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β-Conglycinins among Sources of Bioactives in Hydrolysates of Different Soybean Varieties That Inhibit Leukemia Cells in Vitro

WENYI WANG,[†] NEAL A. BRINGE,[§] MARK A. BERHOW,[#] AND ELVIRA GONZALEZ DE MEJIA*,[†]

Department of Food Science and Human Nutrition, University of Illinois at Urbana−Champaign, Urbana−Champaign, Illinois 61801; The Monsanto Company, St. Louis, Missouri 63167; and Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604

Soybean is a complex matrix containing several potentially bioactive components. The objective was to develop a statistical model to predict the in vitro anticancer potential of soybean varieties based on the correlation between protein composition and bioactive components after simulated gastrointestinal enzyme digestion with their effect on leukemia mouse cells. The IC_{50} values of the hydrolysates of soy genotypes (NB1−NB7) on L1210 leukemia cells ranged from 3.5 to 6.2 mg/mL. Depending on genotype, each gram of soy hydrolysates contained 2.7−6.6 μmol of total daidzein, 3.0−4.7 μmol of total genistein, 0.5−1.3 μmol of glycitein, 2.1−2.8 μmol of total saponins, 0.1−0.2 μmol of lunasin, and 0.1−0.6 μmol of Bowman−Birk inhibitor (BBI). The IC_{50} values calculated from a partial least-squares (PLS) analysis model correlated well with experimental data ($R^2 = 0.99$). Isoflavones and β-conglycinin positively contributed to the cytotoxicity of soy on L1210 leukemia cells. Lunasin and BBI were potent L1210 cell inhibitors ($IC_{50} = 13.9$ and 22.5 μM, respectively), but made modest contributions to the activity of defatted soy flour hydrolysates due to their relatively low concentrations. In conclusion, the data demonstrated that β-conglycinins are among the major protein components that inhibit leukemia cell growth in vitro. Furthermore, it was feasible to differentiate soybean varieties on the basis of the biological effect of their components using a statistical model and a cell-based assay.

KEYWORDS: β-Conglycinin; glycinin; soy peptides; lunasin; Bowman−Birk inhibitor; isoflavone; L1210 leukemia cells

INTRODUCTION

Epidemiological studies have associated soy consumption with lowering cancer risk. One meta-analysis of 18 epidemiological studies indicated that soy consumption may lead to a modest reduction in breast cancer risk (*1*). Another meta-analysis, of two cohort and six case-control studies, associated the consumption of soy foods with a lower risk of prostate cancer in men (*2*). A prospective study in Japan following 13,894 men and 16,327 women for 8 years revealed that increased soy food consumption lowered colon cancer risk in women (*3*). However, the anticancer potential of soy foods is not conclusive, and the chemopreventive components in soy remain to be fully identified (*4*).

Soybean is a complex matrix containing several bioactive components, including isoflavones, saponins, lunasin, Bowman−Birk inhibitor (BBI), and other soy proteins and bioactive peptides. Soy-derived isoflavones (daidzein and genistein but not glycitein) were reported to show growth inhibition on adult T-cell leukemia cell lines ED-40515 and Hut102 at concentrations of 10−30 μM (*5*). Soy saponins extract, BBI (a 71 amino acid protease inhibitor extracted from soybean), and lunasin (a 43 amino acid peptide naturally present in soybean seeds) have also been found to have anticancer capacity or in vitro chemopreventive effects (*6–9*).

Protein is the most abundant component in soybean. On average, soybean contains 40% protein conformed by a complex mixture of different protein types (*10*). In the ExPASy database, to date, there are a total of 1411 protein entries (333 Swiss-Prot entries and 1145 TrEMBL entries) listed for soybean (*Glycine max*). The major components of soy proteins are seed storage proteins known as β-conglycinin and glycinin, which account for 50−70% of total seed proteins (*11, 12*). β-Conglycinin is a trimer with a molecular mass of 150−200 kDa. It

* Address correspondence to this author at 228 ERML, MC-051, 1202 W. Gregory Dr., Urbana−Champaign, IL 61801 [telephone (217) 244-3196; fax (217) 265-0925; e-mail edemejia@uiuc.edu].
[†] University of Illinois at Urbana−Champaign.
[§] The Monsanto Co.
[#] U.S. Department of Agriculture.

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is composed of three subunits, α, α′, and β (*13*). The α and α′ subunits consist of core regions with a high degree of homology (86.8%) and extension regions (α, 125 residues; α′, 141 residues) exhibiting lower homologies (57.3%), whereas the β subunit consists of only a core region that has homology with the α and α′ core regions (75.5 and 71.4%, respectively) (*14*). Glycinin is a hexamer of 320−375 kDa and with five major subunits, G1−G5. Each subunit consists of an acidic chain (about 40 kDa) and a basic chain (about 20 kDa), joined by disulfide bonds. G1−G3 can be grouped as they share 90% sequence homologies (*10*). Similarly, G4 and G5 share 90% sequence homologies. However, sequence homologies between these two groups (G1−G3 and G4−G5) are only 50%. In addition, there are many enzymes (such as lipoxygenase, chalcone synthase, catalase, and urease) in soybean, but only a relatively small number of them exceed 1% of total seed protein (*10*). Upon ingestion, soy proteins are digested to peptides by gastrointestinal enzymes. These peptides have been found to be bioactive and exert anticancer, antihypertensive, hypocholesterolemic, antiobesity, antioxidant, or immunomodulatory activities (*15*).

It has been well accepted that soy exerts health benefits through a concerted action of several components (*16, 17*). However, it is challenging to identify the contribution of each component to the health benefits of soy diet. The objective of this study was to develop a statistical model to predict the in vitro anticancer potential of soybean varieties on the basis of the correlation between protein composition and bioactive components after simulated gastrointestinal enzyme digestion with their effect on leukemia mouse cells and, thus, to identify the relative contribution of the major bioactive soybean components in the inhibition of leukemia cell proliferation.

MATERIALS AND METHODS

Materials. Seven conventional soybean genotypes, NB1−NB7, were provided by the Monsanto Co. (St. Louis, MO). Defatted soy flour for the purification of β-conglycinin and glycinin and soy protein isolate (SPI) were obtained from the Illinois Center for Soy Products, University of Illinois Urbana−Champaign. Tricine sample buffer, 2-mercaptoethanol, and Precision Plus protein standard for SDS-PAGE were purchased from Bio-Rad (Hercules, CA). Gradient 8−25% Phast Gels were purchased from GE Healthcare (Piscataway, NJ). Pepsin (EC 3.4.23.1, 662 units/mg), pancreatin (8× USP, from porcine pancreas), and purified BBI were purchased from Sigma (St. Louis, MO). Synthetic lunasin was purchased from American Peptide Co. (Sunnyvale, CA). Immunoaffinity-purified lunasin (98% purity) from soy and rabbit polyclonal antibody against the lunasin epitope−EKHIMEKIQGRGD-DDDD was provided by Dr. Ben O. de Lumen, University of California−Berkeley. Soy isoflavone concentrates Novasoy 400 (44.70%, predominantly in glycoside form) and Novasoy 700 (73.25%, predominantly in glycoside form) were provided by Archer Daniels Midland Co. (ADM, Decatur, IL). Purified B group soy saponins were provided by Dr. Mark Berhow from the USDA. Mouse leukemia cell line L1210 was obtained from the American Type Culture Collection (Rockville, MD).

Preparation of Defatted Soy Flour. Soybean samples (150 g) were ground using a Thomas-Wiley model 4 mill (Thomas-Wiley, Swedesboro, NJ) with a 2 mm screen. Ground materials were then reground in the same mill using a 1 mm sieve. Samples were extracted using hexane with traditional Soxhlet extractor. Aliquots of approximately 25 g were extracted for a minimum of 5 h. Extracted samples were air-dried for 2−3 h, and samples from the same soybeans were combined.

Preparation of Soy Protein Hydrolysates. In vitro digestions of defatted soy flours (NB1−NB7) and purified glycinin and β-conglycinin were performed in a way to simulate the in vivo enzyme hydrolysis by following Megias's procedure with slight modifications (*18*). Briefly, soy samples were suspended in water (1:20 w/v) and heated at 80 °C for 5 min to reduce bacteria population and to denature lipoxygenase. Then a sequential enzyme digestion was carried out with pepsin [EC 3.4.23.1, 662 units/mg; enzyme/flour, 1:20 (w/w); pH 2] and pancreatin [8× USP; enzyme/flour, 1:20 (w/w); pH 7.5] at 37 °C for 3 h each. Pancreatin (8× USP, from porcine pancreas) converts not less than 200 times its weight of USP potato starch reference standard into soluble carbohydrates and not less than 200 times its weight of casein into peptides. Pancreatin hydrolyzes fats to glycerol and fatty acids, changes proteins into peptides and derived substances, and converts starch into dextrins and sugar. It contains lipase, not less than 16 USP units/mg; protease, not less than 200 USP units/mg; and amylase, not less than 200 USP units/mg. The hydrolysis was stopped by heating at 75 °C for 20 min. The resulting hydrolysate was centrifuged at 27000*g* for 15 min. The supernatant was filtered through a 0.22 μm polyvinylidene fluoride (PVDF) membrane and lyophilized in a FreeZone freeze-dry system (Kansas City, MO). The seven respective hydrolysates of NB1−NB7 were named as NBH1−NBH7. All samples were stored at −80 °C until analysis.

Purification of β-Conglycinin and Glycinin from Defatted Soy Flour. β-Conglycinin and glycinin were purified in our laboratory by following published procedures with some modification (*19, 20*). One hundred grams of defatted soy flour was suspended in 1.5 L of distilled H_2O and the pH adjusted to 7.5 with 2 N NaOH. The solution was centrifuged at 9000*g* for 30 min. For glycinin purification, sodium bisulfite (SBS) (0.98 g/L) was added to the supernatant and the pH was adjusted to 6.4 with 2 N HCl. It was then stored overnight at 4 °C and centrifuged at 6500*g* for 20 min at 4 °C. The precipitate was washed with 40 mL of ice-cold distilled H_2O and labeled as purified glycinin. For β-conglycinin purification, the first step was similar to glycinin purification except the pH was adjusted to 5.9 to obtain high-purity β-conglycinin. After centrifugation, NaCl was added to the supernatant to make a salt concentration of 0.25 M, and the pH was adjusted to 5.0 with 2 N HCl. After 1 h of storage at 4 °C, the supernatant was collected after centrifugation at 9000*g* for 30 min at 4 °C. Two volumes of ice-cold distilled H_2O was added to the supernatant and the pH adjusted to 4.8 with 2 N HCl. The precipitate was collected from 6500*g* for 20 min at 4 °C and washed twice with 40 mL of cold distilled H_2O. Then it was suspended in 25 mL of water and pH adjusted to 7.5. This was designated purified β-conglycinin. Both purified β-conglycinin (>80% purity) and glycinin (>97% purity) were lyophilized and stored at −70 °C until use.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis of Soy Flours and Purified β-Conglycinin and Glycinin. Ground soy flours were extracted with 1× Laemmli SDS buffer, pH 6.8, with 0.07 M DTT (~30 mg/mL). For each sample, 10 μg of total protein was run on a 26-lane 12% homogeneous Bis-Tris Criterion gel. Myosin (193.9 kDa), β-galactosidase (103.6 kDa), bovine serum albumin (59.9 kDa), ovalbumin (41.4 kDa), carbonic anhydrase (27.4 kDa), soybean trypsin inhibitor (20.8 kDa), lysozyme (15.5 kDa), and aprotinin (6.6 kDa) were used as molecular mass markers. The gels were stained in Colloidal Coomassie Blue G-250, destained in distilled water, and imaged using the GS 800 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, CA). Protein quantitation was performed using Bio-Rad Quantity One software. The software was used to determine the relative quantity of each band in the sample lane. Proteins were reported as the relative percent of the total protein bands in the lane. Amino acid sequences of major soy proteins were retrieved from UniProtKB/Swiss-Prot release 54.1 of August 21, 2007. The theoretical molecular weight of each protein was calculated from amino acid sequence with the ProtParam program (http://ca.expasy.org/tools/protparam.html). Identification of protein bands of the seven soy genotypes was confirmed by comparing theoretical molecular weight with experimental data.

Isoflavone and Saponin Analysis. To determine isoflavone and saponin concentrations, the seven soy protein hydrolysates were extracted with a dimethyl sulfoxide/methanol (1:1) solution at room temperature for 4 h, followed by a 15 min sonication at 50 °C and another 1−2 h extraction at room temperature. The extracts were then filtered through a 0.45 μM nylon 66 filter for both isoflavone and saponin analysis. HPLC analysis was conducted on a Hewlett-Packard

series 1100 HPLC system equipped with an Inertsil ODS-3 reverse phase C-18 column (5 μm, 250 × 4.6 mm, with a Metaguard column, from Varian) and a G1316A column oven. The system was controlled by HPChem Station version A.06.01. For isoflavone analysis, a linear water−methanol gradient from 20 to 100% in 53 min was used, with 0.025% trifluoroacetic acid (TFA) added to both solvents. The flow rate was set at 1 mL/min, and the effluent was monitored at 285 nm. Isoflavone concentration was calculated by using the standard curves of pure standards of daidzein, glycitein, and genistein. For saponin analysis, a linear water−acetonitrile gradient from 30 to 50% in 45 min was used, with 0.025% TFA added to both solvents. The flow rate was set at 1 mL/min, and the effluent was monitored at 210 nm. Saponins concentrations were calculated by using standard curves prepared from a characterized mix of B group saponins prepared in the laboratory. The extinction coefficient for soyasaponin I was used to quantitate the A group saponins and the 1,l-dimethyl-4-phenylpiperzinium (DMPP) conjugated B group saponins. Identification of isoflavone and saponin peaks was confirmed by comparison of standard and/or LC-MS analysis (*21*).

Chymotrypsin Inhibitory Units (CTIU) Determination. The concentrations of total chymotrypsin inhibitors in NBs was measured as CTIU by following published procedures (*22*). Briefly, NBs were extracted at pH 9.5−9.8 using dilute sodium hydroxide. A known volume of chymotrypsin solution was mixed with the sample suspension or with a sample blank to measure the base level of protein in the reaction mixtures. Casein was then added as the substrate for chymotrypsin hydrolysis. The hydrolysis was stopped at exactly 10 min by adding trichloroacetic acid. The amount of chymotrypsin inhibitor was calculated from the decrease in trichloroacetic acid soluble protein, as determined by absorbance at 275 nm with a UV spectrophotometer. One chymotrypsin inhibition unit is defined as a decrease of 0.01 absorbance unit at 275 nm in a 1 cm path length cell per 10 mL of final reaction volume after 10 min of reaction.

Enzyme-Linked Immunosorbent Assay (ELISA) for Lunasin and BBI. Lunasin concentration of soy protein hydrolysates was analyzed by ELISA as described previously (*23*) with the following modifications. Rabbit lunasin polyclonal antibody (1:4000 dilution) was used as the first antibody and anti-rabbit alkaline phosphatase (AP) conjugated IgG (1:7000, Amersham Biosciences) as the secondary antibody. The reaction was stopped with 25 μL of 3 N NaOH at 30 min and the absorbance (405 nm) read at 35 min. A similar procedure was used for BBI analysis, except that for BBI, mouse BBI monoclonal antibody (1:1000 dilution, Agdia, Inc., Elkhart, IN) was used as the first antibody and anti-mouse alkaline phosphatase (AP) conjugated IgG (1:2000, Amersham Biosciences) as the secondary antibody. Standard curves were determined using synthetic lunasin and purified BBI as lunasin (ng/mL) = $0.036A_{405} - 0.0832$, $R^2 = 0.97$, and BBI (ng/mL) = $0.0112A_{405} + 0.2363$, $R^2 = 0.95$.

L1210 Leukemia Cell Cytotoxicity Assay. L1210 leukemia is a mouse lymphocytic leukemia cell line. It has been extensively used as a screening model for anticancer drugs (*24, 25*). For the cytotoxicity assay, L1210 leukemia cells were cultured at 37 °C in a 5% humidified CO_2 atmosphere in Minimum Essential Medium (MEM, Invitrogen, Carlsbad, CA) supplemented with 10% bovine serum. The log phase cell suspension (90 μL) was plated on 96-well flat-bottom cell culture plates to make 2000 cells/well. After incubation for 24 h, the L1210 leukemia cells were treated respectively with nonhydrolyzed soy protein (7 mg/mL), soy protein hydrolysates (0.3−8 mg/mL), synthetic lunasin (1−80 μM), purified soy BBI (0.1−500 μM), and Novasoy 400 and Novasoy 700 (total isoflavone 3−203 μM). Etoposide was included as a positive control. The plates were then incubated for 48 h at 37 °C in a 5% CO_2 atmosphere. Cell Counting Kit-8 (Dojindo Inc., Gaithersburg, MD) was used to determine the number of viable cells. The results were expressed as percent viability of treated cells compared with the untreated control.

Partial Least-Squares (PLS) Regression Analysis. PLS regression analyses were carried out using SAS version 9 (with the proc PLS function) to evaluate the relationship between bioactive soy components (X variables, including matrix protein profile, concentrations of genistein, daidzein, glycitein, total saponins, lunasin, and BBI in soy hydrolysates) and the IC_{50} (concentration needed to inhibit 50% of cell growth) values of the seven soy hydrolysates (NBH1−NBH7) on L1210 leukemia cells (Y variable). A regression model, $IC_{50} = XWQ + E$ (where X is dependent variable matrix, W is weight matrix for X, Q is the loading matrix for IC_{50}, and E is the error term), was developed to predict in vitro L1210 leukemia inhibitory activity from soy components and to evaluate the contribution of each component on this activity. Because soy protein hydrolysates contain numerous peptides, it is not possible to consider the contribution of each one, and, most likely, peptides work together through additive and synergistic ways; therefore, isolating one or two peptides has its inherent limitations. For this reason, we considered the matrix protein profile instead. Because saponins, isoflavones, lunasin, and BBI concentrations may change during hydrolysis, their concentrations in the hydrolysates were used in the PLS model.

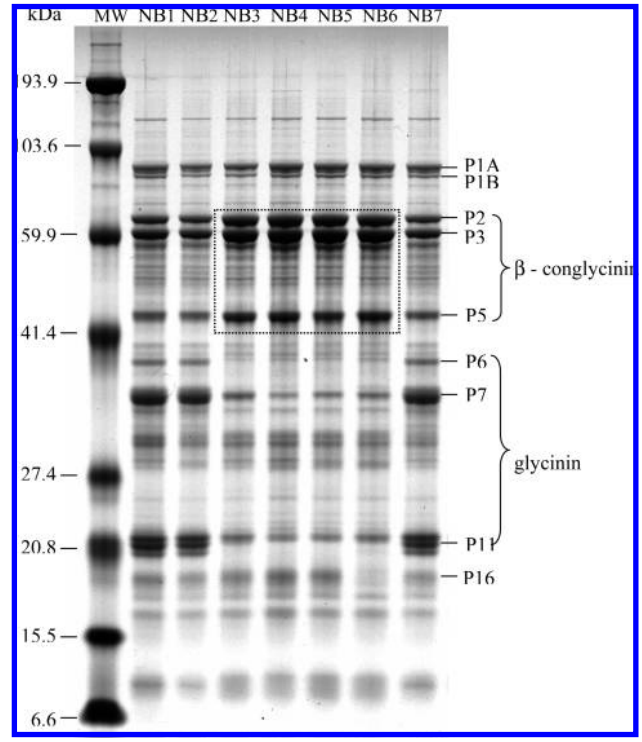

Figure 1. Protein profiles of soy flours from seven soy genotypes (NB1−NB7). For each sample, 10 μg of total protein was run on a 26-lane 12% homogeneous Bis-Tris Criterion gel. Major protein bands were coded and identified as P1A, lipoxygenases 2 and 3; P1B, lipoxygenase 1; P2, α' subunit of β-conglycinin; P3, α subunit of β conglycinin; P5, β subunit of β-conglycinin; P6, glycinin A3 chain; P7, glycinin A1,2,4 chains; P11, glycinin basic chains; P16, Kunitz trypsin inhibitor. The molecular mass markers (lane 1) consisted of myosin (193,916 Da), β-galactosidase (103,624 Da), bovine serum albumin (59,883 Da), ovalbumin (41,374 Da), carbonic anhydrase (27,354 Da), soybean trypsin inhibitor (20,778 Da), lysozyme (15,505 Da), and aprotinin (6598 Da).

Other Statistical Analysis. The concentration needed to inhibit 50% of L1210 leukemia cell growth (IC_{50}) was determined by nonlinear regression (curve fit) using GraphPad Prism software. The difference between the IC_{50} of each sample was evaluated by using one-way ANOVA with Tukey's multiple-comparison test, and $p < 0.05$ was considered to be significant. The differences among bioactive components composition in NBs and NBHs were analyzed by the PROC GLM procedure of SAS (SAS Institute, Cary, NC). The difference between glycinin and β-conglycinin groups was tested by unpaired t test.

RESULTS AND DISCUSSION

Electrophoretic Profile of Seven Soy Genotypes. The average total protein concentration of NB1−NB7 was 45.1 ± 2.3%, ranging from 40.5 to 48.2%. Protein extracts of different soy genotypes showed different protein profiles (**Figure 1**). NB1,

Table 1. Calculated Molecular Masses of Major Soy Proteins[a]

	name	accession no.	no. of aa	mol mass (Da)
β-conglycinin	α′ subunit	gil9967361	559	65142.6
	α subunit	gil9967357	543	63164.8
	β subunit	gil9967359	416	47975.7
glycinin	**G1 precursor**	**P04776**	**495**	**55706.3**
	A1a chain	CHAIN_20−306	287	32646.9
	Bx chain	CHAIN_311−490	180	19955.5
	G2 precursor	**P04405**	**485**	**54390.7**
	A2 chain	CHAIN_19−296	278	31622.8
	B1a chain	CHAIN_301−480	180	19773.2
	G3 precursor	**P11828**	**481**	**54241.7**
	A chain	CHAIN_22−296	275	31483.7
	B chain	CHAIN_297−476	180	19911.4
	G4 precursor	**P02858**	**562**	**63587.1**
	A5 chain	CHAIN_24−120	97	10540.8
	A4 chain	CHAIN_121−377	257	29953.9
	B3 chain	CHAIN_378−562	185	20743.5
	G5 precursor	**P04347**	**516**	**57956.1**
	A3 chain	CHAIN_25−344	320	36392.4
	B4 chain	CHAIN_345−516	172	19049.5

[a] Amino acid sequences of major soy proteins were retrieved from UniProtKB/Swiss-Prot release 54.1 of Aug 21, 2007, and the theoretical molecular weight of each protein was calculated using the ProtParam program (http://ca.expasy.org/tools/protparam.html).

NB2, and NB7 contained both glycinin and β-conglycinin subunits, whereas NB3−NB6 were enriched in β-conglycinin subunits. To identify each band on the protein profile, theoretical molecular masses of major soy proteins were calculated (**Table 1**). The molecular masses of β-conglycinin subunits ranged from 47.9 kDa (β subunit) to 65.1 kDa (α′ subunit), whereas the glycinin subunits ranged from 10.5 kDa (A5 chain) to 36.4 kDa (A3 chain). The acidic and basic chains of glycinin joined together by disulfide bonds to form glycinin precursors (G1−G5). Their molecular masses are also presented in **Table 1**. However, the precursors are not shown in **Figure 1** because the gel was run under reducing conditions.

Table 2 presents the identity of protein bands in **Figure 1** and their molecular masses calculated from the molecular mass marker. The percent contribution of each protein to total proteins is also presented. Nine major protein bands on the SDS-PAGE gel were identified, and the identities of these bands agreed with other publications (*26–28*). The total β-conglycinin and total glycinin content of the seven genotypes ranged from 25.6 to 50.6% and from 3.9 to 37.9%, respectively. On the basis of the percentage of total β-conglycinin and total glycinin, one-way ANOVA analysis divided seven genotypes into two groups, a β-conglycinin featured group (NB3−NB6, total β-conglycinin concentration > 40%), and a glycinin featured group (NB1, NB2, and NB7, total glycinin concentration > 30%).

Glycinin percent in the glycinin featured group (NB1, NB2, and NB7) was comparable to other reported values obtained with SDS-PAGE analysis, such as 36.3−51.3% (*29*) and 31.4−38.3% (*30*). The β-conglycinin featured group was bred for lower glycinin content. A comparison study of soybean cultivars released during the past 60 years indicated that the soy protein composition has not varied substantially through selective breeding (*31*). However, diverse protein compositions are possible using subunit null lines (*32–34*) that have low glycinin profiles similar to those of NB3−NB6.

The seven soy genotypes also contained different amount of lipoxygenase 1 (1.6−2.3%), lipoxygenases 2 and 3 (4.9−6.9%), and Kunitz trypsin inhibitor (1.5−4.9%), as shown in **Table 2**. The total chymotrypsin inhibitory units of NB1−NB7 were 18.5, 11.1, 20.4, 20.0, 25.1, 19.9, and 14.5 IU, respectively.

The difference in protein profiles of each soy genotype provided different amounts of various amino acid sequences (*35*). Therefore, variations in bioactive peptide profiles were expected after gastrointestinal digestion. Thus, the protein composition heterogeneity of seven soy genotypes allowed us to study the effect of matrix proteins on the in vitro anticancer potential of soy hydrolysates.

Isoflavone and Saponin Concentration of Soy Protein Hydrolysates. The objective of this paper was to evaluate the contribution of several soy bioactive components (not only peptides) to the anticancer activity. Therefore, it was important to include isoflavones and saponins in the model, because these compounds have also reported biological activity. Isoflavone and saponin concentrations in the seven soy protein hydrolysates were analyzed to evaluate their role in the bioactivity of the hydrolysates (**Table 3**). The isoflavone concentrations of NBH1−NBH7 ranged from 2.66 to 6.62 μmol/g of hydrolysate for total daidzein, from 3.01 to 4.71 μmol/g for total genistein, and from 0.53 to 1.28 μmol/g for glycitein. Compared to the average of nonhydrolyzed soy flour, hydrolysis did not significantly change total isoflavone concentration or isoflavone distribution as glycoside or aglycone forms (data not shown). This agreed with previous findings (*36*). Daidzein and genistein concentrations in NBH1−NBH7 were more abundant than glycitein. This followed the same trend as in other soy cultivars (*37*).

Total saponin concentrations of soy hydrolysates ranged from 2.09 to 2.77 μmol/g (0.20−0.26%). Comparing these values with saponin concentrations in soy flours (0.80−4.66 μmol/g of soy flour, 0.07−0.42%), an average of 64 ± 15% of saponin was retained through digestion. Saponin concentrations in soy flours were lower than other reported values, which were 0.5−2% (*37*) and 0.6−6.2% of dry weight (*21*), depending on cultivars and growing conditions (*38*). Even though isoflavones and saponins were not significantly affected by hydrolysis, their true concentrations in the hydrolysates were considered in the model.

Lunasin and BBI Concentrations of Soy Protein Hydrolysates. Lunasin and BBI concentrations in soy hydrolysates are presented in **Table 3**. Lunasin concentrations ranged from 0.10 to 0.21 μmol/g of hydrolysate (0.54−1.07 mg/g), which correspond to 3.9−7.6% of reported value in defatted soy flour (2.6 μmol or 14 mg of lunasin per gram of flour) (*23*). This generally agreed with our findings (unpublished) that after simulated gastrointestinal digestion, 3% lunasin remained active. BBI concentration in hydrolysates ranged from 0.12 to 0.63 μmol/g of hydrolysate (0.95−5.02 mg/g), corresponding to 17−91% of the reported highest BBI concentration in nonhydrolyzed soy flours (5.5 mg/g) (*39*). These results showed that lunasin and BBI were digested but not totally eliminated during hydrolysis. Therefore, due to their proven anticancer potential, BBI and lunasin concentrations present in the hydrolysates were included in the correlation study. The structure of BBI linked to the number and distribution of disulfide bridges seems to be a major contributor to prevent structural changes induced by harsh conditions during gastrointestinal digestion (*40*).

Cytotoxicity of Soy Protein Hydrolysates on L1210 Leukemia Cells. Previous results in our laboratory showed that gastrointestinal enzyme digestion greatly increased cytotoxicity of soy protein on L1210 leukemia cells (*41*). At 7 mg/mL, soy protein hydrolysate produced from simulated gastrointestinal enzyme digestion inhibited 80.4% cell growth, whereas a negative control nonhydrolyzed soy protein isolate (obtained from the same defatted soy flour) and nonhydrolyzed defatted soy flour showed no inhibition. This indicated that bioactive

Table 2. Protein Percentage Distribution of NB1−NB7 As Determined by SDS-PAGE

			% of total protein[a]							
code	mol mass (kDa)	proteins identified in soy flours	NB1	NB2	NB3	NB4	NB5	NB6	NB7	LSD ($p < 0.05$)
P1A	92.9	lipoxygenases 2 and 3	5.3 bc	4.9 d	5.0 cd	6.6 a	6.9 a	6.7 a	5.6 b	0.3
P1B	89.1	lipoxygenase	1.7 cd	1.6 d	1.9 bcd	2.0 abc	2.3 a	2.1 ab	1.8 bcd	0.4
P2	66.9	α' subunit of β-conglycinin	8.2 b	8.1 b	15.5 a	16.1 a	16.1 a	15.0 a	8.3 b	1.9
P3	61.1	α subunit of β-conglycinin	12.2 b	12.4 b	21.2 a	21.4 a	23.9 a	21.6 a	12.1 b	3.0
P5	44.9	β subunit of β-conglycinin	5.2 c	5.5 c	12.1 ab	12.3 ab	10.5 b	13.2 a	6.0 c	2.0
P6	38.5	glycinin A3 chain	2.3 a	2.5 a	0.0 b	0.0 b	0.0b	0.0 b	2.7 a	0.5
P7	34.4	glycinin A1,2,4 chains	15.6 a	15.6a	3.4 b	1.5 b	1.7b	3.4 b	14.4 a	2.8
P11	20.5	glycinin basic chains	19.8 a	19.8 a	4.5 b	2.3 b	2.3 b	4.7 b	17.6 a	2.7
P16	18.6	Kunitz trypsin inhibitor	3.5 b	3.4b	4.8 a	4.9 a	4.6a	1.5 c	3.5 b	1.0
		total β-conglycinin	25.6 b	25.9 b	**48.9** a	**49.8** a	**50.6** a	**49.9** a	26.4 b	6.4
		total glycinin	**37.7** a	**37.9** a	7.9 b	3.9 b	4.0 b	8.1 b	**34.7** a	5.7

[a] Means with different letters in the same row are significantly different ($p < 0.05$).

Table 3. Isoflavone, Saponins, Lunasin, and Bowman−Birk Inhibitor (BBI) Concentrations of Seven Soy Protein Hydrolysates (NBH1−NBH7)[a]

		μmol/g of hydrolysate							
code		NBH1	NBH2	NBH3	NBH4	NBH5	NBH6	NBH7	LSD ($p < 0.05$)
					Isoflavones				
IS1	total daidzein	2.66 d	3.05 d	4.44 c	5.37 bc	5.60 ab	6.62 a	4.62 bc	1.08
IS2	total genistein	3.09 a	3.01 a	3.28 a	4.34 a	4.18 a	4.71 a	4.23 a	1.96
IS3	total glycitein	0.56 c	0.53 c	0.66 bc	0.59 c	1.09 a	1.28 a	1.02 ab	0.40
					Saponins				
SP1	total saponins	2.15	2.22	2.77	2.09	2.42	2.31	2.13	*b*
					Peptides				
LNS	lunasin	0.21 a	0.13 c	0.16 b	0.10 d	0.17 b	0.13 c	0.17 b	0.0072
BBI	BBI	0.60 ab	0.12 d	0.57 b	0.27 c	0.63 a	0.25 c	0.24 c	0.039

[a] Means with different letters in the same row are significantly different ($p < 0.05$). Other saponins were below detection limits. [b] Represents only one analysis. Total saponins included saponins I, II, IV, and A1.

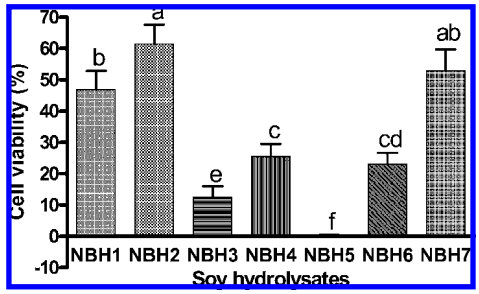

Figure 2. Percent cell viability of L1210 leukemia cells after treatment for 48 h with 6 mg/mL of soy hydrolysates (NBH1−NBH7). L1210 leukemia cells were cultured at 37 °C in a 5% humidified CO_2 atmosphere in Minimum Essential Medium supplemented with 10% bovine serum. Cell Counting Kit-8 was used to determine the number of viable cells. The results are expressed as percent viability of treated cells compared with the untreated control. NBHs with different letters were significantly different ($p < 0.05$, one-way ANOVA with Tukey's multiple-comparison test). Positive control, etoposide, inhibited 90.5% of L1210 leukemia cell growth at 1.0 μM.

peptides released from the hydrolysis might be important for the observed cytotoxicity of soy on L1210 leukemia cells. **Figure 2** presents the percent L1210 leukemia cell viability after 48 h of treatment with 6 mg/mL of soy protein hydrolysates. NBH5 showed the highest cytotoxicity to L1210 leukemia cells (100% inhibition), followed by NBH3 (87.6% inhibition), NBH6 (77.0% inhibition), NBH4 (74.5% inhibition), NBH 1 (53.1% inhibition), NBH7 (47.0% inhibition), and NBH2 (38.5% inhibition). Different hydrolysate samples showed statistically significant different activities ($p < 0.05$), except between the pairs NBH2 and NBH7, NBH4 and NBH6, and NBH1 and NBH7. Soy protein hydrolysates inhibited L1210 leukemia cell growth in a dose-dependent manner, and cytotoxicity results showed that the IC_{50} values of the hydrolysates were 5.6 (NBH1), 6.2 (NBH2), 4.6 (NBH3), 5.0 (NBH4), 3.5 (NBH5), 3.7 (NBH6), and 5.0 (NBH7) mg of dry material/mL, respectively.

Partial Least-Squares (PLS) Regression Analysis. To further understand the correlation between bioactive components in soy and the in vitro cytotoxicity to L1210 leukemia cells, PLS regression analyses were conducted using the variables listed in **Tables 2** and **3**. A two-factor model was found to be adequate to explain 98.8% of the response variation and 71.8% of the predictor variation.

Unlike linear regression, PLS does not give a simple regression formula. The regression model can be built in SAS, and predicted value can be calculated from this model. As shown in **Figure 3A**, the predicted IC_{50} values calculated by the PLS regression model, based on the *X* variables, correlated very well with the experimental values ($R^2 = 0.99$). PLS regression also indicated the importance of each soy component on L1210 leukemia cytotoxicities. **Figure 3B** presents the weights of first and second latent variables (W1 and W2, respectively) for each *X* variable. A high absolute weight value indicates a more important effect of *X* variable on the respective latent variable, where the sign indicates the direction of the impact. **Figure 3B** also shows that most *X* variables were represented in the first factor. P6 (glycinin A3 chain), P7 (glycinin A1,2,4 chains), and P11 (glycinin basic chains) had a positive impact on the first latent variable (W1); the other *X* variables showed a negative impact, except P16 (Kunitz trypsin inhibitor) and LNS (lunasin), which showed a near-zero W1 weight. The contributions of P16 and LNS on the *Y* variable (IC_{50} for L1210 leukemia cells) were reflected on the second latent variable, where absolute W2 scores >0.3 were observed for both. This showed the power of PLS analysis in comparison to simple linear regression. No *X*

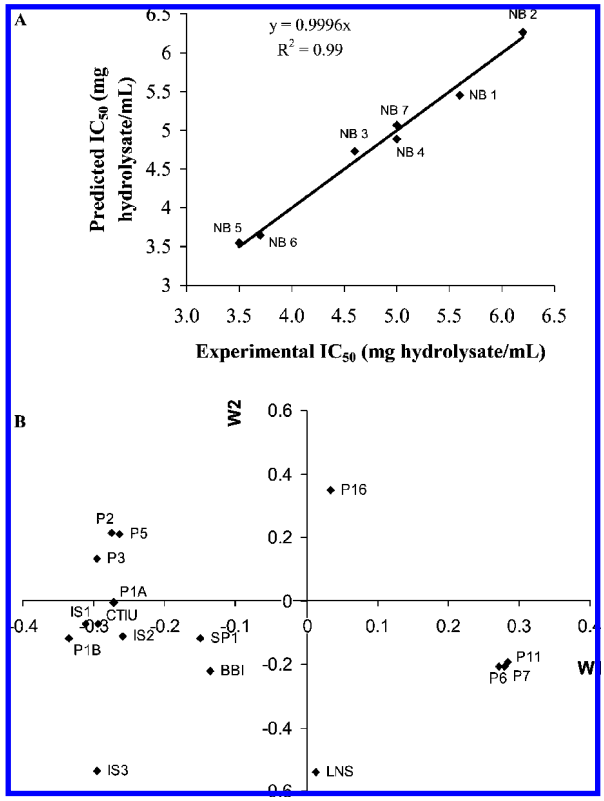

Figure 3. Partial least-squares (PLS) analysis of bioactive components in soy hydrolysates and L1210 leukemia cell inhibitory activities. (**A**) Correlation between experimental and predicted IC_{50} values of soy hydrolysates on L1210 leukemia cells. Log phase L1210 leukemia cells were treated with soy protein hydrolysates NBH1−NBH7 at 0.3−8.0 mg/mL. The experimental IC_{50} values were determined as the concentrations that led to 50% inhibition of treated cells compared with the untreated control. A PLS model was build to calculate predicted IC_{50} values from the matrix profile and concentrations of genistein, daidzein, glycitein, total saponins, lunasin, and BBI in soy hydrolysates. (**B**) Weights of first (W1) and second (W2) latent variables for each *X* variable. A high absolute weight value indicated a more important effect of the *X* variable on the respective latent variable, where the sign indicated the direction of the impact (see **Table 4** for code identification).

variables were weighted at nearly zero for both factors, so none of them were removed to improve the model's predictive capability.

To obtain an overall statistical evaluation of the contribution of each variable to the final activity, regression coefficients (*B*) were calculated from W1 and W2 weights. The higher the absolute *B* value, the higher the impact on cell cytotoxicity; the sign indicates a positive ("+" sign) or negative ("−" sign) effect on IC_{50} values. Variable importance for projection (VIP) of Wold was also calculated to summarize each variable contribution to fit the PLS model for both variables and response (*42*). In this study, a VIP value of <0.9 (*43*) was considered to be small, indicating the variable was not important to the model. **Table 4** presents the *B* and VIP values for each variable, arranged by positive or negative *B* values and then sorted by VIP values. Of 16 variables considered in the model, 12 had a negative *B* value. This meant that higher concentrations of these 12 variables led to lower IC_{50} values, which indicates higher cytotoxicity to L1210 leukemia cells. On the other hand, there were four variables that showed a positive *B* value, indicating a negative correlation with L1210 leukemia cell cytotoxicity. All β-conglycinin subunits α (P3, $B = -0.07$, VIP = 1.10), α' (P2, $B = -0.04$, VIP = 1.05), and β (P5, $B = -0.03$, VIP = 1.01) showed a VIP of >0.9 and a negative regression coefficient, indicating a positive correlation with cytotoxicity. On the other hand, all of the major glycinin chains, basic chains (P11, $B = 0.04$, VIP = 1.07), A1,2,4 chains (P7, $B = 0.04$, VIP = 1.07), and A3 chain (P6, $B = 0.04$, VIP = 1.04) showed a large VIP and a positive regression coefficient. These results indicated that β-conglycinin embeds more active peptides against L1210 leukemia cell growth than glycinin.

Table 4. Estimated Partial Least-Squares (PLS) Weight of First and Second Variables, Regression Coefficients (*B*), and Variable Importance for Projection (VIP)[a]

code	variable	*B*	VIP
IS3	**total glycitein**	**−0.25**	**1.34**
P1B	**lipoxygenase 1**	**−0.15**	**1.23**
IS1	**total daidzein**	**−0.13**	**1.14**
P3	**α subunit of β-conglycinin**	**−0.07**	**1.10**
CTIU	**CTIU**	**−0.12**	**1.07**
P2	**α′ subunit of β-conglycinin**	**−0.04**	**1.05**
P5	**β subunit of β-conglycinin**	**−0.03**	**1.01**
P1A	**lipoxygenases 2 and 3**	**−0.10**	**0.99**
IS2	**total genistein**	**−0.12**	**0.96**
LNS	lunasin	−0.15	0.81
BBI	BBI	−0.11	0.60
SP1	total saponins	−0.08	0.57
P11	**glycinin basic chains**	**0.04**	**1.07**
P7	**glycinin A1,2,4 chains**	**0.04**	**1.07**
P6	**glycinin A3 chain**	**0.04**	**1.04**
P16	Kunitz trypsin inhibitor	0.11	0.54

[a] *B* values were calculated to obtain an overall statistical evaluation of the contribution of each variable to the final activity. The higher the absolute *B* value is, the higher the impact, whetrsd the sign indicates positive ("+" sign) or negative ("−" sign) effect on IC_{50} value. VIP of Wold was calculated to summarize each variable contribution to fit the PLS model for both variables and response (*41*). A VIP value of <0.9 (*42*) was considered to be small, indicating the variable was not important to the model.

All three isoflavones, glycitein, daidzein, and genistein, positively correlated with cytotoxicity ($B = -0.25$, -0.13, and -0.12, respectively) and had high VIP values (1.34, 1.14, and 0.96, respectively), indicating that isoflavones may have contributed to the cytotoxicity to L1210 leukemia cells.

Lunasin ($B = -0.15$) and BBI ($B = -0.11$) had VIP values of 0.81 and 0.60, respectively, indicating only a modest effect on the inhibitory activity of L1210 leukemia cells at the concentrations present in the seven hydrolysates. Total saponins showed a low VIP value (0.57), also indicating a limited contribution to the activity.

The PLS regression study suggested that both isoflavone concentration and the matrix protein composition were important to cytotoxicity; total β-conglycinin embedded more active peptides than total glycinin. BBI and lunasin showed a modest contribution, whereas no correlation between activity and saponin concentration was observed. The model also showed that lipoxygenases and chymotrypsin inhibitor activity (CTIU) positively related to cytotoxicity. To confirm the contributions of β-conglycinin and glycinin hydrolysates, BBI, lunasin, saponin B, and isoflavone on in vitro L1210 leukemia cells cytotoxicity, purified samples of these compounds were tested.

Figure 4A presents the average IC_{50} values of both the glycinin featured group (NB1, NB2, and NB7), and the β-conglycinin featured group (NB3−NB6). The average IC_{50} value of the β-conglycinin featured group (4.2 mg/mL) was significantly lower than that of the glycinin featured group (5.6 mg/mL), also indicating β-conglycinin embeds more active peptides against L1210 leukemia cell growth than does glycinin.

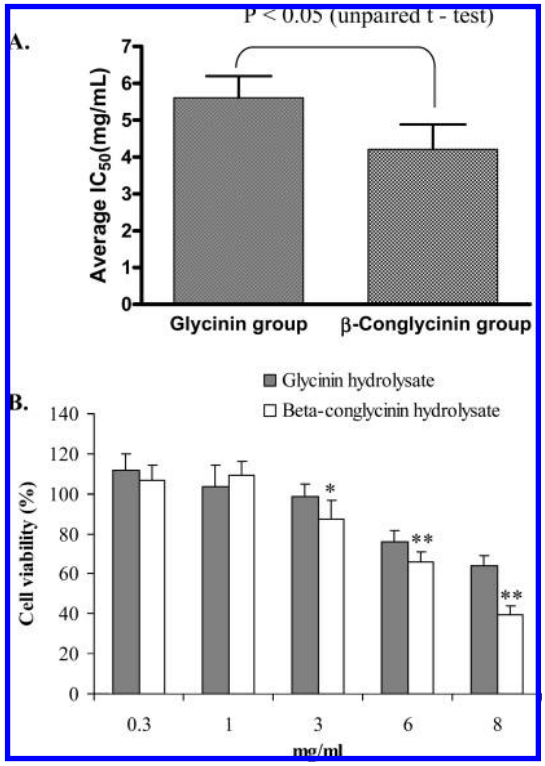

Figure 4. Comparison of cytotoxicity of glycinin and β-conglycinin on L1210 leukemia cells. (**A**) Comparison between average IC_{50} values of NBHs derived from proteins containing >30% glycinin (NBH1, NBH2, and NBH7) and >40% β-conglycinin (NB3−NB6). A significant difference based on unpaired *t* test was observed between these two groups. (**B**) Dose-dependent percent cell viability of purified glycinin and β-conglycinin on L1210 leukemia cells. Glycinin and β-conglycinin were purified from defatted soy flour and subjected to sequential pepsin−pancreatin digestion. The hydrolysates were tested on log phase L1210 leukemia cells for 48 h. Cell Counting Kit-8 was used to determine the number of viable cells. The results are expressed as percent viability of treated cells compared with the untreated control. Significant difference between glycinin and β-conglycinin hydrolysate at certain concentrations was indicated (∗, $p < 0.05$; ∗∗, $p < 0.01$; one-way ANOVA with Tukey's multiple-comparison test).

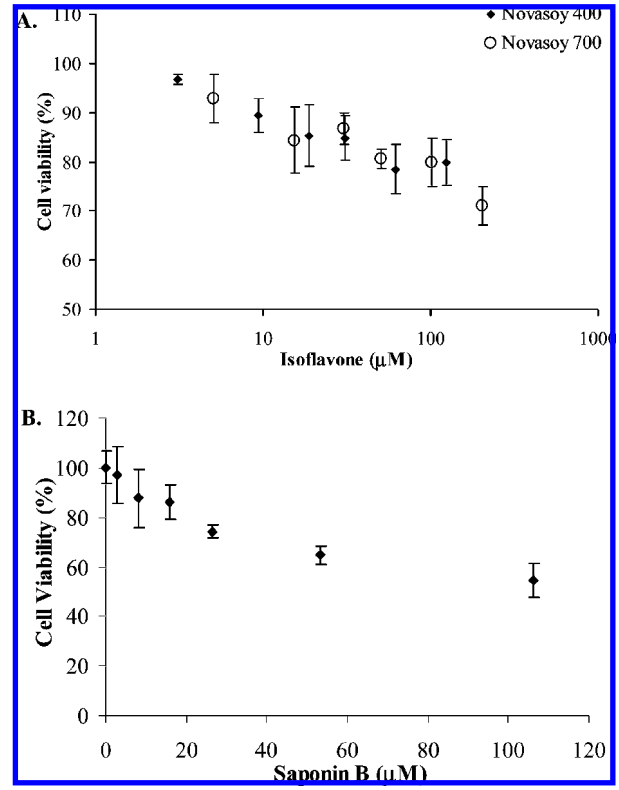

Figure 5. Percent L1210 leukemia cell viability after exposure to soy isoflavone concentrates (**A**) and saponin (**B**). L1210 leukemia cells were treated with various concentrations of soy isoflavone concentrates, Novasoy 400 (44.70% isoflavones) and Novasoy 700 (73.25% isoflavones), and purified saponin B for 48 h. Cell Counting Kit-8 was used to determine the number of viable cells. The results are expressed as percent viability of treated cells compared with the untreated control.

Higher percentage of glycinin subunits correlated with a decreased percentage of more active β-conglycinin and, therefore, led to less cytotoxicity of total protein present in the hydrolysates.

Cytotoxicity of Pure Glycinin and β-Conglycinin Hydrolysates on L1210 Leukemia Cells. To confirm that β-conglycinin embeds more active peptides against L1210 leukemia cell growth than glycinin, purified glycinin and β-conglycinin were hydrolyzed with pepsin and pancreatin following exactly the same procedures as for defatted soy flours. The dose-dependent cytotoxicities of both hydrolysates on L1210 leukemia cells are shown in **Figure 4B**. At concentrations of 3 mg/mL and higher, β-conglycinin hydrolysate showed significantly higher activity than glycinin hydrolysate; these results confirm the previous PLS findings. The difference in the cytotoxicity of β-conglycinin and glycinin may be explained by the difference in amino acid sequence and composition. Within glycinin or β-conglycinin, different subunits share good sequence homology (*15*). However, between glycinin and β-conglycinin, the sequences are largely different. Unique protein sequences in β-conglycinin may embed more potent bioactive peptides. The percentages of acidic (Asp and Glu) and basic (Arg, His, and Lys) amino acids in β-conglycinin are higher than those in glycinin. The aromatic amino acids, Phe and Tyr, are also more abundant in β-conglycinin. On the other hand, glycinin contains much higher percentages of sulfur-containing amino acids Cys and Met (*31*).

Compared with NBHs, purified β-conglycinin and glycinin hydrolysates showed higher IC_{50} values (less cytotoxicity to L1210 leukemia cells), suggesting possible differences in digestion and/or the presence of other active components in NBH1−NBH7, such as isoflavones.

Cytotoxicity of Soy Isoflavone Concentrates and Purified Saponin B on L1210 Leukemia Cells. The PLS study indicated that isoflavone positively correlated to L1210 leukemia cytotoxicity. To test this result with experimental data, the cytotoxicity of soy isoflavone concentrates Novasoy 400 and Novasoy 700 on L1210 leukemia cells was studied. Novasoy 400 and Novasoy 700 contained 44.70 and 73.25% isoflavones, respectively. The isoflavone concentrations in the treatments were up to 124 μM for Novasoy 400 and 203 μM for Novasoy 700. **Figure 5A** presents the dose−response inhibition on L1210 leukemia cells of both soy isoflavone concentrates. At equal molar concentrations for isoflavones, Novasoy 400 and Novasoy 700 showed similar cytotoxicities on L1210 leukemia cells, indicating isoflavones are an active component in the concentrates. At up to 203 μM, isoflavone concentrate inhibited 29% of cell growth. The isoflavone concentrations in NBH1−NBH7 ranged from 6.3 to 12.6 μmol/g of dry material. At the concentrations at which NBH1−NBH7 inhibited 50% of L1210 leukemia cell growth, the isoflavone concentration ranged from 35 to 52 μM, which contributed 14−21% of cytotoxicity. These results indicated that isoflavones and soy peptides worked together to induce cytotoxicity to L1210 leukemia cells. The

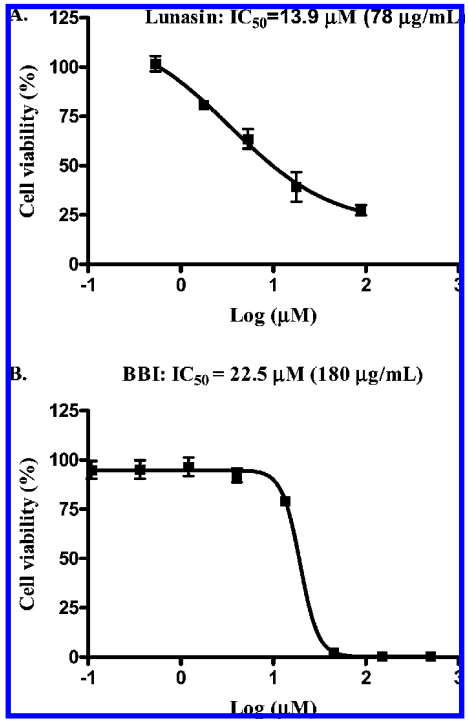

Figure 6. Dose-dependent percent L1210 leukemia cell viability after exposure to lunasin (**A**, 98% purity) and BBI (**B**, 95% purity). L1210 leukemia cells were treated with various concentrations of lunasin and BBI for 48 h. Cell Counting Kit-8 was used to determine the number of viable cells. The results are expressed as percent viability of treated cells compared with the untreated control.

interaction between soy peptides and isoflavones needs to be further studied. It should be noted that the remaining material in the Novasoy isoflavone concentrates has not been characterized. It may contain any number of alcohol soluble proteins, peptides, and carbohydrates that may contribute toward inducing or inhibiting cancer cell cytotoxicity.

Figure 5B presents the dose−response inhibition on L1210 leukemia cells of saponin B. The saponin concentrations in NBH1−NBH7 ranged from 2.09 to 2.77 μmol/g. At the concentration at which NBH1−NBH7 inhibited 50% of L1210 leukemia cell growth, saponin concentration ranged from 8.5 to 13.8 μM, which contributed 12.4−13.7% of cytotoxicity. The small difference in cytotoxicity explained why no significant correlation was observed for saponins in the PLS model.

Cytotoxicity of Pure Lunasin and BBI on L1210 Leukemia Cells. PLS study suggested a modest correlation between lunasin and BBI concentrations and the L1210 leukemia cell cytotoxicity of NBH1−NBH7. To confirm these results, cytotoxicity assay was performed on L1210 leukemia cells with lunasin (98%) and BBI (95%). **Figure 6** presents the dose-dependent inhibition of L1210 leukemia cells by lunasin (**Figure 6A**) and BBI (**Figure 6B**). The IC_{50} values were 78 μg/mL (13.9 μM) for lunasin and 180 μg/mL (22.5 μM) for BBI. Lunasin starts decreasing leukemia cell viability at lower concentrations, whereas BBI presents a sharp reduction on leukemia cell viability within a narrow range of concentrations and at a higher level, suggesting that it is less potent than lunasin.

At the concentrations that the soy hydrolysates inhibited 50% of L1210 leukemia cell growth, lunasin concentrations ranged from 0.48 to 1.07 μM (2.6−6.0 μg/mL), whereas BBI from ranged from 0.7 to 3.4 μM (5.9−26.9 μg/mL). As shown in **Figure 6**, at these ranges, lunasin and BBI showed only 1.9−7.6 and 3.5−7.0% of inhibition of L1210 leukemia cell growth, respectively. These results agreed with the PLS findings. In soy hydrolysates, the concentrations of lunasin and BBI were only enough to show modest activity.

In summary, food is usually a complex system containing many components. Sometimes it is not feasible to test all components (e.g., all peptides in hydrolysates), and often it has limitations to consider components separately. This study used L1210 leukemia cells as a model and demonstrated the usefulness of PLS regression in predicting activities of bioactive components. The results showed that seven soy genotypes had different protein profiles. After hydrolysis, the isoflavones, saponins, lunasin, and BBI concentrations varied among genotypes. Leading from the composition differences, the L1210 leukemia cell inhibitory activity of seven hydrolysates were different, and NBH5 showed the highest cytotoxicity. The correlation study indicated both the isoflavone concentration and the matrix protein composition played an important role in cytotoxicity. β-Conglycinin embeds more active peptides than glycinin in terms of inhibiting L1210 leukemia cell growth. Lunasin and BBI were potent L1210 cell inhibitors (IC_{50} = 13.9 and 22.5 μM, respectively); however, they showed modest contributions, whereas no correlation between L1210 leukemia cell cytotoxicity and saponins was observed at the concentrations present in the soy hydrolysates. Chymotrypsin inhibitory units and β-conglycinins actively contributed to the estimated projection model.

In conclusion, the data demonstrated that β-conglycinins are among the major soybean components that inhibit leukemia cell proliferation in vitro. In addition, the present approach shows that it is feasible to differentiate soybean varieties on the basis of a cell assay.

ABBREVIATIONS USED

BBI, Bowman−Birk inhibitor; CCK-8, Cell Counting Kit-8; CTIU, chymotrypsin inhibitory units; DMPP, 1,l-dimethyl-4-phenylpiperzinium; ELISA, enzyme-linked immunosorbent assay; IC_{50}, concentration needed to inhibit 50% of cell growth; NB, defatted soy flour; NBH, defatted soy flour hydrolysate; PLS, partial least-squares analysis; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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