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Imbibition of Soybean Seeds in Warm Water Results in the Release of Copious Amounts of Bowman–Birk Protease Inhibitor, a Putative Anticarcinogenic Agent

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ABSTRACT: Protease inhibitors play a protective role against pathogenic microorganisms and herbivorous insects. The two predominant protease inhibitors of soybean seeds are the Kunitz trypsin inhibitor (KTI) and Bowman–Birk protease inhibitor (BBI). In this study, we report that soybean seeds incubated in warm water release large amounts of proteins into the surrounding media. Two-dimensional gel electrophoresis analysis of the seed exudates resulted in the separation of 93 distinct protein spots out of which 90 spots were identified by LC-MS/MS. The basic 7S globulin and the BBI are the two predominant proteins found in the soybean seed exudates. In addition to 7S and 11S seed storage proteins, others known to protect the seeds against pathogens and pests including KTI, peroxidase, α-galactosidase, and endo-1.3-β-glucanase were also identified in the seed exudates. Soybean seed exudate obtained by incubating the seeds in warm water was also able to inhibit the growth of human breast cancer cell line MCF-7. Since soybean seeds release large amounts of enzymatically active BBI when immersed in warm water, our procedure could be exploited as a simplified alternative method for the preparation of BBI concentrate which is being used as a cancer chemoprotective agent.

KEYWORDS: *Bowman–Birk protease inhibitor, basic 7S globulin, Kunitz trypsin inhibitor, seed exudate, soybean*

INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) seeds release several proteins when incubated with hot water.[1,2] The basic 7S globulin, which is soluble in high ionic strength salt solutions,[3] was identified as the most abundant protein in the hot water exudates.[1,2] The 7S basic globulin, also known as Bg7S, is synthesized as a precursor protein of 43 kDa and post-translationally cleaved into α (27 kDa)- and β (16 kDa)-subunits that are held together by disulfide bonds.[4,5] This protein contains a consensus ATP-binding motif and reveals autophosphorylation activity and protein kinase activity.[6,7] Curiously, the 7S basic globulin is able to bind insulin and insulin-like growth factors suggesting that this protein may function as a hormone receptor protein.[7]

Proteins similar to the soybean basic 7S globulin have also been reported from hot water exudates of azuki-bean (*Vigna angulariz* (Willd.) Ohwi and Ohashi), cowpea (*Vigna unguiculata* (L.) Walp.), mung-bean (*Vigna radiate* (L.) R. Wilczek), winged-bean (*Psophocarpus tetragonolobus* (L.) DC.), common-bean (*Phaseolus vulgaris* L.) and jack-bean (*Canavalia ensiformis* DC).[2] The wide distribution of the basic 7S globulins among different legumes suggests that this protein may have an important function. Proteins homologus to soybean basic 7S globulin have been shown to inhibit endoglucanases belonging to glycoside hydrolase families 11 and 12.[8] Therefore, it was suggested that the basic 7S globulins may play an important role in plant defense against phytopathogens.[9]

Germinating seeds release proteins into the rhizosphere that inhibit the growth of microorganisms.[10] For example, several antifungal proteins including β-1,3-glucanase, cystatin, vicilin, and lipid transfer proteins have been identified in cowpea (*Vigna unguiculata* L.) seed exudates.[11] Protease inhibitors can also control pathogenic microorganisms and herbivorous insects.[12] Soybean seeds store two major types of protease inhibitors, Kunitz trypsin inhibitor (KTI) and Bowman–Birk protease inhibitor (BBI).[13−15] These two protease inhibitors account for about 6% of the total protein of soybean seeds.[16] Given their abundance and their protective role against insects and phytopathogens, one would expect that these proteins would be released when the seeds are incubated in warm water. However, surprisingly, in an earlier study, protease inhibitors were not detected in soybean seed exudates even though they were readily found in the winged-bean seed exudates.[2] In this study, we have employed a proteomic approach to identify soybean seed proteins that were released when incubated in warm water. We demonstrate, contrary to the previous study, that soybean seeds release large amounts of protease inhibitors along with several other defense related proteins when incubated in warm water. The BBI released in the warm

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water strongly inhibits both trypsin and chymotrypsin and also inhibits the proliferation of MCF7 breast cancer cells.

MATERIALS AND METHODS

Collection of Seed Exudates. Soybean (*Glycine max* L. Merr. cv.Williams 82) seeds were obtained from Missouri Seed Improvement Association, Columbia, MO. Seeds were disinfected by incubating the seeds first with 50% ethanol for 5 min, followed by incubation in 2.5% sodium hypochlorite for 5 min. After extensive washes in running water, intact seeds were carefully selected and transferred to 250 mL flasks. Typically 50 seeds were incubated with 50 mL of sterile water or buffer in a temperature controlled incubator shaker set at 50 rpm (New Brunswick Scientific, Edison, NJ). We first tested the effect of temperature on the release of the proteins by incubating the seeds for 24 h at 4, 24, 37, and 50 °C, respectively. Incubation at 4 and 24 °C resulted in a limited release of proteins, while incubation at 37 and 50 °C released large amounts of proteins into the incubating media. Since the highest amount of proteins was released when the seeds were incubated at 50 °C, all the subsequent experiments were carried out at this temperature. A time-course experiment was also conducted to monitor the release of proteins from soybean seeds. Soybean proteins released into the surrounding solution at 0.5, 1, 2, 4, 6, 8, and 16 h after incubation at 50 °C were recovered by decanting through two layers of cheesecloth into sterile 50 mL plastic tubes. When this solution was placed on ice for a few minutes, a significant amount of precipitated proteins settled at the bottom of the tubes. The denatured proteins were recovered by centrifugation at 12000*g* for 15 min, and the clear supernatant containing the released proteins from the seeds was transferred to new 50 mL plastic tubes.

SDS–PAGE Analysis of Soybean Seed Exudates. Soybean seed exudates collected at defined periods were concentrated by acetone precipitation, or small aliquots (200 μL) were directly mixed with an equal volume of 2× SDS-sample buffer [120 mM Tris-HCl, pH 6.8, 4% SDS (w/v), 20% glycerol (v/v), and 10% 2-mercaptoethanol]. The samples were placed in a boiling water bath for 5 min and clarified by centrifugation (15800*g*, 5 min). The resulting supernatant was transferred to clean Eppendorf tubes. Proteins were separated on a 15% SDS–PAGE[17] using a Hoeffer SE 260 minigel apparatus according to the manufacturer's recommendations (GE Healthcare). Separation was achieved with a constant 20 mA per gel and a run time of 1.5 h. Gels were removed from the cassette and placed immediately in Coomassie Brilliant Blue G-250.

Immunoblot Analysis. Soybean seed exudate proteins were first resolved by SDS–PAGE followed by electrophoretic transfer to nitrocellulose membranes (Protran, Schleicher & Schuell Inc., Keene, NH). Following the transfer, the membranes were blocked with 5% milk in Tris-buffered saline (TBS, pH 7.3) for 1 h and incubated with either KTI[18] or BBI antiserum[19] or preimmune serum at 1:5000 dilution overnight at room temperature with gentle rocking. The membrane was washed four times with TBS containing 0.05% Tween-20 (TBST) for 10 min each wash and incubated for 2 h in affinity purified goat antirabbit IgG-horseradish peroxidase (HRP) conjugate (Bio-Rad Laboratories, Hercules, CA, USA) at a 1:3000 dilution. Following this step, the membrane was washed four times with TBST as described above. Immunoreactive polypeptides were detected with an enhanced chemiluminescent substrate (Super Signal West Pico Kit; Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's protocol.

2-D Electrophoresis. For 2-DE analysis, soybean seed exudate was first concentrated by acetone precipitation. Precipitated proteins were recovered by centrifugation at 8000 rpm for 20 min at 4 °C. The protein pellet was briefly air-dried and resuspended in 4 mL of 100 mM Tris-Cl, pH 8.8, containing 0.9 M sucrose, 0.4% β-mercaptoethanol, and protease inhibitor cocktail. Protein samples were purified by phenol extraction and dissolved in a small volume of 7 M urea, 2 M thiourea, 1% CHAPS, 2% C7BzO, and 100 mM DTT as previously described.[20] For standard IEF, protein estimation was performed following the method of Bradford[21] and 300 μg of protein sample loaded per strip using in-gel rehydration. Linear gradient, 13 cm IPG strips (GE Healthcare) were brought to a rehydration volume of 250 μL with 7 M urea, 2 M thiourea, 1% CHAPS, and 2% C7BzO with a final concentration of the following: 5% glycerol, 2.2% 2-HED, and 0.25% 4–7 IPG buffer or 0.5% 3–10 IPG buffer. Strips were then passively rehydrated with the entire rehydration solution containing protein sample at 22 °C for 15 h prior to focusing. Isoelectric focusing was performed with a Protean II IEF (BioRad) as described earlier.[20] Following electrophoresis, gels were fixed in 5:4:1 (methanol/water/acetic acid) for 1 h, followed by staining in Colloidal Coomassie Blue G-250 for 24 h.

Protein Identification. A small gel piece of each protein for identification was excised with a 1.5 mm Spot Picker (The Gel Company, San Francisco, CA, USA) from a Colloidal Coomassie Blue G-250 stained gel, washed briefly in distilled water, and then destained completely in a 50% (v/v) solution of acetonitrile containing 25 mM ammonium bicarbonate. After a 100% acetonitrile wash, the protein contained in the acrylamide gel was subjected to digestion using 20 μL (10 μg/mL) of modified porcine trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate. The peptides were analyzed by using Thermo Fisher Scientific LTQ Orbitrap XL hybrid ion trap, Orbitrap mass spectrometer (ThermoFisher Scientific, San Jose, CA). Initially, peptides were separated by reverse phase chromatography on a 100 × 0.18 mm BioBasic-18 column using a 30-min gradient from 5 to 40% ACN in 0.1% formic acid at a flow rate of 3 μL/min. The instrument's operation was detailed in an earlier publication.[22] Protein identification was performed using the Mascot search engine (http://www.matrixscience.com) or the Protein Prospector search engine (http://www.prospector.ucsf.edu), which uses a probability based scoring system.[23] NCBI nonredundant *Glycine max* database and UniProtKB *Glycine max* database, both with a peptide mass tolerance of 20 ppm, were selected as the primary databases to be searched. Positive identifications of proteins by the MS analysis required a minimum of two unique peptides, with at least one peptide having a significant ion score.

Trypsin and Chymotrypsin Inhibitor Assays. Fifty milligrams of seed powder was extracted by the addition of 1 mL of 100 mM Tris-Cl, pH 8.0, followed by vortexing for 10 min. Extract was clarified at 16000*g* for 10 min and 100 μL (for KTI) or 400 μL (for BBI) of extract removed and diluted to 1 mL with extraction buffer. KTI assays were performed in 100 mM sodium phosphate, pH 7.6, using 0.23 mM *N*-benzoyl-L-arginine ethyl ester (BAEE; Sigma B-4500) dissolved in sodium phosphate buffer. BBI assays were performed in 100 mM Tris-Cl, pH 8.0, with 25 mM calcium chloride, using 0.55 mM *N*-benzoyl-L-tyrosine ethyl ester (BTEE; Sigma) dissolved in 64% methanol as substrate. Assay solution was zeroed, then 50 U of trypsin (dissolved in 1 mM HCl solution) or 0.1 U of α-chymotrypsin (Sigma; dissolved in 1 mM HCl solution) added to begin the reaction. The change in absorbance was monitored at 256 nm for at least 3 min. Trypsin inhibitor units (TIU) or chymotrypsin inhibitor units (CIU) were calculated as the amount of inhibitor that reduced the absorbance per minute of the standard reaction by 0.01.[24] For accuracy,the reaction was measured in the linear portion in the 40–60% inhibition range.

Cell Proliferation Assay. Human breast cancer cell line MCF-7 was obtained from American type culture collection (ATCC) and maintained in RPMI-1640 containing 5% fetal bovine serum (FBS), 100 U/mL of penicillin and 100 mg/mL streptomycin. For cell proliferation experiments, MCF-7 cells (1×10^5 cell/well) were plated in media containing 10% FBS on a 24-well plate. After a day, the medium was changed and incubated with or without various concentrations of test compounds. Exudates obtained from soybean seeds incubated at 50 °C for 8 h was used to test its inhibitory effect on the human breast cancer cell line MCF-7. Soybean seed exudate was used at a concentration ranging from 0 to 3.2 mg/mL. The soybean isoflavone genistein (25 μM) was used as positive control while bovine serum albumin (400 μM) was used as a negative control. Cells were treated for 72 h with daily change of fresh media containing the test compounds. Subsequently, cell proliferation was determined by the sulforhodamine B (SRB) method following published protocols.[25,26]

RESULTS

Soybean Seed Exudate Contains Both KTI and BBI. SDS–PAGE analysis was performed to examine the proteins released into the surrounding media when soybean seeds were incubated in 50 °C water (Figure 1). Unfractionated soybean seed exudate contained several proteins ranging in molecular weights from 7 to 100 kDa (Figure 1, lane 1). The two most abundant proteins in this fraction had molecular weights of 27 and 16 kDa. These two major proteins have been identified as the subunits of the basic 7S globulin.[2] In addition to these proteins, several other proteins were also detected. Prominent among them were several low molecular weight proteins that ranged from 7 to 14 kDa. We also separated the unfractionated soybean seeds exudate into a pellet and supernatant fraction by centrifugation and examined their respective protein profiles by SDS–PAGE (Figure 1, lanes 2 and 3). The 7 to 14 kDa low molecular weight proteins were prominent in the supernatant fraction (Figure 1, lane 2). Interestingly, the 27 and 16 kDa proteins were mostly found in the pellet fraction indicating that the 7S basic globulins are denatured in warm water (Figure 1, lane 3).

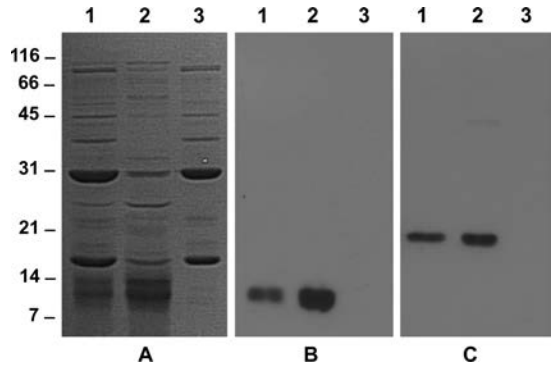

Figure 1. Identification of KTI and BBI in soybean seed exudates. Proteins released from soybean seeds incubated in warm water (50 °C) for 16 h were collected and separated on 15% SDS–PAGE gel and stained with Coomassie Brilliant Blue (panel A) or electrophoretically transferred to a nitrocellulose membrane and probed with BBI peptide antibody (panel B) or KTI antibody (panel C). Immunoreactive proteins were detected using anti rabbit IgG-horseradish peroxidase conjugate followed by chemiluminescent detection. Lane 1, total protein; lane 2, proteins remaining in the supernatant; lane 3, precipitated proteins. Molecular weight markers are shown on the left and designated in kDa.

Previous studies have shown that germinating seeds often release protease inhibitors which can inhibit pathogen growth.[10,27–30] The presence of protease inhibitors in soybean exudates was examined by immunoblot analysis using antibodies raised against the purified BBI and KTI (Figure 1B and C, respectively). The BBI antibody recognized a broad band (10–12 kDa), while the KTI antibody reacted strongly against a 21 kDa protein. Neither of these protease inhibitors was detected in the pellet fraction (Figure 1B and C, lanes 3).

Since the immunoblot analysis revealed the presence of protease inhibitors in soybean seed exudates, we wanted to examine if these proteins are enzymatically active. KTI and BBI activities were measured using *N*-benzoyl-L-arginine ethyl ester and *N*-benzoyl-L-tyrosine ethyl ester as substrates, respectively. Both trypsin and chymotrypsin inhibitor activities were readily detected in the seed exudates (Figure 2). The seed exudate contained higher amounts of chymotrypsin inhibitor activity when compared with trypsin inhibitor activity. Most of the protease inhibitory activity in the soybean seed exudates remained in the supernatant fraction and only trace amounts of trypsin and chymotrypsin inhibitory activities were detected in the pellet fraction (Figure 2).

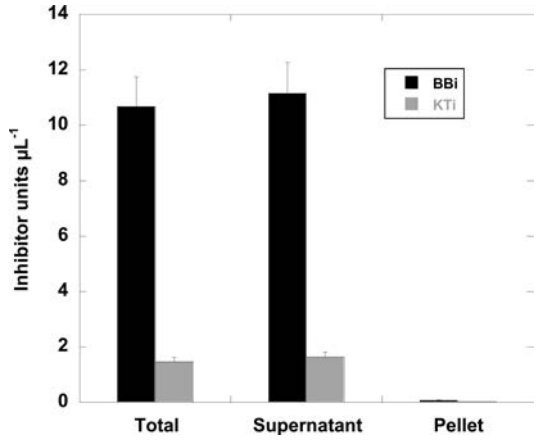

Figure 2. Trypsin and chymotrypsin inhibitor activity of soybean seed exudates. Trypsin and chymotrypsin inhibitor activity was measured under standard assay conditions using *N*-benzoyl-L-arginine ethyl ester and *N*-benzoyl-L-tyrosine ethyl ester as substrates, respectively. The activity is expressed as inhibitor units and presented as the mean ± SE (n = 3). Lane 1, total protein; lane 2, proteins remaining in the supernatant; lane 3, precipitated proteins.

Influence of Incubation Period on the Release of Soybean Proteins. A time-course experiment was conducted to monitor the release of proteins from soybean seeds (Figure 3). An examination of the Coomassie stained gel revealed very little release of seed proteins in the exudates collected at 0.5, 1 and 2 h (Figure 3). After 4 h of incubation at 50 °C, there was a substantial release of proteins in the exudate. Prominent among them were the 27 and 16 kDa 7S basic globulin subunits and a 12 kDa protein (Figure 3). Measurement of chymotrypsin inhibitor activity in the seed exudates collected from 1 to 8 h

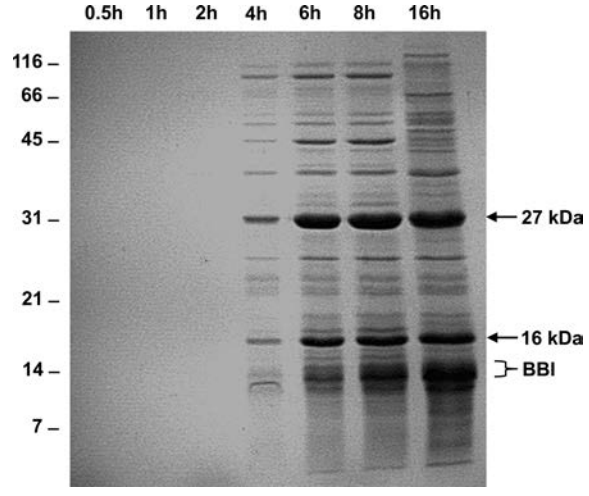

Figure 3. Time course release of proteins from soybean seeds. Proteins released from soybean seeds incubated in warm water (50 °C) were collected at 0.5, 1, 2, 4, 6, 8, and 16 h and separated on 15% SDS–PAGE gel. Resolved proteins were visualized by staining with Coomassie Brilliant Blue. Molecular weight markers are shown on the left and designated in kDa.

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after incubation revealed a steady increase in the enzyme activity (Figure 4). Chymotrypsin inhibitor activity was not detected from seed exudates collected at 0.5 h after incubation in warm water. Maximum BBI activity was detected at 4−8 h after incubation at 50 °C. Interestingly, prolonged incubation at 50 °C resulted in a significant drop in chymotrypsin inhibitor activity (Figure 4). Extended incubation of BBI at 50 °C may lead to denaturation of the enzyme resulting in a loss of biological activity.

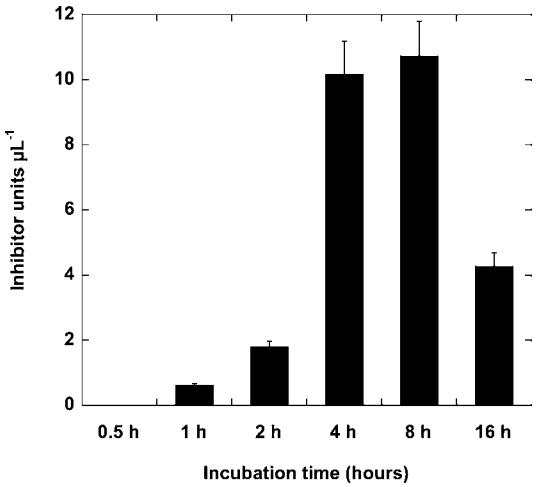

Figure 4. Chymotrypsin inhibitor activity from soybean seed exudates. Proteins released from soybean seeds incubated in warm water (50 °C) were collected at 1, 2, 4, 6, 8, and 16 h. Chymotrypsin inhibitor activity was measured under standard assay conditions using *N*-benzoyl-L-tyrosine ethyl ester as substrate. The activity is expressed as inhibitor units and presented as the mean ± SE (n = 3).

Identification of Soybean Proteins Released in Warm Water by Mass Spectrometry. To resolve and identify the proteins present in the soybean seed exudate, 2-D gel electrophoresis was performed (Figure 5). An examination of the colloidal Coomassie blue stain revealed more than 90 well separated protein spots. These spots were excised from the gel and subjected to LC-MS/MS to identify these proteins. An analysis of the MS data revealed that a significant number of proteins in the seed exudate represented the soybean seed 7S and 11S globulins (Table 1). The two abundant protein spots (spot #42 and #70) were identified as the subunits of the 7S basic globulin. A cluster of acidic proteins in the molecular weight range of 12 to 10 kDa was identified as BBI (Table 1). Spots #47−50 were identified as KTI. Several defense-related proteins including disease resistance protein (spots #19 and 20), peroxidase (spot #21), α-galactosidase (spot #23), and endo-1.3-β-glucanase (spot #41) were also found in the seed exudates. Interestingly, the seed exudate also contained Napin-type 2S albumin (spot #67).

Soybean Seed Exudates Inhibit the Proliferation of MCF7 Breast Cancer Cells. Protein identification coupled with the measurement of chymotrypsin inhibitor activity clearly demonstrates that BBI is a major component of soybean seed exudate. Previous studies have shown that BBI has chemopreventive activity against different types of cancer.[31−36] Consequently, we examined if soybean seed exudates obtained by incubating soybean seeds at 50 °C for 8 h had any effect on human breast cancer cell line MCF-7. We initially tested different concentration of exudates (0−3.2 mg/mL) obtained from soybean seeds incubated at 50 °C for 8 h on cell proliferation of MCF-7 cells. Lower concentrations of soybean seed exudate (0.2 to 0.8 mg/mL) showed no inhibitory effect (data not shown), but a higher concentration (3.2 mg/mL) inhibited the MCF-7F growth, similar to the levels obtained with 25 μM genistein (Figure 6), a soybean isoflavone known to inhibit cancer cell growth.[37,38]

DISCUSSION

Soybean seeds when imbibed in warm water releases several proteins into the surrounding media. Five proteins (S1, S2, S3,

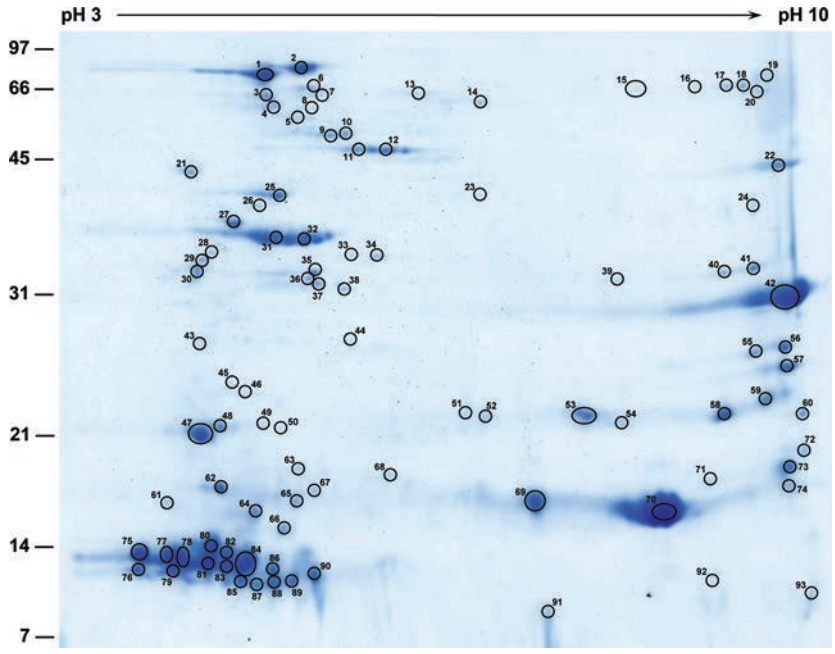

Figure 5. 2-DE separation of seed proteins released in warm water. Soybean seeds were incubated in water for 16 h at 50 °C. Released proteins were concentrated and separated by isoelectric focusing on IPG strips (13 cm, pH 3−10) then by 2D SDS−PAGE on a 16% gel. The gels were stained with Colloidal Coomassie Blue G-250. The numbers at the top of circles point to different proteins that were identified by LC-MS/MS.

Table 1. Proteins Identified from Imbibed Soybean Seed Exudate after 2D-PAGE Separation and Peptide Mass Fingerprinting (LC-MS/MS)[a]

spot #	protein ID	protein score	peptides matched	% cov.	M_r E/T	pI E/T	MS/MS-fit DB: acc. #	NCBI BLAST acc. #
1	α-subunit of β-conglycinin (*G. max*)	1251	44	36	77000/63184	4.80/4.92	NCBInr: gil9967357	
2	α′-subunit of β-conglycinin (*G. max*)	1254	36	35	82000/65160	5.10/5.23	NCBInr: gil9967361	
3	α-subunit of β-conglycinin (*G. max*)	994	27	31	65000/63184	4.80/4.92	NCBInr: gil9967357	
4	α-subunit of β-conglycinin (*G. max*)	871	28	34	62000/63184	4.90/4.92	NCBInr: gil9967357	
5	α′-subunit of β-conglycinin (*G. max*)	745	18	27	60000/65160	5.00/5.23	NCBInr: gil9967361	
6	α′-subunit of β-conglycinin (*G. max*)	1093	29	38	66000/65160	5.15/5.23	NCBInr: gil9967361	
7	α′-subunit of β-conglycinin (*G. max*)	1038	26	35	65000/65160	5.20/5.23	NCBInr: gil9967361	
8	α′-subunit of β-conglycinin (*G. max*)	958	26	31	62000/65160	5.10/5.23	NCBInr: gil9967361	
9	α′-subunit of β-conglycinin (*G. max*)	1364	34	43	50000/65160	5.30/5.23	NCBInr: gil9967361	
10	β-amylase (*G. max*)	353	8	15	51000/53983	5.40/5.31	NCBInr: gil169913	
11	β-subunit of β-conglycinin (*G. max*)	*833*	24	44	49000/48331	5.60/5.70	NCBInr: gil63852207	
12	β-subunit of β-conglycinin (*G. max*)	*818*	25	45	49000/48331	5.90/5.70	NCBInr: gil63852207	
13	sucrose binding protein (*G. max*)	*162*	6	13	65000/60523	6.10/6.40	NCBInr: gil548900	
14	glycinin G1 (*G. max*)	*25*	1	2	62000/56299	6.75/5.90	NCBInr: gil121276	
15	glycinin G1 (*G. max*)	56	1	2	66000/56299	8.10/5.90	NCBInr: gil121276	
16	glycinin G1 (*G. max*)	1118	27	37	67000/64351	8.80/5.90	NCBInr: gil18641	
17	glycinin G1 (*G. max*)	156	3	7	68000/56299	9.10/5.90	NCBInr: gil121276	
18	disease resistance protein/LRR protein-related (*G. max*)	95	2	3	68000/54311	9.25/8.71	NCBInr: gil 223452534	
19	disease resistance protein/LRR protein-related (*G. max*)	523	16	23	77000/54311	9.50/8.71	NCBInr: gil 223452534	
20	disease resistance protein/LRR protein-related (*G. max*)	499	11	22	65000/54311	9.40/8.71	NCBInr: gil 223452534	
21	seed coat peroxidase isozyme (*G. max*)	173	4	10	43000/30980	4.05/5.07	NCBInr: gil1125104	
22	basic 7S globulin (*G. max*)	*325*	11	31	44000/46394	9.55/8.70	UniProtKB: P13917	
23	putative uncharacterized protein fragment (*G. max*); [83% homology with alpha-galactosidase 1 (*Pisum sativum*)]	*117*	4	12	42000/34898	6.70/8.40	UniProtKB: C6TFG7	[UniProtKB: Q5ZP79]
24	α-subunit β-conglycinin fragment (*G. max*)	61	3	8	42000/45019	9.30/5.60	UniProtKB: Q84UB3	
25	putative uncharacterized protein fragment (*G. max*); [99% homology with glycinin (*G. max*)]	*285*	8	27	42000/48541	4.95/5.50	UniProtKB: C6T7B0	[UniProtKB: Q9SB12]
26	putative uncharacterized protein fragment (*G. max*); [99% homology with glycinin (*G. max*)]	*113*	3	10	41000/48541	4.70/5.50	UniProtKB: C6T7B0	[UniProtKB: Q9SB12]
27	putative uncharacterized protein fragment (*G. max*); [99% homology with glycinin (*G. max*)]	*70*	2	4	39000/48541	4.40/5.50	UniProtKB: C6T7B0	[UniProtKB: Q9SB12]
28	putative uncharacterized protein fragment (*G. max*); [99% homology with glycinin (*G. max*)]	*27*	1	4	35000/48541	4.15/5.50	UniProtKB:C6T7B0	[UniProtKB: Q9SB12]
29	7S storage protein α-subunit (*G. max*)	*117*	5	16	34500/27368	4.10/5.90	UniProtKB:Q39816	
30	glycinin (*G. max*)	634	17	23	33000/64351	4.05/5.21	NCBInr: gil18641	
31	glycinin G2 (*G. max*)	625	25	35	36000/54927	4.90/5.46	NCBInr: gil121277	
32	glycinin G1 (*G. max*)	544	16	27	36000/56299	5.05/5.90	NCBInr: gil121276	
33	α′-subunit of β-conglycinin (*G. max*)	506	14	17	34500/65160	5.50/5.23	NCBInr: gil9967361	
34	α′-subunit of β-conglycinin (*G. max*)	904	23	34	34500/65160	5.75/5.23	NCBInr: gil9967361	
35	chain A, agglutinin (*G. max*)	408	13	32	33000/27555	5.10/5.15	NCBInr: gil6729836	
36	chain A, agglutinin (*G. max*)	320	8	28	32000/27555	5.05/5.15	NCBInr: gil6729836	
37	chain A, agglutinin (*G. max*)	350	8	28	31500/27555	5.15/5.15	NCBInr: gil6729836	
38	chain A, agglutinin (*G. max*)	360	8	28	31000/27555	5.45/5.15	NCBInr: gil6729836	
39	no match							
40	endo-1.3-β-glucanase (*G. max*)	473	14	33	32000/36638	9.05/8.88	NCBInr: gil38640795	
41	unknown (*G. max*); [endo-1.3-β-glucanase (*G. max*)]	*209*	8	21	32500/36678	9.40/8.90	NCBInr: gil 255641166	[NCBInr: gil 38640795]
42	basic 7S globulin (*G. max*)	*140*	5	15	30000/46394	9.80/8.70	UniProtKB: P13917	
43	α-subunit of β-conglycinin (*G. max*)	86	4	3	28000/63184	4.10/4.92	NCBInr: gil9967357	
44	unknown (*G. max*); [dehydro-ascorbate reductase (*G. max*)]	244	6	21	28000/23530	5.50/5.81	NCBInr: gil 255627415	[NCBInr: gil 68131811]
45	glycinin G2 (*G. max*)	*127*	4	9	26000/54391	4.40/5.50	UniProtKB: P04405	
46	putative uncharacterized protein (*G. max*)	*25*	1	14	25500/16371	4.50/9.70	UniProtKB: C6T7B0	no match
47	Kunitz trypsin inhibitor (*G. max*)	706	38	54	21000/20310	4.05/5.00	UniProtKB: C6TCR8	
48	Kunitz trypsin inhibitor (*G. max*)	425	16	41	22500/22817	4.15/4.97	NCBInr: gil125722	
49	Kunitz trypsin inhibitor (*G. max*)	125	2	17	22500/22947	4.70/5.24	NCBInr: gil 168259036	
50	Kunitz trypsin inhibitor (*G. max*)	125	2	17	22000/22947	4.85/5.24	NCBInr: gil 168259036	
51	glycinin A2B1a subunit precursor (*G. max*)	96	2	9	23500/25085	6.50/5.50	NCBInr: gil169967	

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Table 1. continued

spot #	protein ID	protein score	peptides matched	% cov.	M_r E/T	pI E/T	MS/MS-fit DB: acc. #	NCBI BLAST acc. #
52	glycinin A2B1a subunit precursor (*G. max*)	95	2	9	23000/25085	6.70/5.50	NCBInr: gil169967	
53	7S storage protein α-subunit (*G. max*)	*84*	3	13	23000/27368	7.75/6.20	UniProtKB: Q39816	
54	7S storage protein α-subunit (*G. max*)	*80*	3	12	22500/27368	8.00/6.20	UniProtKB: Q39816	
55	unknown (*G. max*); [R-14 protein, basic secretory protein (*G. max*)]	175	4	12	26500/25336	9.30/8.72	NCBInr: gil 255627849	[NCBInr: gil 27764542]
56	P24 oleosin isoform (*G. max*)	120	7	15	27000/23487	9.70/8.01	NCBInr: gil1709459	
57	24 KDa protein SC24, seed coat protein (*G. max*)	666	31	47	26000/24611	9.75/9.14	NCBInr: gil18448973	
58	glycinin A2B1a subunit (*G. max*)	*171*	5	25	24000/24873	9.00/5.50	NCBInr: gil169967	
59	dehydrin (*G. max*)	269	6	40	23500/23720	9.50/5.87	NCBInr: gil37495455	
60	basic 7S globulin (*G. max*)	*55*	2	5	23000/46394	10.00/8.70	UniProtKB: P13917	
61	soybean trypsin inhibitor (*G. max*)	224	5	22	17000/20310	3.80/4.61	NCBInr: gil13318877	
62	unknown (*G. max*); [stress-induced protein SAM22, allergen Gly m4 (*G. max*)]	721	26	85	18000/16808	4.15/4.73	NCBInr: gil 255633070	[NCBInr: gil 134194]
63	unknown (*G. max*); [ML domain-containing lipid recognition protein (*A. thaliana*)]	133	5	21	19000/18273	5.00/5.48	NCBInr: gil 255629055	[NCBInr: gil 18399355]
64	seed maturation protein PM22 (*G. max*)	716	25	54	17000/16735	4.60/5.16	NCBInr: gil4585271	
65	seed maturation protein PM22 (*G. max*)	663	25	54	17500/16735	5.00/5.16	NCBInr: gil4585271	
66	seed maturation protein PM22 (*G. max*)	575	21	54	16500/16735	4.90/5.16	NCBInr: gil4585271	
67	Napin-type 2S albumin 1 precursor (*G. max*)	171	4	23	18000/18393	5.10/6.00	NCBInr: gil4097894	
68	dehydrin (*G. max*)	299	6	40	19000/23720	5.90/5.87	NCBInr: gil37495455	
69	basic 7S globulin (*G. max*)	*205*	7	23	17500/46394	7.20/8.70	UniProtKB: P13917	
70	basic 7S globulin 2 precursor (*G. max*)	781	38	23	16000/47859	8.25/8.58	NCBInr: gil 351727625	
71	basic 7S globulin (*G. max*)	*177*	6	20	18500/46394	9.00/8.70	UniProtKB: P13917	
72	β-conglycinin β-subunit(*G. max*)	*400*	12	31	20000/48331	10.00/5.70	UniProtKB: Q50JD8	
73	basic 7S globulin (*G. max*)	*132*	5	14	19000/46394	9.90/8.70	UniProtKB: P13917	
74	basic 7S globulin	57	3	25	18000/14111	9.90/4.83	NCBInr: gil225878	
75	Bowman–Birk proteinase inhibitor D-II (*G. max*)	61	3	24	13000/12343	3.50/4.98	NCBInr: gil18572	
76	Bowman–Birk proteinase inhibitor (*G. max*)	83	2	59	11500/5097	3.50/6.67	NCBInr: gil4191566	
77	Bowman–Birk proteinase inhibitor (*G. max*)	94	2	59	13000/5097	3.75/6.67	NCBInr: gil4191566	
78	Bowman–Birk proteinase inhibitor D-II (*G. max*)	*181*	2	30	12500/9468	4.00/4.90	NCBInr: gil124033	
79	Bowman–Birk proteinase inhibitor D-II (*G. max*)	96	2	30	11500/10316	3.85/4.93	NCBInr: gil122033	
80	Bowman–Birk proteinase isoinhibitor D-II (*G. max*)	93	2	24	14000/12343	4.20/4.98	NCBInr: gil18572	
81	Bowman–Birk proteinase isoinhibitor D-II (*G. max*)	205	7	48	12500/12343	4.15/4.98	NCBInr: gil18572	
82	Bowman–Birk proteinase isoinhibitor D-II (*G. max*)	78	2	24	13000/12343	4.30/4.98	NCBInr: gil18572	
83	Bowman–Birk proteinase isoinhibitor D-II (*G. max*)	226	10	48	12000/12343	4.30/4.98	NCBInr: gil18572	
84	Bowman–Birk proteinase isoinhibitor D-II (*G. max*)	199	7	48	12500/12343	4.60/4.98	NCBInr: gil18572	
85	Bowman–Birk proteinase isoinhibitor D-II (*G. max*)	102	4	24	11500/12343	4.55/4.98	NCBInr: gil18572	
86	Bowman–Birk proteinase isoinhibitor D-II (*G. max*)	80	2	24	12500/12343	4.85/4.98	NCBInr: gil18572	
87	Bowman–Birk proteinase isoinhibitor D-II (*G. max*)	126	4	33	11500/12343	4.75/4.98	NCBInr: gil18572	
88	Bowman–Birk proteinase isoinhibitor D-II (*G. max*)	115	4	24	11500/12343	4.85/4.98	NCBInr: gil18572	
89	Bowman–Birk proteinase isoinhibitor D-II (*G. max*)	130	8	30	11500/12343	4.95/4.98	NCBInr: gil18572	
90	Bowman–Birk proteinase isoinhibitor D-II (*G. max*)	79	4	24	12000/12343	5.05/4.98	NCBInr: gil18572	
91	late embryogenesis-abundant protein (*G. max*)	351	7	46	9000/11485	7.40/5.52	NCBInr: gil1762955	
92	no match							
93	no match							

[a]Spot identification numbers correspond to those proteins labeled in Figure 5. [b]Molecular weight (M_r) and isoelectric point (pI) values are given as experimental/theoretical values. Protein scores (nonitalics) represent those searches performed via Mascot. Protein scores (italics) represent those searches performed via Protein Prospector. Peptides matched, % coverage, and accession numbers within each respective database are given. Searches were confined to the *Glycine max* database unless no clear match could be found. Putative uncharacterized proteins from the soybean database were blasted against the NCBInr all-plant database, and the resulting homologous proteins and accession numbers are given in brackets. The database used was National Center for Biotechnological Information (NCBI nonredundant).

S4, and S5) were reported to be released from soybean seeds immersed in warm water.[2] On the basis of N-terminal amino acid sequencing and immunoblot analysis, the S1, S2, and S4 were identified as the subunits of the 7S basic globulin. The N-terminal sequence of the S5 was found to be homologous to a hydrophobic protein of soybean, while the S3 protein revealed no homology to any known sequences in the protein database.[2] Lupin seeds also secrete large amounts of conglutin γ, a glycoprotein similar to soybean 7S basic globulin, when incubated in warm water.[39] The results of our study also confirms that the 7S basic globulin is the most abundant protein in the soybean seed exudate. Unlike the previous study, we were able to detect several additional proteins in the soybean seed exudates. 2D-gel electrophoresis followed by LC-MS/MS resulted in the identification of 90 proteins including those which are known to play a protective role against plant pathogens. Several other studies have documented the release of antimicrobial proteins and peptides during seed germination in both legume and nonleguminous plants.[10,39−42]

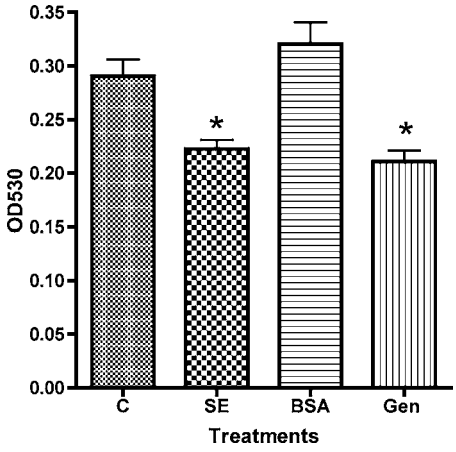

Figure 6. Effect of soybean seed exudates on the proliferation of MCF7 breast cancer cells. Cells were treated with 3.2 mg/mL of protein exudates (SE) obtained from soybean seeds incubated at 50 °C for 8 h or 400 μm bovine serum albumin (BSA) or 25 μm genistein (GE). After 24 h of incubation, antiproliferation effects of the test compounds were determined as described in Materials and Methods. *, $p < 0.005$ as compared with the control.

Soybean seed releases large amounts of basic 7S globulin when incubated in warm water. Proteins homologous to soybean 7S basic globulin have been shown to inhibit the activity of endoglucanases belonging to glycoside hydrolase families 11 and 12. Recently, the crystal structure of basic 7S globulin was elucidated and found to be similar to that of xylanase inhibitor protein from wheat.[8] This study suggested that the soybean 7S basic globulin is a xyloglucan-specific endo-β-1, 4-glucanase inhibitor protein (XEGIP). Since β-linked glucans are important components of the plant cell wall and are targeted by bacterial and fungal endo-β-glucanases for degradation, the release of the7S basic globulin (XEGIP) to the rhizosphere would protect the germinating seedling against plant pathogens. However, Yoshizawa and his associates[9] could not detect any inhibitory activity of the 7S basic globulin on either glycoside hydrolase family members 11 and 12. This observation raises doubt on the role of 7S basic globulin protein protecting the soybean plants against fungal and bacterial pathogens. However, one cannot exclude the possibility that the soybean 7S basic globulin, which reveals distinct biochemical and biophysical features, may inhibit other glycoside hydrolase family members.

In our study, we identified the presence of significant amounts of seed storage proteins (7S β-conglycinin and 11S glycinin) in the seed exudates. Even though it is possible that these storage proteins may have been released from the seeds due to cell damage, their role in defense against pathogens cannot be ignored. It should be pointed out that vicilins (7S storage proteins) from soybean, cowpea (*Vigna unguiculata*), jack bean (*Canavalia ensiformis*), adzuki bean (*Vigna angulariz*), common bean (*Phaseolus vulgaris*), and lima bean (*Phaseolus lunatus*) are known to interfere with the development of the cowpea weevil (*Callosobruchus maculatus*) larvae and prevent fungal spore germination.[28,29] It has been proposed that the toxic effect of vicilins is due to its ability to interact with glycoproteins present in the digestive tracts of insects.[43] Soybean seed exudates also contain two protease inhibitors: Kunitz inhibitors and Bowman–Birk inhibitors. These two serine protease inhibitors have been extensively studied and are known to play an important defensive role against insects and pathogens.[12] They bind to digestive proteases of phytophagous insects and impede protein digestion resulting in delayed growth and development and reduced fecundity and mortality.[44–46]

Bowman–Birk inhibitor concentrate (BBIC), a crude extract prepared from defatted soybeans, has been shown to affect various forms of cancer.[31–36] Currently, BBIC is being tried in human clinical trials to treat several forms of cancer.[47–49] The results from these clinical trials are promising, and there is increasing evidence that BBIC can be exploited as an effective chemopreventive agent. The preparation of BBIC has been previously described, and it is time-consuming and involves the use organic solvents.[31] The abundance of BBI in soybean seed exudates by incubating the seeds in warm water provides a simple and alternative method to isolate this low molecular weight proteins. Our results demonstrate that BBI in the seed exudates retains chymotrypsin inhibitor activity and also inhibits the proliferation of MCF7 breast cancer cells. It will be interesting to examine if the BBI prepared by incubating the seeds in warm water is as efficacious as the BBIC prepared following the previously published elaborate procedures using organic solvents.

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Notes

Product names are necessary to report factually on available data; however, the University of Missouri and the USDA neither guarantees nor warrants the standard of product, and the use of the name by the University of Missouri and the USDA implies no approval of the product to the exclusion of others that may be suitable.

The authors declare no competing financial interest.

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