preferably 100% by polypeptide weight said lunasin polypeptide and less than 2% , preferably less than 0.5%, more preferably less than 0.1%, most preferably 0% by polypeptide weight Bowman-Birk Inhibitor polypeptide. The formulations may be delivered or administered by any of a wide variety of convenient delivery methods well known in the art, (see, e.g. Goodman and Gilman's
5 The Pharmacological Basis of Therapeutics, Ninth Edition) including oral ingestion, by topically contacting skin using well known techniques for dermal delivery, by introducing into retained physiological fluids such as blood, synovial fluid, interstitial fluid, etc.

The invention also provides methods for making the subject formulations by purifying lunasin polypeptides to the requisite purity, and combining said lunasin polypeptide with a pharmaceutically
10 acceptable excipient in an orally active unit dosage. The lunasin source material may be soybeans, a recombinant lunasin polypeptide expression system, a synthetically produced lunasin, or extract or fraction thereof. Suitable excipients and dosages are readily determined empirically as guided by existing BBIC data.

According to an aspect of the invention, there is provided the use of a lunasin polypeptide
15 comprising the amino acid sequence of SEQ ID NO:1 or an active fragment thereof comprising at least the Arg-Gly-Asp motif followed by at least an hexa-Asp/Glu motif in the manufacture of a formulation for cancer chemoprevention, wherein said formulation comprises at least 50% by polypeptide weight said lunasin polypeptide and less than 10% by polypeptide weight Bowman-Birk Inhibitor polypeptide.

The formulation may be for oral administration of a lunasin polypeptide.

20 The formulation may be for topical administration of a lunasin polypeptide by contacting skin with said formulation.

The formulation may be for introduction of said formulation into a retained physiological fluid for administration of a lunasin polypeptide.

According to another aspect of the invention, there is provided a method for making a
25 formulation as described herein comprising the steps of: purifying the lunasin polypeptide to at least 50% by polypeptide weight said lunasin polypeptide, and to less than 10% by polypeptide weight Bowman-Birk Inhibitor polypeptide, and combining said lunasin polypeptide with a pharmaceutically acceptable excipient in an orally active unit dosage.

- 4a -

DETAILED DESCRIPTION OF PARTICULAR EMBODIMENTS OF THE INVENTION

The following descriptions of particular embodiments and examples are offered by way of illustration and not by way of limitation. Unless contraindicated or noted otherwise, in these
5 descriptions and throughout this specification, the terms “a” and “an” mean one or more, the term “or” means and/or and polynucleotide sequences are understood to encompass opposite strands as well as alternative backbones described herein.

We have shown that a small subunit peptide of a 2S albumin isolated from soybean seed, which we named lunasin, has an antimitotic effect when expressed in mammalian cells (Galvez AF and
10 de Lumen BO, 1999 and US Pat No 6,107,287). Unlike other antimitotic agents that disrupt microtubule function, we have shown that when the *lunasin* gene is transfected and expressed inside the cell, lunasin preferentially binds to hypoacetylated chromatin in *lunasin*-transfected cells, leading to the displacement of kinetochore proteins during mitosis.

The lunasin polypeptide also contains a functional RGD (arg-gly-asp) motif. When
15 exogenously applied to mammalian cell cultures, lunasin through the RGD tripeptide binds to cell membrane integrins and subsequently gets into the cytoplasm through membrane turnover. The relatively small amounts of lunasin that get into the nucleus by passive diffusion and at prometaphase (when nuclear membrane breakdown occurs) are sufficient to

WO 00/66625

PCT/US00/11657

effectively bind regions of hypoacetylated chromatin. However, unlike *lunasin*-transfected cells that constitutively express lunasin in high amounts, internalized lunasin appears not to affect kinetochore assembly as the cells undergo normal mitosis. Instead, lunasin inhibits the transformation of normal embryo fibroblast cells into cancerous tumors by carcinogenic agents. The binding affinity of lunasin to regions of deacetylated nucleosomes is consistent with an anticarcinogenic role of lunasin as a surrogate tumor suppressor by preventing chromatin acetylation and oncogene activation in cells with mutated tumor suppressor genes. The cell adhesion property of lunasin also confers an added benefit of preventing the spread of cancer by competitively binding to membrane integrins required by metastatic cells for attachment to the extracellular matrix and for proliferation.

We also demonstrated that the protease inhibitor Bowman-Birk Inhibitor (BBIC) derived from soybean, which has been shown to be chemopreventive against different types of cancers in *in vitro* and *in vivo* animal models, contains lunasin as a major component. In fact, the removal of lunasin from BBIC by immuno-depletion significantly reduced the ability of BBIC to inhibit carcinogen-mediated transformation of C3H cells. In addition, using equimolar amounts, lunasin was found to be more effective than BBIC in preventing carcinogen-induced tumor formation. These findings indicate that lunasin is an, if not the, active anticarcinogenic constituent of BBIC.

Accordingly, as used herein the term lunasin refers to compounds comprising the natural soybean lunasin polypeptide (coincidentally purified and sequenced by Odani et al., 1987 (Ser Lys Trp Gln His Gln Gln Asp Ser Cys Arg Lys Gln Leu Gln Gly Val Asn Leu Thr Pro Cys Glu Lys His Ile Met Glu Lys Ile Gln Gly Arg Gly Asp Asp Asp Asp Asp Asp Asp Asp, SEQ ID NO:1) and active fragments thereof comprising at least the Arg-Gly-Asp motif followed by at least an hexa- Asp/Glu motif. Preferred lunasin polypeptides comprise residues 33-43, preferably 21-43, more preferably 10-43 and most preferably 1-43 of SEQ ID NO:1. Exemplary lunasin-del compounds (again using N→C nomenclature convention) shown to be effective at inhibiting carcinogenesis in *in vitro* and animal models include:

- lunasin-del-1: MRG - residues 32-43 of SEQ ID NO:1 fusion
- lunasin-del-2: α -tubulin - residues 32-43 of SEQ ID NO:1 fusion
- lunasin-del-3: β -tubulin - residues 27-42 of SEQ ID NO:1 fusion
- lunasin-del-4: MAP2 - residues 5-43 of SEQ ID NO:2 fusion
- lunasin-del-5: Mapmodulin - residues 32-43 of SEQ ID NO:1 fusion

WO 00/66625

PCT/US00/11657

lunasin-del-6: GFP - residues 32-43 of SEQ ID NO:1 fusion

lunasin-del-7: MAP4 - residues 23-42 of SEQ ID NO:1 fusion

lunasin-del-8: FLAGG - residues 9-43 of SEQ ID NO:1 fusion

lunasin-del-9: CYCLIN A - residues 32-43 of SEQ ID NO:1 fusion

5 lunasin-del-10: CYCLIN B1 - residues 32-43 of SEQ ID NO:1 fusion

lunasin-del-11: CYCLIN B2 - residues 19-42 of SEQ ID NO:1 fusion

lunasin-del-12: CYCLIN B3 - residues 13-43 of SEQ ID NO:1 fusion

lunasin-del-13: SH2 - octa-aspartate fusion

lunasin-del-14: SH3 - octa-aspartate fusion

10 lunasin-del-15: SEQ ID NO:1, residues 27-34 - MRG-octa-aspartate fusion

lunasin-del-16: SEQ ID NO:1, residues 27-34 - SEQ ID NO:1, residues 27-34 - octa-aspartate fusion

lunasin-del-17: MRG-tetra-aspartate-tetra glutamate fusion

15 Lunasin Peptide Inhibits Carcinogen-mediated Transformation of C3H 10T1/2 Mouse Fibroblast Cells Into Tumor Cells. Experiments done using the same *in vitro* transformation assay used to determine the chemopreventive property of BBIC showed that the exogenous application of the lunasin peptide to as low as 10 nM (10 nM to 10mM) inhibits the transformation of normal mouse embryo fibroblast cells (C3H 10T1/2) into tumorous foci by carcinogenic agents, MCA (3-methylcholanthrene) and DMBA (7,12-

20 dimethylbenz[a]anthracene). Deletion of the acidic carboxyl end (lunasin-del-C) and the RGD cell adhesion motif (lunasin-GRG) removes the inhibition effect indicating that these domains are essential.

Lunasin is a Major Component of the Bowman-Birk Protease Inhibitor (BBIC) from Soybean, a Known Cancer Preventive Substance. Protease inhibitors, unlike other potential

25 classes of cancer preventive that have been studied, have several features that we found to be also true for lunasin. The most dramatic difference is their ability to affect carcinogenesis in an irreversible way. When the administration of BBIC is stopped in either *in vitro* or *in vivo* experiments, malignant cells or tumors do not arise in the assay systems used

(Kennedy,1994). In contrasts, chemopreventive agents such as vitamin E, b-carotene and

30 retinoids have a reversible effect on *in vitro* systems - transformed cells do arise when these chemopreventive agents are removed from carcinogen-treated cultured cells. Protease inhibitors are also unlike other chemopreventive agents in that they are effective at extremely

WO 00/66625

PCT/US00/11657

low levels (nM) while the other agents are effective only at very high levels (mM). The dose response of BBIC suppression of carcinogenesis is unusual in that dose levels of BBIC have the same suppressive effects over a range varying over several orders of magnitude. Thirdly, the striking ability of protease inhibitors to suppress so many different cancers makes them different from most chemopreventive agents. Their anticancer properties are not restricted to specific organs/tissues.

Lunasin, like BBIC, belongs to the same class of 2S albumins family and both are prepared in similar ways. We found by Western blot analysis that lunasin is a major component of commercially available BBIC preparations (Sigma). In an exemplary blot, lane 3 which contained a crude preparation of trypsin inhibitor (Sigma T 9128, Type II-S Soybean soluble powder) showed a major protein band at 16 kDa that lighted up in the immunoblot. BBI is an 8 kDa protein that is known to dimerize in solution. Lane 4 containing BBIC (Sigma T9777, Bowman-Birk Inhibitor, from soybean lyophilized powder) showed two protein bands, 8 kDa and 16 kDa, which both lighted up in immunoblot. Lunasin which has 2 cysteine residues can form disulfide linkages with BBI (as a monomer and a dimer) which has 14 cysteine residues. This clearly shows that lunasin is found in BBIC as a major component. Furthermore, we showed that removal of lunasin from BBIC by lunasin antibody affinity column and by resin treatment significantly reduced the ability of BBIC to inhibit transformation of C3H cells. This evidence together with the ability of lunasin to inhibit carcinogen-mediated transformation of C3H cells indicates that lunasin is a major if not the major active chemopreventive molecule in BBIC. In fact, our data indicate that BBIC may act to protect lunasin from being digested in the gastrointestinal tract. The ability of lunasin to get inside mammalian cells then modulate chromatin function now provides a rational mechanism to explain the chemopreventive property of BBIC as well as guidance for the use in lunasin as a nutraceutical or nutritional supplement, in particular as an anticarcinogenic agent. For example, substituting lunasin for BBI at comparable dosages and routes of administration provides efficacy in cell and animal studies (see, US Pat Nos. 5,618,679; 5,616,492; 5,614,198; 5,505,946; 5,376,373; 5,338,547).

Chromatin Modification and Tumor Suppression. Our hypothesis is that the chromatin-binding ability of lunasin is the underlying mechanism behind the antimitotic property of the *lunasin* gene and the cancer preventive property of lunasin peptide. A series of studies strongly suggest that chromatin modification is linked with tumor

suppression pathways. (DePinho,1998; Brehm et al,1998; Magnaghi-Jaulin,1998; Pazin and Kadonaga,1997; Hassig et al,1997; Laherty et al,1997). For instance, the retinoblastoma protein Rb is complexed with E2F (a family of transcription factors controlled by Rb) and HDAC1 (the major mammalian histone deacetylase which modulates chromatin structure) in mammalian cells. Rb represses E2F-regulated promoter by recruiting HDAC1 which deacetylates core histones causing a tighter association between DNA and nucleosomes, thus impairing the binding of transcription factors to recognition elements in DNA. The Rb/HDAC/E2F complex could be the target for transforming viruses leading to the release of suppression and a fully transformed state. The binding of lunasin to the hypoacetylated region of chromatin may suppress tumorigenesis in a different context. While deacetylation causes tighter association of DNA with nucleosomes in condensed chromosomes leading to a change in higher order structure, it also exposes positive charges on lysine residues that would allow lunasin to bind to histone terminal sequences and prevent the reversible acetylation/deacetylation process. Consequently, gene expression is more or less permanently repressed. Animal studies show that lunasin effectively survives digestion, gets absorbed and ends up in the tissues. Our evidence shows that lunasin gets internalized in the cell via the RGD motif (i.e. there is no internalization when the -RGD- motif is deleted) and gets inside the nucleus through passive diffusion because of its 4kDa size and at prometaphase when the nuclear envelope breaks down. Eventually, lunasin binds to the core histones of the nucleosomes and inhibits transformation of the cell in the presence of carcinogens by repressing transcription as described above.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WO 00/66625

PCT/US00/11657

SEQUENCE LISTING

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1 5 10 15

Val Asn Leu Thr Pro Cys Glu Lys His Ile Met Glu Lys Ile Gln Gly
20 25 30

Arg Gly Asp Asp Asp Asp Asp Asp Asp Asp Asp
35 40

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CLAIMS

1. Use of a lunasin polypeptide comprising the amino acid sequence of SEQ ID NO:1 or an active fragment thereof comprising at least the Arg-Gly-Asp motif followed by at least an hexa-Asp/Glu motif in the manufacture of a formulation for cancer chemoprevention, wherein said formulation comprises at least 50% by polypeptide weight said lunasin polypeptide and less than 10% by polypeptide weight Bowman-Birk Inhibitor polypeptide.
2. The use according to claim 1, wherein the formulation comprises at least 90% by polypeptide weight said lunasin polypeptide and less than 1% by polypeptide weight Bowman-Birk Inhibitor polypeptide.
3. The use according to claim 1 or 2, wherein the lunasin polypeptide comprises residues 33-43 of SEQ ID NO:1.
4. The use according to claim 1 or 2, wherein the lunasin polypeptide comprises residues 21-43 of SEQ ID NO:1.
5. The use according to claim 1 or 2, wherein the lunasin polypeptide comprises residues 10-43 of SEQ ID NO:1.
6. The use according to claim 1 or 2, wherein the lunasin polypeptide comprises residues 1-43 of SEQ ID NO:1.
7. The use according to claim 1 or 2, wherein the lunasin polypeptide consists of residues 1-43 of SEQ ID NO:1.
8. The use according to any one of claims 1 to 7 wherein said formulation is for oral administration of a lunasin polypeptide.

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- 11 -

9. The use according to any one of claims 1 to 7, wherein said formulation is for topical administration of a lunasin polypeptide by contacting skin with said formulation.

5 10. The use according to any one of claims 1 to 7, wherein said formulation is for introduction of said formulation into a retained physiological fluid.