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Lunasin Concentration in Different Soybean Genotypes, Commercial Soy Protein, and Isoflavone Products

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Lunasin is a unique and novel cancer preventive peptide originally isolated from soy. Information on lunasin concentration of soybean cultivars and commercial soy proteins would be useful in developing lunasin-enriched cultivars and soy products. We report the development of an enzyme-linked immunosorbent assay (ELISA) method to identify lunasin and quantify the variations in concentration in 144 selected, diverse soybean accessions from the U.S. Department of Agriculture Soybean Germplasm Collection, several commercially available soy protein fractions and isoflavone-enriched products. With synthetic lunasin and monoclonal antibody, ELISA shows a linear concentration range of 24−72 ng/mL, good reproducibility, a detection limit of 8 ng/mL, and a recovery of 90% on spiked soy samples. Lunasin concentrations in the tested materials range from 0.10 to 1.33 g/100 g flour. Differences that exceeded 100% have been observed among accessions of similar maturity that were grown in the same environment, indicating that genetic differences in soybeans exist for lunasin. The mean of 23 major ancestral lines of U.S. cultivars is similar to the mean of 16 modern cultivars selected to represent the current diversity of the crop, but the highest values were found within the ancestral and exotic accessions. Soy protein concentrate, isolate, and hydrolyzate contain 2.81 ± 0.30, 3.75 ± 0.43, and 4.43 ± 0.59 g lunasin/100 g flour, respectively, while soy flour and soy flakes contain 1.24 ± 0.22 g lunasin/100 g flour. Isoflavone-enriched products contain very little or no lunasin. The relative mass (M_r) of lunasin in the samples is 5.45 ± 0.25 kDa. The wide range of lunasin concentrations within the *Glycine max* species indicates that the levels of this important bioactive peptide can be genetically manipulated. Furthermore, soy isolates and hydrolyzed soy proteins contain the highest concentrations of lunasin.

KEYWORDS: Bioactive peptides; synthetic lunasin; soybean genotypes; U.S. Department of Agriculture germplasm

INTRODUCTION

Soybeans contain a variety of anticarcinogenic phytochemicals including protease inhibitors, phytate, phytosterols, saponins, and isoflavones (*1*). These compounds, among others, may contribute to the role of soy in the prevention and/or treatment of cancer. Recently, there has been an increased interest in the potential health benefits of bioactive polypeptides and proteins from soybeans. Bioactive peptides may exist naturally or be derived from soy protein hydrolyzates. These peptides may act as physiological modulators during the gastrointestinal digestion of soy products (*2*). In addition, they may also be a potential source of bioactive ingredients for designing functional foods. Many bioactive peptides have in common structural properties that include a relatively short peptide residue length (e.g., 2−9 amino acids), possessing hydrophobic amino acid residues in addition to proline, lysine, or arginine groups (*3*). Bioactive peptides are also resistant to the action of peptidases. Immunomodulatory peptides derived from tryptic hydrolyzates of rice and soybean proteins act to stimulate superoxide anions, which trigger nonspecific immune defense systems (*4*). Peptides in fermented soy products bind well to bile acid and also contain angiotensin-converting enzyme inhibitory activity (*5*), suggesting a promising prospect of exploitation as a functional food. Soybean peptides have antioxidant activity (*6*) and antiobesity effects (*7*). Soybeans also contain bioactive proteins that exhibit anticancer activity, including lectins (*8*) and the most recently discovered peptide, lunasin (*9*).

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Lunasin is a unique and novel cancer preventive peptide originally isolated from soy and more recently from barley (*10*). Its efficacy against chemical carcinogens and oncogenes has been demonstrated in cell cultures and in a skin cancer model in mice. Researchers from the University of California, Berkeley (*11*), have isolated and cloned a cDNA encoding a posttranslationally processed 2S albumin (Gm2S-1) from midmaturation soybean seeds. This unique 43 amino acid soybean peptide, whose carboxyl end contains nine Asp residues, an Arg-Gly-Asp cell adhesion motif, and a helix with structural homology to a conserved region of chromatin-binding proteins (*12*), is now known as lunasin (*13*). Lunasin from soybeans appears to have potential as a novel cancer chemopreventive agent (*14*). Clearly, further research is essential to confirm these preliminary observations and possible health uses, including its role in chronic disease prevention.

The objective of this research is to develop immunoassay techniques to identify and quantify the variation of lunasin in 144 selected and diverse soybean accessions from the U.S. Department of Agriculture Soybean Germplasm Collection and a number of commercially available soy proteins and isoflavone-enriched products.

MATERIALS AND METHODS

Biological Materials. We tested 144 soybean lines from the U.S. Department of Agriculture Soybean Germplasm Collection at the University of Illinois. These included 105 exotic accessions that represented nine countries, including 15 provinces from China, and all 13 maturity groups (000 to X), as well as 23 major ancestral lines of current U.S. cultivars and 16 recently released U.S. cultivars selected to represent the currently used genetic diversity. Synthetic lunasin derived from *Glycine max* (L.) Merr. and monoclonal antibody for lunasin, developed in mice (*12*), were provided by FilGen BioSciences and were used for an enzyme-linked immunosorbent assay (ELISA) procedure that included goat anti-mouse IgG and alkaline phosphatase conjugate. Commercial soy proteins and isoflavone-enriched products were obtained from manufacturers or bought from local groceries and health food stores.

Chemicals. The reagents used were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise noted. Hexane used for sample defatting was nonspectro grade and of greater than 85% purity (American Burdick & Jackson, Muskegon, MO). Bovine serum albumin (BSA) was obtained with a minimum of 98% purity. Tris[hydroxymethyl]aminomethane hydrochloride (Tris-HCl) was biotechnology performance certified, minimum 99% purity. Phosphate-buffered saline (PBS) with polyoxyethylene sorbitan monolaurate (Tween 20), pH 7.4, was obtained in pouches with the proper concentrations (0.01 M PBS, 0.05% Tween 20). The *p*-nitrophenyl phosphate (pNPP) liquid substrate system was purchased ready to use. Anti-mouse IgG (whole molecule) conjugated with alkaline phosphatase was developed in goat and affinity isolated. Other reagents, such as analytical grade NaOH, H_2SO_4, and HCl, used occasionally to adjust buffers and to stop the color substrate reaction, were also purchased from Sigma Chemical. All electrophoresis and Western blot chemicals were purchased from Bio-Rad laboratories (Hercules, CA). The polypeptide sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) molecular mass standard purchased from Bio-Rad consisted of a mixture of triosephosphate isomerase (26625 Da), myoglobin (16950 Da), α-lactalbumin (14437 Da), aprotinin (6512 Da), insulin *b* chain oxidized (3496 Da), and bacitracin (1423 Da).

Lunasin Quantification by ELISA. *A. Protein Extraction from Soybean Genotypes.* The dried mature seeds were kept in paper envelopes at 4 °C in a dry environment. The seeds were ground into flour with a Wiley Intermediate laboratory mill (Thomas Scientific, NJ) fitted with a mesh 40 to obtain a homogeneous particle size material and stored at −20 °C until used. This ground meal material was then defatted overnight (14−16 h) in a Soxhlet extractor using hexane (T_{vap} = 67.5 °C) as the solvent. The defatted meal was air-dried in a hood for 8 h and kept at 4 °C until further use. The extraction procedure consisted of placing 0.05 g of the defatted flour and 1 mL of the extracting buffer (0.05 M Tris-HCl buffer, pH 8.2) in an Eppendorf tube. After the mixture was mixed, the samples were placed in an ultrasonic bath (Bransonic model 2510, Branson Ultrasonic Corporation, Danbury, CT) for 70 min, mixing again every 10 min to avoid settlement. The temperature of the water bath was controlled at 40 °C by using a recirculating bath (Endocal model RTE-9, Neslab Intruments, Portsmouth, NH). At least two independent extractions were performed for each sample. Following extraction, the samples were centrifuged at 20000*g* for 30 min at 4 °C in an Eppendorf Centrifuge (model 5417R, Brinkmann Instruments, Westbury, NY), and the obtained supernatant was transferred to an Eppendorf tube. This material was then diluted (1:25600) with extracting buffer for ELISA analysis. ELISA titration curves were obtained (data not shown), and sample dilution was determined based on the least variance in a good absorbance range.

To investigate the best extracting conditions, the effect of different treatments was tested as follows: (i) addition of 10% w/w polyvinylpolypyrrolidone (0.1 g PVPP in 1 g flour) and (ii) addition of 1% v/v mixture of water soluble protease inhibitor cocktail (Sigma P2714), with broad specificity for the inhibition of serine, cysteine, aspartic, and metallo-proteases. A sample was also quantified as a control, without boiling or adding PVP or protease inhibitor cocktail. All samples were extracted using 1 g of defatted flour in 10 mL of PBS buffer and then magnetically stirred for 3.5 h at room temperature.

B. Lunasin Extraction in Soy Proteins and Isoflavone-Enriched Products. Several commercial samples were chosen based on availability and extracted using a 0.1 M PBS buffer, pH 7.4. They came in one of two forms: either as a defatted powder or as texturized flakes, which were ground to a fine homogeneous particle size using a mortar. The isoflavone commercial products, either tablets or capsules, were extracted using 0.1 M PBS buffer, pH 7.4, plus 10% PVP (w/w). All samples were kept at 4 °C until analysis.

One gram of sample was added to 10 mL of the extracting buffer (0.1 M PBS, pH 7.4) and magnetically stirred for a period of 2 h. After that time, the samples were centrifuged for 30 min in an Eppendorf microcentrifuge at 20000*g* and 4 °C. The supernatant was removed, and proper dilutions were prepared for each sample for ELISA analysis. Because the samples came from different parts of the commercial process and were different in composition, dilutions differed from 1:25600 to 1:102400. Each sample was coated on the ELISA plate the same day it was extracted. The remaining supernatant was stored at 4 °C.

C. ELISA Procedure. A competitive antigen-coated (indirect) ELISA for lunasin was developed. Aliquots (100 μL) of each diluted sample were added to the well of Nunc Maxisorp 96 well plates, especially designed for ELISA, with high affinity for proteins. A standard curve of pure synthetic lunasin (prepared in distilled water) was also added (100 μL/well) using at least seven concentrations in the range of 24−72 ng/mL every time samples were run. All samples, standards, and blanks were coated in triplicate. At least two independent extractions were run for each sample and served as a reliable duplicate.

Coated plates were left at 4 °C overnight (14 h) and were washed with 0.01 M PBS and 0.05 M Tween-20 six times the next morning. Immediately after that, the plates were blocked with a BSA blocking solution (5% BSA in 0.05 M TBS-1% Tween), with 300 μL per well. Blocking took place for an hour, after which the plates were washed again with 0.01 M PBS and 0.05 M Tween-20. An amount of 50 μL of a 1:1000 dilution of the lunasin monoclonal antibody was added to each well and incubated for 1 h. After the wells were washed with 0.01 M PBS, pH 7.4, which contained 0.05% Tween-20 buffer, 50 μL of anti-mouse IgG and alkaline phosphatase conjugate (1:2000 dilution) were added and incubated for another hour. After they were washed again, 100 μL of pNPP, the color substrate of choice for alkaline phosphatase, was added and the plates were placed in the ELISA plate reader (Elx 808 IU from Biotek Instruments, Winooski, VT) at 405 nm and read after 20 min. The reaction was stopped with 100 μL of 3 N NaOH at 30 min and read again at 35 min. All washing procedures were done with 300 μL of the washing solution, six times per well at the lowest dispensing rate (150 μL/well/s) and aspiration rate (5 mm/s) to avoid protein detachment. All antibody dilutions were made in

3% BSA, 1% Tween, and 0.05 M TBS buffer. The total protein concentration was measured at 280 nm using a BSA standard curve (ϵ = 0.66 mL/cm g) in a Beckman DU640 spectrophotometer (Beckman Coulter, Fullerton, CA). Lunasin was expressed as mg/g of extracted protein and mg/g of flour.

Lunasin Identification. *Gel Electrophoresis and Western Blot Procedures.* The identity of lunasin in the soybean genotypes and commercial products was established by Western blot analysis. Samples were centrifuged (20000*g*) to eliminate any precipitate that might have formed during freezing or thawing. The supernatant (50 μL) was added to 150 μL of tricine sample buffer (Bio-Rad Laboratories) and boiled for 5 min. Also, 50 μL of synthetic lunasin solution (1 mg/mL) was added to 150 μL of sample buffer and boiled for 5 min. One well was also loaded with a broad-range protein standard (Bio-Rad) and another one with a lunasin solution (1 mg/mL), which served as a comparative standard. After the samples and standard had cooled to room temperature, 10 μL per sample was loaded into 16.5% Tris-Tricine polypeptide ready gels (Bio-Rad Laboratories). The gels were run in Mini Protean-3 Cells (Bio-Rad Laboratories) using Tris-Tricine-SDS buffer as the running buffer. The conditions were set at 55 mA constant, and gels were run for 100 min (60−125V). For electrophoresis, the gels were stained with Coomassie Brilliant G-250. The gels were fixed for 30 min in methanol/acetic acid/water (10:40:50%) and destained with acetic acid/water (40%) for 1 h and then washed once again with freshwater, left overnight, and read in a Kodak Image Station 440 CF, where the respective molecular masses and band intensities were recorded. Each protein band was normalized by dividing its intensity by the intensity of the lunasin standard on the same gel (the intensities are given as net intensities, where the value of the background is subtracted from the total). The values for both MW and net intensity were averaged between duplicates and then normalized as described above.

Unstained gels were soaked in 100 mL of blotting buffer, pH 8.3, which consisted of 10% (v/v) methanol, 25 mM Tris base, and 192 mM glycine, for 15 min. The membrane used was the Immun-Blot PVDF membrane (Bio-Rad Laboratories). After the apparatus was filled with enough blotting buffer to cover the sandwich (about 1 L), conditions were set at 40 V constant (110−140 mA) and allowed to transfer for 60 min. After the transfer was completed, the membrane was removed and washed twice with TBS buffer (20 mM Tris, 500 mM NaCl, pH 7.5). The membrane was then blocked with 25 mL of a blocking buffer (2% nonfat dry milk, 1% Tween-20 in TBS) for 1 h. The membrane was then washed twice for 15 min with TTBS buffer (20 mM Tris, 500 mM NaCl, 0.1% Tween-20, pH 7.5), incubated with the lunasin antibody (polyclonal against lunasin, developed in rabbit) at 1:3000 dilution in antibody buffer (2% nonfat dry milk in TTBS), and incubated for 1 h. After it was washed twice with TTBS buffer for 15 min, the membrane was incubated for 1 h, with an anti-rabbit secondary antibody at 1:3000 dilution developed in goat that had been conjugated to alkaline phosphatase (Bio-Rad Laboratories). The membrane was washed three times with TTBS and prepared for detection using an Immun-Star Chemiluminescent Substrate solution (Bio-Rad Laboratories) following the manufacturer's recommendations. Finally, the intensity of the bands was read in a Kodak Image Station 440 CF (Kodak, United States), with two captures and a total exposure time of 2 min. A standard curve of synthetic lunasin was obtained (y = 212713x − 7237, R^2 = 0.963), with a detection limit of 8.5 μg/mL lunasin.

Statistical Analysis. One-way analysis of variance, with Dunnet and linear trend post-test, was used for statistical analysis. A probability (P) value < 0.05 indicated a significant difference.

RESULTS AND DISCUSSION

Lunasin Analysis. The standard curve for the ELISA method is shown in **Figure 1**. This method has good reproducibility, with a recovery from spiked soybean samples of 90% and a detection limit of 8 ng/mL or 1.47 nM. This is better than that for quantitative Western analysis, which has a detection limit of 1.56 μM (**Figure 2**). The equation for the lunasin standard curve is y = 0.0117x − 0.0485, R^2 = 0.963. There was no significant difference in the concentration of lunasin in the presence of either PVP (10% w/w) or protease inhibitor cocktail (1% v/v) during the protein extraction of the soy samples.

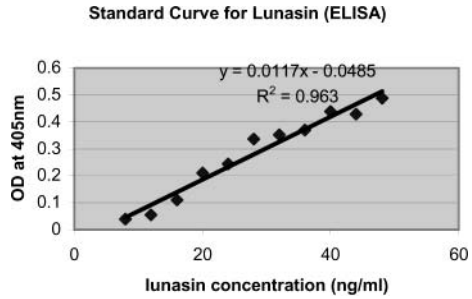

Figure 1. Standard curve of lunasin using ELISA. Eleven solutions of synthetic lunasin in the range 8–48 ng/mL prepared in distilled water gave a high linearity (R^2 = 0.963) when read in the ELISA reader 20 min after the addition of the color reagent, pNPP.

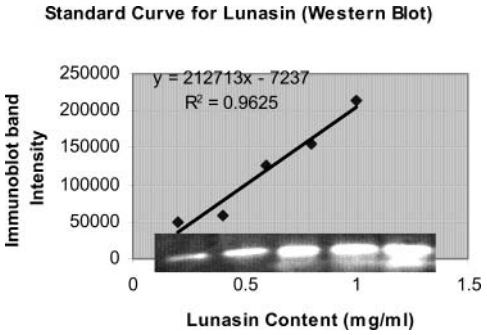

Figure 2. Standard curve of lunasin using Western blot analysis with 1:3000 dilution of the primary polyclonal antibody against lunasin developed in rabbit and 1:3000 of the goat anti-rabbit secondary antibody conjugated to alkaline phosphatase. A volume of 10 μL of five solutions in the range of 0.2–1 mg/mL of lunasin in Tricine sample buffer gave a high linearity (R^2 = 0.963) after Western blot development with chemiluminescent substrate.

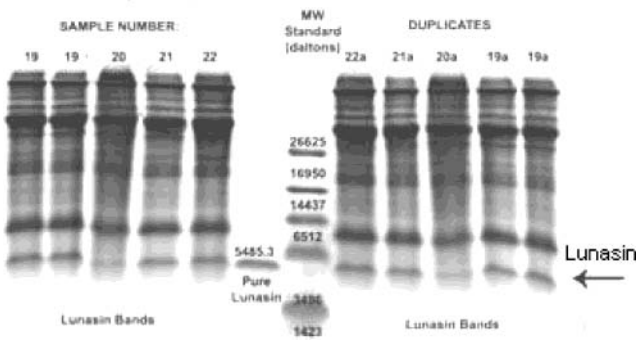

Figure 3. Coomassie Blue staining of protein extracts from different soybean products. M = polypeptide SDS–PAGE Mr marker; L = 2.5 μg of lunasin per well; 19, 20, 21, and 22 are commercial soy concentrates. The 16.5% Tris-Tricine gel was loaded with 10 μL of sample, MW standard, or lunasin in Tricine sample buffer. The lunasin band shows in all samples. The MW standard is a mixture of triosephosphate isomerase (26625 Da), myoglobin (16950 Da), α-lactalbumin (14437 Da), aprotinin (6512 Da), insulin *b* chain oxidized (3496 Da), and bacitracin (1423 Da).

The relative mass (M_r) of lunasin in the studied samples was 5.45 ± 0.25 kDa (**Figure 3**). This value is in agreement with the M_r of lunasin based on the amino acid sequence reported (*13*).

Lunasin in Different Soybean Genotypes. Lunasin concentrations in defatted soybean flour from the different geno-

Table 1. Lunasin Concentration of Exotic, Ancestral, and Modern Soybean Cultivars[a]

class	N	mg/g extracted protein[b]	mg/g flour	range[c] of mg/g flour
exotic[d]	95	5.9 ± 3.3	5.3 ± 2.9	1.0–13.3
ancestral	33	7.2 ± 1.9	6.5 ± 1.6	3.6–10.1
modern	16	6.4 ± 1.9	6.0 ± 1.7	3.3–9.5

[a] Samples were extracted with 0.05 M Tris buffer, pH 8.2, as described in the Materials and Methods section and run in ELISA. The readings from the samples were converted to concentration using a pure lunasin standard curve in each ELISA plate. [b] Mean ± SD, based on a BSA standard curve ($y = 0.66x$). [c] Range of lunasin concentration (in mg/g flour in cultivars from that category). [d] This includes primate cultivars from China, Japan, and South and North Korea.

types studied ranged from 0.1 to 1.3 g/100 g flour. Because our results are from unreplicated samples that were grown in different environments, we cannot separate the genetic and environmental effects and thus cannot make final conclusions about the genetic diversity of lunasin concentrations in soybean seeds. However, these data provide the first evidence regarding the potential genetic variation of lunasin in soybeans (**Table 1**).

The mean lunasin value of 33 ancestral lines is 6.5 mg/g, and the mean value of 16 recently released cultivars is 6.0 mg/g. The range of values among the ancestral lines (3.6−10.1 mg/g) is larger than those of current modern cultivars (3.3−9.5 mg/g), but the difference is accounted for by only three lines. Arksoy is the ancestral line with the highest lunasin concentration (10.1 mg/g defatted flour), and there are two lines, PI 594512 and PI 594726, with also high concentrations, 11.8 and 13.3 mg/g defatted flour, the highest value found among the cultivars.

The samples analyzed were grown during different years and thus exposed to a variety of environmental conditions. However, all samples were kept at 4 °C during storage. Among the U.S. cultivars, Cisne had the lowest and Kunitz has the highest concentration of lunasin. Both lines were developed at the University of Illinois. Kunitz is a near-isogenic line developed through five cycles of backcrossing with Williams-82 as the recurrent parent and PI 157440 as the donor parent of the *ti* allele, which eliminates the Kunitz trypsin inhibitor. By pedigree, Williams provides half of the genes to one of the parents of Cisne. PI 240664 has a much higher lunasin concentration than any of the other lines and is the only line in these samples that was grown in a subtropical environment. The large differences in lunasin concentration that we observed among these lines suggest that real genetic differences do exist. These results also suggest that over 50 years of selection for high yield and desirable agronomic traits has had little effect on lunasin concentration and that modifying lunasin concentration among high-yielding cultivars should be possible. Interestingly, the Bowman−Birk inhibitor null accession PI 321393 *Glycine latifolia* contains no lunasin.

There was little difference among the mean values of samples from primitive cultivars from China, Japan, and the Korean peninsula, but there is substantial variation among samples within each country. The greatest variation is observed among the accessions from China. This is not surprising, since most of the accessions analyzed are from China, and Li and Nelson (*15*) also observed greater variation for DNA markers in primitive Chinese cultivars as compared to similar lines from Korea and Japan. However, the variation among the Chinese accessions does not exceed the variation found in the U.S. ancestral lines. There may be a relationship between origin and lunasin concentration among the primitive Chinese cultivars (*16*). Accessions from Guangxi and Hunan averaged 10.2 and 9.1 mg lunasin/g defatted flour, whereas accessions from Heilongjiang and Shaanxi averaged only 2.2 and 2.9 mg/g, respectively. Other workers have also found genetic associations related to the origin of primitive Chinese cultivars. If these data are confirmed, they will provide screening strategies for future research to find greater variation in lunasin concentrations among exotic soybean accessions.

Table 2. Minimum and Maximum Values for Lunasin Concentration (mg/g Extracted Protein and mg/g Defatted Flour) in Each Maturity Group[a]

maturity group[b]	N	mg/g extracted protein[c] min	mg/g extracted protein[c] max	mg/g flour min	mg/g flour max	mg/g flour mean ± SD
0	9	2.0	11.0	1.8	9.5	5.1 ± 2.7
00	6	1.1	10.2	1.0	7.8	4.7 ± 2.3
000	2	6.3	7.2	6.2	5.8	6.0 ± 0.3
I	6	2.4	7.3	2.2	7.1	4.5 ± 2.1
II	20	2.0	9.8	2.1	8.9	5.4 ± 2.0
III	19	2.2	9.8	1.9	9.7	4.7 ± 2.3
IV	21	1.7	8.8	1.5	7.6	4.5 ± 1.9
V	13	2.2	12.6	1.7	10.4	5.7 ± 3.1
VI	16	1.8	13.2	1.5	11.8	6.4 ± 3.3
VII	14	2.7	11.3	2.3	10.4	6.5 ± 2.4
VIII	9	2.5	11.2	2.2	11.4	7.7 ± 2.5
IX	5	5.2	14.0	6.3	13.3	8.9 ± 2.8
X	4	6.9	9.9	6.4	10.1	8.1 ± 1.6

[a] Samples were extracted with 0.05 M Tris buffer, pH 8.2, as described in the Materials and Methods section and run in ELISA. The readings from the samples were converted to concentration using a pure lunasin standard curve in each ELISA plate. On the basis of BSA standard curve ($y = 0.66x$). [c] Cultivars grown in different latitudes, with X being the closest to the equator.

As with the U.S. ancestral lines and cultivars, there is no strong association between maturity group and lunasin concentration; however, the four samples with the highest lunasin concentrations were all in maturity groups IX and X and were produced in the subtropical environment of Costa Rica. **Table 2** presents the minimum and maximum values of lunasin in each maturity group for all of the soybean accessions analyzed. The samples for similar maturity groups were grown at the same latitude. All of the samples in maturity groups 000 to IV were grown in Urbana, IL. Those in maturity groups V through VIII were grown in Stoneville, MS, and those in maturity groups IX and X were grown in Costa Rica. Samples with high or low values were found in each of the locations.

Overall, the large differences in lunasin concentration of the cultivars indicate that genetic variations do exist. The lunasin concentration does not seem to be strongly related to maturity or environment in which the plant is grown, but these results do not provide the definitive answer. Differences between the U.S. ancestral lines and current cultivars are very small, so selection for yield has not indirectly changed lunasin concentration. Variation among U.S. ancestral lines is as great as variation among the larger samples of exotic accessions. Relatively closely related U.S. cultivars can have very large differences in lunasin concentrations.

Lunasin in Commercial Soy Proteins and Isoflavone-Enriched Products. The lunasin concentrations of commercially available soy proteins and isoflavone-enriched products are presented in **Tables 3** and **4**, respectively. Soy protein concentrate (60−76% protein), isolated soy protein (84.6−88.2% protein), and hydrolyzed soy protein (82% protein) contained 2.8 ± 0.3, 3.8 ± 0.4, and 4.4 ± 0.6 g lunasin/100 g flour, respectively. These values represent 7.0 ± 0.9, 8.1 ± 0.8, and 7.7 ± 0.6% of the extracted protein, respectively. Soy flour (48%

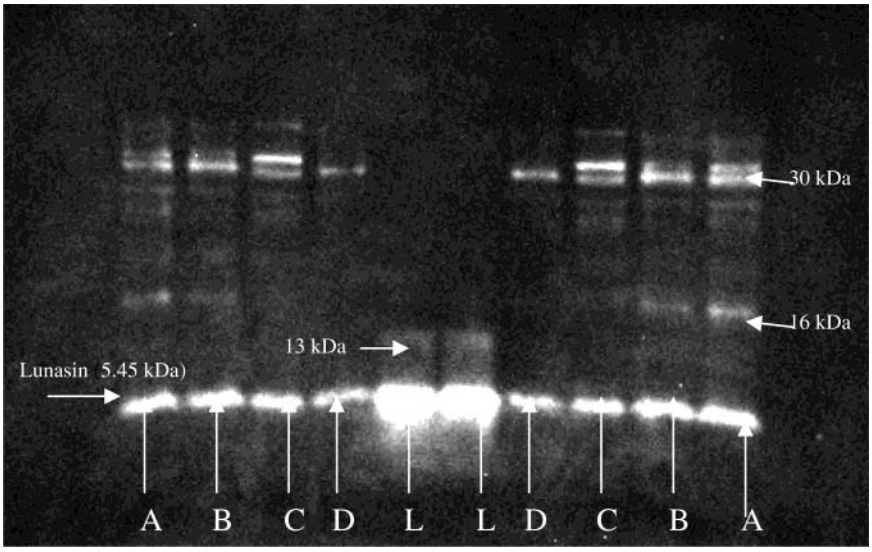

Figure 4. Gel of different commercial samples showing the band for lunasin by Western blot analysis. A = hydrolyzed soy protein; B = isolated soy protein; C = soy flakes; D = soy protein concentrate; L = pure lunasin standard (2.5 μg of lunasin per well). Wells on the 16.5% Tris-Tricine gel were loaded with 10 μL of sample or lunasin in Tricine sample buffer. The proteins were transferred to a PVDF membrane and blocked and incubated with a 1:3000 dilution of the primary polyclonal antibody against lunasin developed in rabbit, and a 1:3000 dilution of the goat anti-rabbit secondary antibody conjugated to alkaline phosphatase. Finally, the membrane was developed with the chemiluminescent substrate for 5 min and read in the Kodak Image Station 440 CF.

Table 3. Lunasin Concentrations of Commercially Available Soy Proteins[a]

	mg lunasin/ g extracted protein[b]		mg lunasin/ g flour	
sample	mean	SD	mean	SD
soy flour (48% protein)	20	4	14	3
soy flakes (50% protein)	16	3	13	2
soy flour (50% protein)	21	2	12	2
soy powder	34	3	14	2
soy protein concentrate (60–76% protein)	70	9	28	3
isolated soy protein (84.6–88.2% protein)	81	8	38	4
hydrolyzed soy protein (82% protein)	77	6	44	6

[a] Samples were extracted with 0.1 M PBS buffer, pH 7.4, as described in the Materials and Methods section and run in ELISA. The readings from the samples were converted to concentration using a pure lunasin standard curve in each ELISA plate. On the basis of BSA standard curve ($y = 0.66x$).

protein) and soy flakes (40−50% protein) contained a combined average value of 1.3 ± 0.2 g lunasin/100 g flour, equivalent to 1.6 ± 0.3 g lunasin/100 g extracted protein.

A soy saponin concentrate and a textured soy concentrate had insignificant amounts of lunasin. A lunasin-enriched flour presented the highest concentration of 27.3 mg/g solid material.

The evidence gathered so far on the health benefits of isoflavones has led to the appearance of soy isoflavone-enriched products on the market (*17*). The lack of data on the lunasin contents of these products led us to analyze for such products, and the results are shown in **Table 4**. These products contain very little or no lunasin except for two products (Soy Care for Menopause and Low Isoflavone), which have values in the low range of lunasin contents found in the soy proteins. This is likely due to the low solubility of lunasin in ethanol, which was used to extract isoflavones (*18*).

These results indicate the effects of processing on lunasin concentrations of commercially available soy protein fractions. The lunasin concentration in other commercial soy products has been recently reported by Jeong et al. (*14*).

Table 4. Lunasin Contents of Commercial Isoflavone Products[a]

sample	lunasin content (mg/g solid material)
Now extra Strength Soy Isoflavones, 60 mg (soy isoflavone extract, rice flour)	1.0 ± 0.16
Vitamin World Soy Isoflavones, 23 mg (soy extract)	1.4 ± 0.37
PhytoNutramins Isoflavones, 11 mg (isoflavones from soybean flour)	3.1 ± 0.22
TwinLab Soy Germ Isoflavone Caps, 10 mg	4.8 ± 0.62
Soy Care for Menopause, 25 mg (soy extract, soy protein concentrate)	11.6 ± 0.37
Low Isoflavone	15.1 ± 0.40
High Isoflavone (GCP)	ND
Nature's Resource Soy Balance, 65 mg	ND
NovaSoy Soy Isoflavones, 50 mg	ND
NovaSoy Soy Isoflavone Concentrate	ND
PhytoNutramins Genistein Plus	ND

[a] ND = nondetectable levels of lunasin.

Figure 4 shows the results of a Western blot performed on several commercial soy proteins and synthetic lunasin standards. The pure lunasin standard shows two bands, one at 5.45 kDa and the other around 13 kDa, that appears to have a binding site for the polyclonal antibody and thus gives a signal. Hydrolyzed soy protein and isolate also give a distinct band at 16 kDa that might correspond to a trimer of lunasin. Interestingly enough, and in accordance with that hypothesis, all products show bands around the 30 kDa size that might be lunasin hexamers. If true, it would be interesting to determine the nature of the interaction among these monomers.

In summary, the variations in lunasin concentration observed in a large number of cultivars indicate that the levels of lunasin in soybeans can be genetically manipulated. It is also clear that large-scale processing of soy to produce different soy protein fractions influences lunasin concentration.

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LITERATURE CITED

(1) Messina, M. J.; Barnes, S. The role of soy products as cancer preventive agents. *J. Natl. Cancer Inst.* **1991**, *83*, 541−546.

(2) Korhonen, H.; Pihlanto, A. Food-derived bioactive peptides: Opportunities for designing future foods. *Curr. Pharm. Des.* **2003**, *9* (16), 1297−1308.

(3) Kim, S. E.; Kim, H. H.; Kim, J. Y.; Kang, Y. I.; Woo, H. J.; Lee, H. J. Anticancer activity of hydrophobic peptides from soy proteins. *BioFactors* **2000**, *12* (1−4), 151−155.

(4) Kitts, D. D.; Weiler, K. Bioactive proteins and peptides from food sources: Applications of bioprocesses used in isolation and recovery. *Curr. Pharm. Des.* **2003**, *9* (16), 1309−1323.

(5) Shin, Z.-I.; Yu, R.; Park, S.-A.; Chung, D. K.; Ahn, C.-W.; Nam, H.-S.; Kim, K.-S.; Lee, H. J. His-His-Leu, an angiotensin I converting enzyme inhibitory peptide derived from Korean soybean paste, exerts antihypertensive activity in vivo. *J. Agric. Food Chem.* **2001**, *49*, 3004−3009.

(6) Pena-Ramos, E. A.; Xiong, Y. L. Antioxidant activity of soy protein hydrolysates in a liposomal system. *J. Food Sci.* **2002**, *67* (8), 2952−2956.

(7) Nakamori, T. Antiobesity effects of soy proteins and soy peptides. *Food Style* **2002**, *21* (5), 86−88.

(8) Abe, M.; Suzuki, O.; Tasaki, K.; Tominaga, K.; Wakasa, H. Analysis of lectin binding properties on human Burkitt's lymphoma cell lines that show high spontaneous metastasis to distant organs in SCID mice. *Pathol. Int.* **1996**, *46*, 977−983.

(9) de Lumen, B. O.; Galvez, A. F. Soybean protein nutraceuticals. United States Patent No. 6,391,848 B1, May 21, 2002, *49*, 1069−1086.

(10) Jeong, H. J.; Lam, Y.; de Lumen, B. O. Barley lunasin suppresses ras-induced colony formation and inhibits core histone acetylation in mammalian cells. *J. Agric. Food Chem.* **2002**, *50*, 5903−5908.

(11) Galvez, A. F.; Revilleza, M. J. R.; De Lumen, B. O. A novel methionine-rich protein from soybean cotyledon: Cloning and characterization of cDNA (accession No. AF005030). Plant Gene Register #PGR97-103. *Plant Physiol.* **1997**, *114*, 1567−1569.

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

(13) Galvez, A. F.; de Lumen, B. O. A soybean cDNA encoding a chromatin-binding peptide inhibits mitosis of mammalian cells. *Nat. Biotechnol.* **1999**, *17*, 495−500.

(14) Jeong, H. J.; Park, H. J.; Lam, Y.; de Lumen, B. O. Characterization of lunasin isolated from soybean. *J. Agric. Food Chem.* **2003**, *51*, 7901−7906.

(15) Li, Z.; Nelson, R. L. Genetic diversity among soybean accessions from three countries measured by RAPDs. *Crop Sci.* **2001**, *41*, 1337−1347.

(16) Li, Z.; Nelson, R. L. RAPD marker diversity among soybean and wild soybean accessions from four Chinese provinces. *Crop Sci.* **2001**, *41*, 1337−1347.

(17) IFT Weekly Newsletter. Fortified foods market reaches $18 billion. April 17, 2002.

(18) Liu, K. *Soybeans: Chemistry, Technology, and Utilization*; Chapman and Hall: New York, 1997.

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