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Immunological Investigation for the Presence of Lunasin, a Chemopreventive Soybean Peptide, in the Seeds of Diverse Plants

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ABSTRACT: Lunasin, a 44 amino acid soybean bioactive peptide, exhibits anticancer and anti-inflammatory properties. All soybean varieties that have been examined contain lunasin. It has also been reported in a few other plant species including amaranth, black nightshade, wheat, barley, rye, and triticale. Interestingly, detailed searches of transcriptome and DNA sequence databases of cereals failed to identify lunasin-coding sequences, raising questions about the authenticity of lunasin in cereals. To clarify the presence or absence of lunasin in cereals and other plant species, an immunological investigation was conducted utilizing polyclonal antibodies raised against the first 20 amino acid N-terminal peptide (SKWQHQQDSCRKQLQGVNLT) and a 15 amino acid C-terminal peptide (CEKHIMEKIQGRGDD) of lunasin. Protein blot analyses revealed the presence of proteins from several plants that reacted against the lunasin N-terminal peptide antibodies. However, the same proteins failed to react against the lunasin C-terminal peptide antibodies. These results demonstrate that peptides identical to soybean lunasin are absent in seeds of diverse plants examined in this study.

KEYWORDS: *cereal, legume, lunasin, soybean*

■ INTRODUCTION

Soybean is one of the most important field crops in the United States along with maize and wheat. Although soybean was initially cultivated as a forage crop in the United States, during the past century it has become an important source of vegetable oil and a dominant protein source for humans and animals throughout the world. Soybean meal is a widely available, cost-effective source of quality protein. Soybean proteins provide adequate amounts of all essential amino acids with the exception of methionine. Research conducted during the past few decades has established that certain compounds in soybean seeds can positively influence human health.[1] It is now established that regular consumption of soybean could lower the risk of breast, colon, and prostate cancer.[2] Due to the health-promoting effects of soy, the Food and Drug Administration (FDA) in 1999 approved a health claim stating that consumption of 25 g of soy protein per day may reduce the risk of heart disease. Additionally, it has been shown that soybeans can significantly lower cholesterol and triglycerides.[3,4] Consequently, the intake of soybeans in Western countries has steadily increased among health-conscious consumers.

Soybean contains several phytochemicals and bioactive peptides that have been demonstrated to have a positive effect on human health.[5] Prominent among them is a 44 amino acid bioactive peptide called lunasin.[6,7] In 1987, Odani and associates purified this peptide and elucidated its amino acid sequence (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-Asp).[6] Because of an unusually high number of aspartic acid residues, the researchers named it "soybean aspartic acid-rich peptide". They also highlighted the presence of the putative cell attachment sequence Arg-Gly-Asp in this peptide. Nearly a decade later de Lumen and associates cloned *Gm2S-1*, a gene encoding a methionine-rich protein.[7] This 2S albumin is synthesized as a precursor protein and contains a signal peptide, a methionine-rich larger subunit, an aspartic acid-rich small subunit corresponding to lunasin, and a linker peptide. Subsequently, it was demonstrated that lunasin could arrest cell division of mammalian cells by preferential binding of the poly-D carboxyl end of lunasin to highly basic histones.[8−10] The RGD cell attachment motif was reported to facilitate tumor cell attachment to the extracellular matrix.[11] Research has now demonstrated that lunasin can regulate cholesterol levels and possesses antioxidative, anti-inflammatory, and anticancerous properties.[12−15]

All soybean varieties that have been examined contain lunasin, and its concentration in the seed can be influenced by environmental factors.[16] A screen of 144 soybean accessions by enzyme-linked immunosorbent assay revealed the concentration of lunasin can range from 0.1 to 1.3 g/100 g of flour.[17] Subsequent studies have shown that this bioactive peptide also occurs in several other plants. In the past few years lunasin has been reported in barley, wheat, rye, oat, triticale, *Solanum nigrum*, and *Amaranthus hypochondriacus*.[18−26] Interestingly,

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Table 1. Immunological Detection of Lunasin and Lunasin-like Peptides in the Seeds of Diverse Plants

common name	botanical name	family	reactivity against lunasin antibodies	
			N-terminal	C-terminal
adzuki bean	*Vigna angulariz*	Fabaceae	(−)	(−)
agathi keerai	*Sesbania grandiflora*	Fabaceae	(−)	(−)
alfalfa	*Medicago sativa*	Fabaceae	(−)	(−)
amaranth	*Amaranthus hypochondracus* PI558499	Amaranthaceae	(+)	(−)
amaranth	*Amaranthus hypochondracus* PI511721	Amaranthaceae	(+)	(−)
amaranth	*Amaranthus hypochondracus* PI477915	Amaranthaceae	(+)	(−)
anise	*Pimpinella anisum*	Apiaceae	(+)	(−)
barley	*Hordeum vulgare* subsp. *vulgare* GSHO577	Poaceae	(+)	(−)
barley	*Hordeum vulgare* subsp. *vulgare* GSHO475	Poaceae	(+)	(−)
barley	*Hordeum vulgare* subsp. *vulgare* CIho15773	Poaceae	(+)	(−)
black bean	*Phaseolus coccineus*	Fabaceae	(−)	(−)
black cumin	*Nigella sativa*	Ranunculaceae	(+)	(−)
black gram	*Vigna mungo*	Fabaceae	(−)	(−)
brazil nut	*Bertholletia excelsa*	Lecythidaceae	(−)	(−)
bush lima	*Phaseolus lunatus*	Fabaceae	(−)	(−)
cabbage	*Brassica oleracea*	Brassicaceae	(−)	(−)
canola	*Brassica napus*	Brassicaceae	(−)	(−)
cardamom	*Elettaria cardamomum*	Zingiberaceae	(−)	(−)
carrot	*Daucus carota*	Apiaceae	(−)	(−)
chickpea	*Cicer arietinum*	Fabaceae	(−)	(−)
coffee	*Coffea arabica*	Rubiaceae	(−)	(−)
coriander	*Coriandrum sativum*	Apiaceae	(+)	(−)
cowpea	*Vigna unguiculata*	Fabaceae	(−)	(−)
cucumber	*Cucumis sativus*	Cucurbitaceae	(−)	(−)
cumin	*Cuminum cyminum*	Apiaceae	(−)	(−)
datura	*Datura stramonium*	Solanaceae	(+)	(−)
dill	*Anethum graveolens*	Apiaceae	(−)	(−)
Egyptian spinach	*Corchorus olitorius*	Malvaceae	(−)	(−)
fenugreek	*Trigonella foenum-graecum*	Fabaceae	(−)	(−)
flax	*Linum usitatissimum*	Linaceae	(+)	(−)
hazelnut	*Corylus avellana*	Betulaceae	(−)	(−)
jackfruit	*Artocarpus heterophyllus*	Moraceae	(−)	(−)
kidney bean	*Phaseolus vulgaris*	Fabaceae	(−)	(−)
leek	*Allium porrum*	Liliaceae	(−)	(−)
lupine	*Lupinus mutabilis*	Fabaceae	(−)	(−)
soybean	*Glycine max*	Fabaceae	(+)	(+)
mung bean	*Vigna radiata*	Fabaceae	(−)	(−)
nightshade	*Solanum nigrum* PI 381289	Solanaceae	(+)	(−)
nightshade	*Solanum nigrum* PI 304600	Solanaceae	(+)	(−)
nightshade	*Solanum nigrum* PI 381290	Solanaceae	(+)	(−)
nightshade	*Solanum retrofleum* PI 634755	Solanaceae	(+)	(−)
nightshade	*Solanum retrofleum* PI 636106	Solanaceae	(+)	(−)
oat	*Avena sativa* Clav 2866	Poaceae	(+)	(−)
oat	*Avena sativa* PI 51202	Poaceae	(+)	(−)
oat	*Avena sativa* PI 546035	Poaceae	(+)	(−)
okra	*Abelmoschus esculentus*	Malvaceae	(+)	(−)
onion	*Allium cepa*	Liliaceae	(−)	(−)
parsley	*Petroselinum crispum*	Apiaceae	(+)	(−)
peanut	*Arachis hypogaea*	Fabaceae	(−)	(−)
pinto bean	*Phaseolus vulgaris*	Fabaceae	(−)	(−)
pistachio	*Pistacia vera*	Anacardiaceae	(−)	(−)
potato bean	*Apios priceana*	Fabaceae	(−)	(−)
pumpkin	*Cucurbita pepo*	Cucurbitaceae	(−)	(−)
radish	*Raphanus sativus*	Brassicaceae	(−)	(−)
red gram	*Cajanus cajan*	Fabaceae	(−)	(−)
rye	*Secale cereal* subsp. *cereale* PI445987	Poaceae	(+)	(−)
rye	*Secale cereal* subsp. *cereale* PI445999	Poaceae	(+)	(−)
rye	*Secale cereal* subsp. *cereale* PI542469	Poaceae	(+)	(−)
sesame	*Sesamum indicum*	Pedaliaceae	(+)	(−)

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Table 1. continued

common name	botanical name	family	reactivity against lunasin antibodies	
			N-terminal	C-terminal
spinach	*Spinacia oleracea*	Amaranthaceae	(+)	(−)
sun flower	*Helianthus annuus*	Asteraceae	(+)	(−)
tomato	*Solanum lycopersicum*	Solanaceae	(−)	(−)
triticale	*Triticosecale* ssp. PI256033	Poaceae	(+)	(−)
triticale	*Triticosecale* ssp. PI358312	Poaceae	(+)	(−)
triticale	*Triticosecale* ssp. PI615414	Poaceae	(+)	(−)
triticale	*Triticosecale* ssp. PI634537	Poaceae	(+)	(−)
watercress	*Nasturtium officinale*	Cruciferae	(−)	(−)
wheat	*Triticum* subsp. *aestivum* CItr13986	Poaceae	(+)	(−)
wheat	*Triticum* subsp. *aestivum* PI294994	Poaceae	(+)	(−)
wheat	*Triticum* subsp. *aestivum* PI372129	Poaceae	(+)	(−)
white beans	*Phaseolus vulgaris*	Fabaceae	(−)	(−)
zucchini	*Cucurbita pepo*	Cucurbitaceae	(−)	(−)

like soybean, lunasin from these diverse plant species was also found to function as a cancer-chemopreventive peptide. The occurrence of lunasin in such diverse plants suggests that this peptide has been evolutionarily conserved, perhaps due to an essential function in plants. However, recent studies have questioned the origin of lunasin in cereals.[27,28] Interestingly, lunasin-encoding sequences were not found in extensive searches of trancriptome and DNA sequence database of wheat and other cereals.[27] Additionally, investigations based on chemical (LC-ESI-MS) and molecular analyses also failed to detect the lunasin peptide or lunasin-related sequences in wheat samples.[28] Thus, the reported occurrence of lunasin in cereals and other plant species remains inconclusive. To clarify this issue, we have carried out an immunological investigation to confirm the presence or absence of lunasin in cereals and other plant species by utilizing polyclonal antibodies specific to the first 20 amino acid N-terminal peptide (SKWQHQQDSCRKQLQGVNLT) and a 15 amino acid C-terminal peptide (CEKHIMEKIQGRGDD) of lunasin. The results of our study demonstrate that lunasin is not present in cereals and seeds of several other plant species investigated in this study.

■ MATERIALS AND METHODS

Reagents. Acrylamide, bis-acrylamide, ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), Coomassie Brilliant Blue R-250, and goat anti-rabbit IgG−horseradish peroxidase (HRP) conjugate were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). β-Mercaptoethanol was purchased from Sigma-Aldrich (St. Louis, MO, USA). Super Signal West Pico Kit was obtained from Pierce Biotechnology, Rockford, IL, USA.

Seed Material. Seeds of the *Avena*, *Hordeum*, *Triticum*, and *Triticosecale* accessions were obtained from the National Small Grains Collection, USDA-ARS, Aberdeen, ID, USA. Seeds of *Solanum nigrum* and *Solanum retroflexum* were obtained from the Plant Genetic Resources Conservation Unit, USDA-ARS, Griffin, GA, USA. Flax seeds were obtained from U.S. National Plant Germplasm Systems (NPGS), Ames, IA, USA. Seeds of most other plants (Table 1) were from our laboratory collections or purchased from a local grocery store.

Lunasin Peptide. Soy lunasin was synthesized by United BioSystems Inc. (Herndon, VA, USA). The lyophilized lunasin peptide was directly dissolved in sodium dodecyl sulfate (SDS) sample buffer [60 mM Tris-HCl, pH 6.8, 2% SDS (w/v), 10% glycerol (v/v), and 5% 2-mercaptoethanol (v/v)] at a concentration of 0.1 mg/mL and was used in Western blot analysis as an internal control.

Lunasin Peptide Antibodies. Peptides corresponding to the first 20 amino acid region of the N-terminal (SKWQHQQDSCRKQLQGVNLT) and a 15 amino acid region of the C-terminal (CEKHIMEKIQGRGDD) were synthesized, and 10 mg of each peptide was conjugated with 5 mg of KLH protein. Polyclonal antibodies to these peptides were raised in rabbits by Proteintech Group Inc. (Chicago, IL, USA). All applicable international, national, and institutional guidelines for the care and use of animals were followed. Rabbits received boost injections 28, 42, 60, and 78 days after the initial antigen injection. After the final bleed, the antiserum was purified by affinity chromatography. The affinity-purified antibodies were stored in small aliquots at −80 °C until used. The titer of the purified antibodies was evaluated by enzyme-linked immunosorbent assay (Supplemental Figure 1).

Extraction of Albumin and Total Protein from Seeds. Dry seeds were ground to a fine powder utilizing a mortar and pestle. The albumin protein fraction was obtained from 100 mg of seed powder by extraction with 1 mL of 50 mM Tris-HCl, pH 6.8, 1 mM EDTA, and 0.1 mM phenylmethanesulfonyl fluoride. The extracted albumin fraction was recovered by precipitation with 3 volumes of acetone. Precipitated proteins were recovered by centrifugation, and the resulting pellet was air-dried and resuspended in sodium dodecyl sulfate (SDS) sample buffer. Total proteins were isolated from 20 mg (for legume seeds) or 40 mg (cereals and other seeds) of seed powder by extraction with 1 mL of SDS sample buffer followed by 5 min of boiling. The samples were clarified by centrifugation, and the clear supernatants were used as the source of total seed protein. Protein concentration was determined according to the method of Bradford using BSA as a standard.

SDS-PAGE Analysis. Albumin and total seed proteins were resolved with 15% gels run using a Hoeffer SE 250 mini-Vertical electrophoresis apparatus (GE Healthcare). Prior to electrophoretic analysis, protein samples were heated in a boiling water bath for 3 min. Electrophoresis was conducted at 20 mA/gel until the tracking dye reached the bottom of the gel. Gels were removed from the cassette, and separated proteins were visualized by staining the gels overnight with Coomassie Blue R-250. Gels were destained with 50% methanol and 10% acetic acid for 20 min followed by incubation with 10% acetic acid until the background was clear. Gels were scanned using an Epson V700 Perfection scanner (Long Beach, CA, USA) controlled through Adobe Photoshop.

Western Blot Analysis. Total seed proteins or albumins were first resolved on 15% SDS-PAGE gels as previously described.[29] Resolved proteins were electrophoretically transferred to nitrocellulose membranes (Protran, Schleicher & Schuell Inc., Keene, NH, USA). The effectiveness of the protein transfer was monitored by staining the nitrocellulose membrane with Ponceau S. Following this, the nitrocellulose membrane was incubated with 3% dry milk powder dissolved in Tris-buffered saline (TBS; pH 7.5) for 1 h at room temperature with gentle rocking. Western blot analyses were carried out under standard and nonstringent conditions. For standard conditions, the nitrocellulose membrane was incubated with either

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N-terminal or C-terminal lunasin peptide antibodies that were diluted 1:20,000 in TBS containing 3% dry milk powder. Nonspecific binding was eliminated by washing the membrane four times (10 min each wash) with TBS containing 0.05% Tween-20 (TBST). For non-stringent conditions, the nitrocellulose membrane was incubated with lunasin peptide antibodies that were diluted 1:5000 in TBS containing 3% dry milk powder followed by washing the membrane four times (10 min each wash) with TBS containing 0.01% Tween-20. Bound antibodies were detected by incubating the nitrocellulose membrane with 1:20,000 (standard conditions) or 1:10,000 (nonstringent condition) dilution of goat anti-rabbit IgG−horseradish peroxidase conjugate antibody (Bio-Rad) for 1 h. Following this, the membrane was washed four times in TBST, as noted above. Immunoreactive polypeptides were visualized by incubation of the membrane with an enhanced chemiluminescent substrate (Super Signal West Pico Kit; Pierce Biotechnology, Rockford, IL, USA).

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS). Protein bands reacting with soybean N-terminal lunasin antibody were excised from Coomasssie-stained SDS-PAGE gels and extensively washed in distilled water. Coomassie stain from the gel pieces was removed by treating them with a 50% solution of acetonitrile containing 25 mM ammonium bicarbonate. In-gel digestion of protein bands with trypsin and MALDI-TOF-MS analysis of tryptic peptides were carried out as described earlier.[30]

■ RESULTS

Titer and Specificity of Lunasin Peptide Antibodies. We have previously developed a fast and efficient procedure to obtain large qualities of lunasin by preferential extraction of soybean seed powder with 30% ethanol followed by calcium precipitation.[31] This procedure results in a protein fraction named lunasin protease inhibitor concentrate (LPIC) that is enriched in lunasin, Bowman−Birk protease inhibitor, and Kunitz trypsin inhibitor. The titer of lunasin antibodies was examined by Western blot analysis using LPIC and chemically synthesized lunasin peptide (Figure 1). Lunasin C-terminal peptide antibody was able to recognize the lunasin peptide even when the antibody was used at 1:100,000 dilution (Figure 1). Similar results were obtained with the N-terminal lunasin peptide antibody (data not shown). For all subsequent Western blot analyses, the lunasin peptide antibody was used at 1:20,000 dilution. To test the specificity of lunasin antibodies raised separately against the N-terminal and C-terminal regions of the lunasin, protein blot analyses were performed using LPIC and soybean total seed proteins. Chemically synthesized lunasin peptide was also included as a positive control. The N-terminal peptide lunasin antibodies reacted strongly against a 5 kDa peptide corresponding to the lunasin peptide (Figure 2).

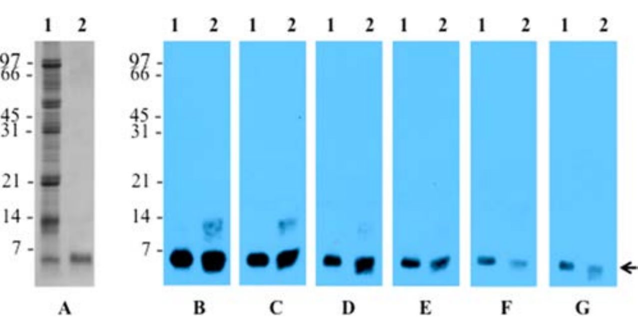

Figure 1. Titer of lunasin peptide antibodies. Proteins were separated on 15% SDS-PAGE gels. Resolved proteins were visualized by staining with Coomassie Brilliant Blue (A) or electrophoretically transferred to a nitrocellulose membrane, cut into several strips, and probed with 1:5000 (B), 1:10,000 (C), 1:20,000 (D), 1:30,000 (E), 1:50,000 (F), and 1:100,000 (G) dilution of C-terminal lunasin antibody (B to G). Immunoreactive proteins were detected using anti-rabbit IgG−horseradish peroxidase conjugate followed by chemiluminescent detection. Lanes: 1, 30% ethanol extracted proteins; 2, synthetic lunasin peptide. Molecular weight markers are shown and designated in kDa.

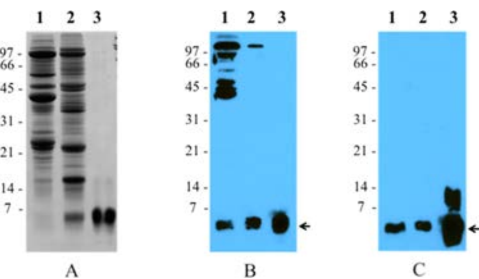

Figure 2. Specificity of lunasin peptide antibodies. Two identical 15% SDS-PAGE gels were used to resolve soybean (*Glycine max* PI 247138) seed proteins. One gel was visualized by staining with Coomassie Brilliant Blue (A), and the other gel was electrophoretically transferred to a nitrocellulose membrane and probed with the N-terminal lunasin antibody (B) or C-terminal lunasin antibody (C). Immunoreactive proteins were detected using anti-rabbit IgG−horseradish peroxidase conjugate followed by chemiluminescent detection. Lanes: 1, total seed proteins; 2, 30% ethanol extracted proteins; 3, synthetic lunasin peptide. Molecular weight markers are shown on the left and designated in kDa. The position of the lunasin is indicated with an arrow.

Additionally, the N-terminal antibody also reacted against a few high molecular weight proteins from the soybean total seed fraction (Figure 2). The C-terminal lunasin antibodies reacted specifically against the 5 kDa lunasin peptide (Figure 2). Unlike the N-terminal lunasin antibodies, the C-terminal lunasin antibodies did not react against any other proteins from soybean total seed fraction, indicating that this antibody is highly specific to lunasin.

Search for Lunasin in Wheat, Barley, Rye, Triticale, Solanum, and Amaranth. Previous studies have demonstrated the presence of lunasin in cereals (barley, oat, wheat, rye, and triticale), *Solanum nigrum*, and *Amaranthus hypochondriacus*.[18−26] However, recent studies have raised questions about the presence of lunasin in cereals.[27,28] To verify if lunasin is present in these plants, we performed Western blot analysis using antibodies specific to the N-terminal and C-terminal regions of lunasin. Albumins isolated from these plants were transferred to nitrocellulose membranes and incubated with 1:20,000 diluted N-terminal and C-terminal antibodies followed by incubation with HRP-conjugated secondary antibodies used at 1:20,000 dilutions. Under these conditions, a strong reaction with a 5 kDa protein corresponding to the lunasin peptide was detected only from soybean seed proteins (Figure 3). Interestingly, no reaction was detected with any seed proteins isolated from any other seed sources including amaranth, a pseudo cereal (Figure 3). A previous study has shown the presence of lunasin in the glutelin fraction from amaranth.[26] Western blot analysis revealed a band at 18.5 kDa, and MALDI-TOF analysis showed that this peptide matched >60% of the soybean lunasin peptide sequence.[26] However, under our experimental conditions we were unable to detect proteins in amaranth seeds reacting with the lunasin antibody. Similar results were also obtained when total seed proteins were

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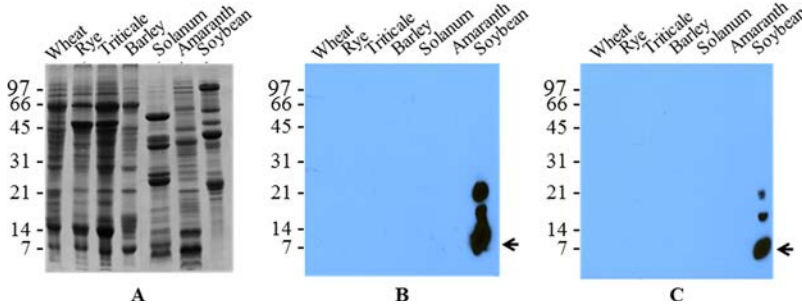

Figure 3. Western blot analysis of lunasin. Three identical 15% SDS-PAGE gels were used to resolve albumins from wheat (*Triticum aestivum* subsp. *aestivum* Cltr 13986; lane 1), rye (*Secale cereale* subsp. *cereale* PI 445987; lane 2), triticale (*Triticosecale* spp. PI 634537; lane 3), barley (*Hordeum vulgare* subsp. *vulgare* GSHO 577; lane 4), Solanum (*Solanum nigrum* PI 381289; lane 5), amaranth (*Amaranthus hypochondriacus* PI 558499; lane 6) and soybean (*Glycine max* PI 247138; lane 7). One gel was visualized by staining with Coomassie Brilliant Blue (A), and the other two gels were used for Western blot analysis. Panels B and C were probed with N-terminal lunasin antibody and C-terminal lunasin antibody, respectively. Western blot analysis was performed under standard conditions (lunasin antibody used at 1:20,000 dilution; washing buffer with 0.05% Tween 20). Immunoreactive proteins were detected using anti-rabbit IgG−horseradish peroxidase conjugate used at 1:20,000 dilution followed by chemiluminescent detection. Molecular weight markers are shown on the left and designated in kDa, and the position of the lunasin is indicated with an arrow.

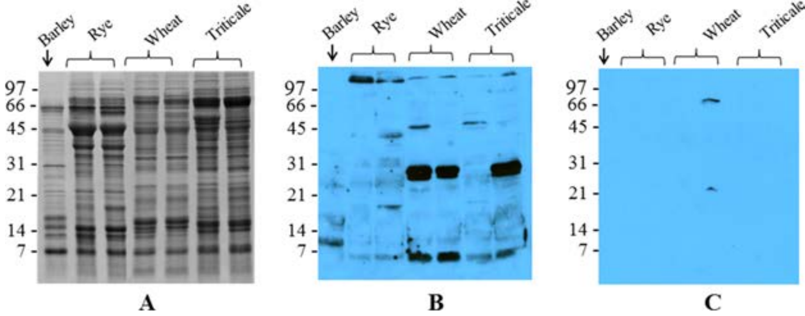

Figure 4. Western blot analysis of lunasin in cereals. Albumins from barley, rye, wheat, and triticale were separated on a 15% SDS-PAGE and visualized by staining with Coomassie Brilliant Blue (A). Proteins visible in panel A were electrophoretically transferred to nitrocellulose membranes and probed with N-terminal lunasin antibody (B) or C-terminal lunasin antibody (C). Western blot analysis was performed under nonstringent conditions (lunasin antibody used at 1:5000 dilution, washing buffer with 0.01% Tween 20). Immunoreactive proteins were detected using anti-rabbit IgG−horseradish peroxidase conjugate (used at 1:10,000 dilution) followed by chemiluminescent detection. Molecular weight markers are shown on the left and designated in kDa. Lanes: 1, barley (*Hordeum vulgare* subsp. *vulgare* GSHO 577); 2, rye (*Secale cereale* subsp. *cereale* PI 445987); 3, rye (*Secale cereale* subsp. *cereale* PI 445999); 4, wheat (*Triticum aestivum* subsp. *aestivum* Cltr 13986); 5, wheat (*Triticum aestivum* subsp. *aestivum* PI 294994); 6, triticale (*Triticosecale* spp. PI 256033); 7, triticale (*Triticosecale* spp. PI 358312).

used instead of albumins in Western blot analyses (data not shown). Our results suggest that under the experimental conditions used in this study lunasin does not appear to be present in cereals.

Seed proteins isolated from additional cereal cultivars belonging to distinct genotypes (Table 1) were also employed in Western blot analysis to verify the presence of lunasin in these plants. None of these cereal proteins reacted against either the lunasin N-terminal or C-terminal antibodies, suggesting that lunasin is not present in these cereals or present at levels that are too low for detection by the lunasin antibodies used in this study. Additional Western blot analyses were performed under less stringent conditions by reducing the primary and secondary antibody dilutions to 1:5000 and 1:10,000, respectively, as well as lowering the detergent concentration in the protein blot buffers from 0.05 to 0.01%. Under these conditions the lunasin N-terminal specific antibodies reacted with few proteins of different molecular weights present in wheat, rye, barley, and triticale (Figure 4). Interestingly, a positive reaction against a 5 kDa low molecular weight protein from rye, wheat, and triticale was detected (Figure 4). However, under identical conditions no reaction with these proteins was detected when lunasin C-terminal specific antibodies were employed in Western blot analyses. Even prolonged exposure of nitrocellulose membrane to X-ray film (>30 min) did not result in any positive reaction with any seed proteins, suggesting the lack of lunasin in these cereals.

Lunasin in Other Plants. We also investigated the presence of lunasin in seeds of several plant sources (Table 1). For this purpose we isolated the albumin fraction from the seeds of 55 commonly consumed or medicinal plants and subjected them to Western blot analysis under less stringent conditions. Of 72 seed samples examined, a positive reaction with lunasin N-terminal antibodies was detected with 35 seed samples (Table 1). The sizes of the proteins recognized by the lunasin N-terminal specific antibodies were heterogeneous (Figure 4). Some of these positively reacting proteins corresponded to abundant seed proteins. Most of these proteins failed to react with the lunasin N-terminal specific antibodies when Western blot analysis was carried out under stringent conditions. Seed proteins isolated from coriander, sesame, anise, black cumin, okra, sunflower, castor bean, and flax showed a strong reaction against the lunasin N-terminal specific antibodies (Figure 5). However, none of these seed proteins showed any reaction when the lunasin C-terminal antibodies were employed.

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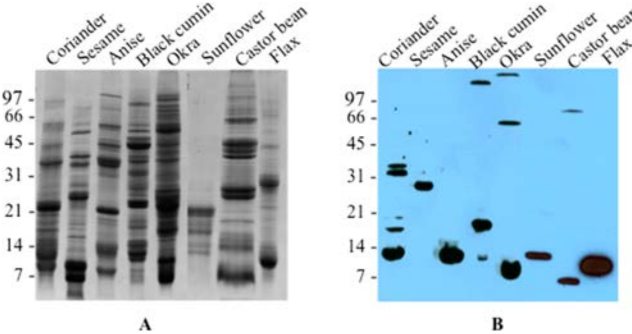

Figure 5. Detection of lunasin-like peptides in seeds of different plants. Two identical 15% SDS-PAGE gels were used to resolve albumins from coriander (lane 1), sesame (lane 2), anise (lane 3), black cumin (lane 4), okra (lane 5), sunflower (lane 6), castor bean (lane 7), and flax (lane 8) separated on a 15% SDS-PAGE and visualized by staining with Coomassie Brilliant Blue (A). Resolved proteins from the other gel were electrophoretically transferred to nitrocellulose membrane and probed with N-terminal lunasin antibody (B). Western blot analysis was performed under nonstringent conditions (lunasin antibody used at 1:5000 dilution, washing buffer with 0.01% Tween 20). Immunoreactive proteins were detected using anti-rabbit IgG−horseradish peroxidase conjugate (used at 1:10,000 dilution) followed by chemiluminescent detection. Molecular weight markers are shown on the left and designated in kDa.

Lunasin-like Peptide Present in Flax. In the process of screening for the presence of lunasin in the seeds of diverse plants, we observed a 7 kDa protein in the flax seed protein extract that was recognized by the N-terminal lunasin antibody (Figure 5). Unlike the positively reacting proteins from other tested plants, the immunoreactivity of the 7 kDa flax protein to the N-terminal lunasin antibody was retained even when the Western blot was performed under stringent conditions. We also investigated the presence of lunasin in additional flax varieties representing different genotypes by Western blot analysis (Figure 6). All of the tested flax varieties contained the 7 kDa protein that reacted specifically against the N-terminal lunasin antibody (Figure 6). To identify the 7 kDa protein, we excised this immunoreactive protein band from SDS-PAGE gels, digested it with trypsin, and analyzed it with matrix-assisted laser desorption ionization time-of flight mass spectrometry. On the basis of the mass spectra of the tryptic peptides, the 7 kDa protein was identified as conlinin, the 2S storage protein of flaxseed. The amino acid sequences of lunasin and conlinin were aligned using LALIGN,[33] revealing 41% identity and 66% similarity in a 32 amino acid overlap (Figure 7).

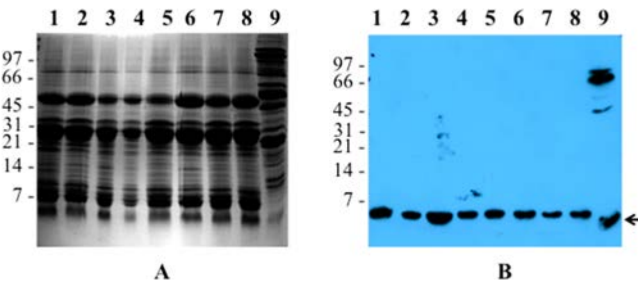

Figure 6. Detection of lunasin-like peptides in flax. Total seed proteins from eight flax genotypes and soybean cultivar Williams 82 were separated on two identical 15% SDS-PAGE gels. One gel was stained with Coomassie Brilliant Blue (A), and the other gel was used for Western blot analysis utilizing N-terminal lunasin antibody (B). Western blot analysis was performed under standard conditions (lunasin antibody used at 1:20,000 dilution, washing buffer with 0.05% Tween 20). Immunoreactive proteins were detected using anti-rabbit IgG−horseradish peroxidase conjugate (used at 1:20,000 dilution) followed by chemiluminescent detection. Molecular weight markers are shown on the left and designated in kDa. Numbers (1−8) at the top of the figure refer to the flax genotypes (lanes: 1, Royal; 2, Norstar; 3, Linott; 4, Victory; 5, Culbert; 6, Linore; 7, Flor; 8, Marine; 9, soybean cv. Williams 82).

DISCUSSION

Lunasin was originally identified and purified from soybean as an aspartic acid-rich peptide.[6] Subsequently, numerous studies have demonstrated the chemopreventive properties of lunasin and its potential use as a cancer therapeutic agent.[8−13] Because of the health-promoting properties of lunasin, efforts were made to identify this bioactive peptide in other plant species. de Lumen and his associates in a series of papers reported the occurrence of lunasin in barley, wheat, and rye and additionally demonstrated that lunasin isolated from these cereal seeds was bioactive and could play an important role in cancer prevention.[18−22] Additionally, the occurrence of lunasin in triticale, oat, and millet has also been reported.[23,24,34] Matrix-assisted laser desorption ionization (MALDI) and LC-ESI-MS were employed to elucidate the peptide sequence of lunasin from barley and wheat.[18−21] Barley and wheat lunasin partial sequences matched exactly the soybean lunasin sequences, which is remarkable given the fact that soybean lunasin belongs to the 2S albumin storage protein family and 2S albumins are not reported in cereals.[35] This observation led to in-depth searches of transcriptome and DNA sequence databases of wheat and a few other cereals, which indicated that sequences encoding lunasin are absent in cereals.[27] Additionally, an investigation based on chemical and molecular analyses also confirmed that lunasin-related sequences are absent in 36 wheat extracts examined in that study.[28] In our study, we have utilized peptide antibodies raised in rabbits against the N- and C-terminal regions of the lunasin to verify previous claims for the presence of this bioactive peptide in cereals. The complete failure of the lunasin C-terminal antibody to recognize any cereal proteins in Western blot analyses even under non-stringent conditions demonstrates that lunasin is not present in cereals or occurs at concentrations below the detection limit of Western blot analysis. In this context it should be pointed out that a chemical and molecular investigation of 12 wheat varieties representing both nondwarf and dwarf genotypes also failed to detected lunasin in wheat.[28]

Earlier studies have used peptide antibodies to detect the presence of lunasin in cereals. Unfortunately, most of these published papers do not provide details on the lunasin antibodies used in their respective studies. In some cases mouse monoclonal antibody was used, and in some cases polyclonal lunasin antibodies were used. However, it is not clear if the antibodies used in Western blot analyses were raised against the entire lunasin peptide or certain defined regions of the lunasin. Furthermore, the specificity of the lunasin antibodies in these studies has not been demonstrated. In our study we have used antibodies specific to the N-terminal and C-terminal regions of the lunasin peptide (sequences shown under Materials and Methods).[32] On the basis of the results of our study, it is clear that the N-terminal lunasin antibody, in addition to reacting against the lunasin peptide, also reacted

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Figure 7. Alignment of soybean lunasin with flax conlinin amino acid sequences. The sequences were aligned with William Pearson's *lalign* program (http://embnet.vital-it.ch/software/LALIGN_form.html). Identical amino acid residues are shown in green boxes, and similar residues are shown in yellow boxes.

nonspecifically against a few other proteins under nonstringent conditions. Nonspecific binding can be reduced or eliminated by increasing the concentration of Tween-20 in Western blot buffers. When we performed Western blot analysis in the presence of 0.5% Tween-20, most of the nonspecific binding with cereal proteins was drastically reduced (compare Figure 2B to Figure 3B). It is therefore surprising to note that Western blot analyses performed under much more stringent conditions (1% Tween 20) than employed in our study (0.05% Tween 20) still revealed a strong reaction against a single protein having a similar size to that of soybean lunasin in cereals.[20] This discrepancy could be attributed to differences in the quality of the lunasin antibodies. Another possibility is that lunasin does not occur universally in all cereals but is restricted to certain cereal cultivars. However, these possibilities appear unlikely because the cereals used in our study represented different geographic locations and the lunasin antibodies employed in the current study were able to detect nanogram quantities of lunasin even under stringent conditions.

The list of lunasin-containing plants has gradually expanded to include bladder-cherry, black nightshade, jimson weed, amaranth, and lupine.[20,25,26,36,37] We have examined the occurrence of lunasin in some of these plants by Western blot analysis. Results from Western blot analysis using the C-terminal lunasin antibodies failed to detect the presence of lunasin in the seeds of these plants. Our observations indicate that lunasin is not present in these plants or accumulates at levels below the detection limits. A screen for the presence of lunasin in the seeds of diverse plants revealed that proteins reacting with the N-terminal lunasin antibody are present in sunflower, castor bean, coriander, sesame, anise, black cumin, and okra. Interestingly, the C-terminal lunasin antibody recognized none of these proteins. Additionally, the positive reaction with the N-terminal lunasin antibody was not detected when the Western blot was performed under stringent conditions. These observations indicate that the positive reaction detected with the N-terminal lunasin antibody may be due to nonspecific binding of the antibodies or the limited amino acid sequence homology with the N-terminal region of the lunasin peptide. A recent study investigated the presence of lunasin in traditional Italian legumes by Western blot analysis and found no evidence of its presence in these legumes.[38] Interestingly, this study also reported positive reactions to high molecular weight proteins with lunasin antibodies. Positively reacting proteins were identified by mass spectrometry as subtilisin inhibitor, legumin A2, phaseolin, phytohemagglutinin, pathogenesis-related protein, lipoxygenase-3, lipoxygenase-2, provicillin, seed bitin-containing protein SBP65, and albumin-1 C. Alignment of soybean lunasin sequences with the immunoreactive proteins revealed various levels of amino acid sequence homology. On the basis of these observations it was concluded that lunasin-like peptides could be released from high molecular seed proteins during sourdough fermentation.[38]

Like soybean, flax seeds are rich in oil and contain several health-promoting compounds. Consumption of flax seeds has been shown to promote the immune response as well as reduce the risk of cancer and cardiovascular diseases.[39] The health-promoting attributes are credited to the presence of bioactive compounds such as α-linolenic acid, lignans such as secoisolariciresinol diglucoside (SDG), and mucilage in flax seeds.[40] However, it is not known if any bioactive peptides present in flax seeds could also play a role in the prevention of cancer and heart disease. In this regard it is interesting to note that our Western blot analysis using the soybean N-terminal lunasin antibody reveals the presence of lunasin-like peptide in flax seeds. Mass spectrometry has identified the 7 kDa protein as conlinin, the 2S albumin of flax seed. Flax seeds contain two abundant groups of storage proteins, 11−12S globulins and 2S albumins.[41] Molecular analysis has shown the presence of two conlinin genes, conlinin 1 (*cnl*1) and conlinin 2 (*cnl*2) in the flax genome.[42] These two genes encode proteins with a molecular mass of about 19 kDa and reveal 88% amino acid identity between them.[42] A comparison of the soybean lunasin and flax conlinin amino acid sequences reveals significant homology (40.6% identity and 65.6% similarity) in 32 amino acid overlap. This apparent homology could explain the strong reactivity of the soybean N-terminal lunasin peptide antibody against the 7 kDa lunasin-like peptide released from the mature conlinin, presumably by proteolysis.

The results of our study, in combination with earlier papers, clearly demonstrate that lunasin is not present in cereals. However, lunasin-like peptides may be present in the seeds of some plants. Most of the proteins recognized by the soybean lunasin antibodies have molecular weight much higher than the soybean lunasin. It is likely that lunasin-like peptides could be released from the high molecular weight precursor proteins by the action of proteolytic enzymes during microbial fermentation or during gastrointestinal digestion.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b00445.

ELISA assay to determine the titer for lunasin N-terminal and C-terminal peptide antibodies (PDF)

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Notes

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