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Quantification of Gly m 4 Protein, A Major Soybean Allergen, By Two-Dimensional Liquid Chromatography with Ultraviolet and Mass Spectrometry Detection

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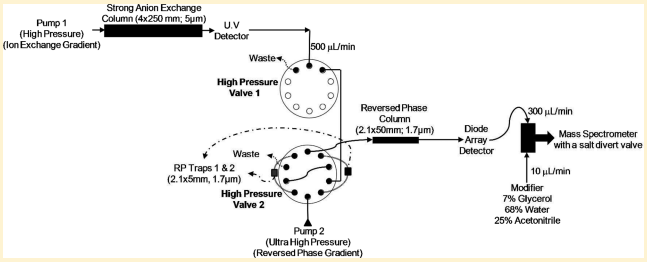
ABSTRACT: Soybean (*Glycine max*) is considered a major allergenic food. Gly m 4 is one of several soybean allergens that has been identified to cause an allergic reaction, typically the symptoms are localized effects including the skin, gastrointestinal tract, or respiratory tract. Soybean allergens are considered a complete food allergen in that they are capable of inducing specific IgE as well as eliciting a range of severity from mild rashes up to anaphylaxis. In this study, we have isolated, purified, and characterized an endogenous Gly m 4 protein. The endogenous protein has 88.0% sequence homology with the theoretically predicted Gly m 4 sequence. Following detailed characterization, an assay was developed for quantification of endogenous Gly m 4 using two-dimensional liquid chromatography with ultraviolet and mass spectrometric detection (2DLC−UV/MS). A linear relationship ($R^2 > 0.99$) was observed over the concentration range of 12.5−531.7 μg/mL. Over the linear range, the assay recoveries (percent relative error, % RE) ranged from −1.5 to 10.8%. The assay precision (percent coefficient of variation, % CV) was measured at three different Gly m 4 levels on each of the 4 days and did not exceed 11.2%. The developed method was successfully applied to quantify Gly m 4 level in 10 commercial soybean lines. To the best of our knowledge, this represents the first quantitative assay for an intact endogenous Gly m 4 protein.



Soybean (*Glycine max*) is considered a major allergenic crop and is one of the eight major food or food groups which account for 90% of allergenic responses.[1] Over 16 soybean allergens have been described in the literature.[2] The International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-Committee has officially accepted some of these allergens as major soybean allergens.[3] These soybean allergens include Gly m 1 (soybean hydrophobic protein),[4] Gly m 2 (defensin),[5] Gly m 3 (profilin),[6] Gly m 4 (PR-10 protein),[7] Gly m 5 (β-conglycinin, 7S globulin), and Gly m 6 (glycinin, 11S globulin).[3] Soybean allergy is most prevalent in children where the reactions may be systemic; however, typically these symptoms are most common to the skin and gastrointestinal and respiratory tracts and can be attributed to IgE-mediated responses.[8] The symptoms range from mild skin irritation to severe anaphylaxis. Approximately 0.4% of children and 0.3% of adults in North America have soybean allergies.[9] As soybean is a major oilseed crop that is often incorporated into products such as beverages, processed foods, and pharmaceuticals, consumers allergic to soybean must avoid soy products in order to prevent an allergic reaction. Currently there is a need for accurate and sensitive methods to identify and quantify seed allergens to provide the means to compare allergen concentrations among seed varieties and understand the contribution of both genetic and environmental factors. Furthermore, these advanced technical approaches have the potential to be applied to understanding exposure levels, tolerance thresholds, and provide allergen expression in genetically modified seeds and breeding lines before commercial release.

In this study, we focused on developing a quantitative assay for determination of Gly m 4 protein in soybeans. Gly m 4 is a 157 amino acid sequence with a low molecular weight of about 17 kDa. The physiological function of Gly m 4 is still unknown, although Gly m 4 was first identified at the mRNA level as one of the genes in the family of stress-induced, developmentally regulated soybean genes.[10] The mRNA was reported to be expressed under stress conditions such as starvation and is thus also called SAM22 (starvation-associated message 22). The mRNA was shown to accumulate predominately in the roots of soybean seedlings and in roots and leaves of mature plants. SAM22 accumulation was observed in senescent leaves and can be induced on young leaves by wounding. In addition, it was observed that multiple copies of sequences related to SAM22 exist in the soybean genome. Moreover, the recombinant Gly m 4 (rGly m 4) was expressed and its allergenic activity confirmed in clinical and immunological studies.[11]

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Gly m 4 also belongs to the pathogenesis-related proteins of class 10 (PR-10) in higher plants. They form a multigenic family with more than 50 amino acid sequences of plant PR-10s being registered in the genome data banks. The PR-10 proteins are common panallergens in the family of Fagales pollens (e.g., Alder, Beech, Chestnut, Hornbeam) and may be present in a number of vegetables and fruits (e. g., apple, pear, lupine, and hazelnut).[12,13] Gly m 4 is also known to have high homology to the major birch pollen allergen Bet v 1.[11] The three-dimensional structure of the rGly m 4 and Bet v 1 is nearly identical, thereby causing high cross-reactivity of both allergens with the same IgE antibodies originally derived against Bet v 1, as shown by immunoblot inhibition and histamine release assays.[11]

The role of Gly m 4 is still obscure because accurate quantification of the protein is difficult. Many of the previous studies are challenged by extensive sample preparation or inadequate and nonspecific *in vitro* bioassays. Previously, immuno-based approaches, such as IgE-immunoblotting and enzyme-linked immunosorbent assays (ELISA), have been used for identification and quantification of soy allergens.[14,15] Although immunoassays generally are highly sensitive and provide a high throughput, they suffer from limitations such as sera availability, high variability, and a limited range of specificity. Moreover, the development of antibodies for multiple soybean allergens is a time-consuming process. Others have investigated endogenous soybean food allergens by using a 2-dimensional gel electrophoresis approach followed by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry.[16] This approach is labor intensive, and it is not clear how much of the variation in quantification is due to experimental precision versus biological variability. Another quantitative proteomic technique utilizes isotopically labeled synthetic analogues of tryptic signature peptides as internal standards to determine the per mass unit amount of one or more proteins within a complex matrix.[17,18] Although sensitive and robust, this approach may pose several challenges such as (a) selecting a suitable peptide whose sequence is specific only to the protein of interest,[18] (b) the potential contribution of peptides from isoforms and other proteins with substantial homology, (c) the potential significant difference in the behavior of the internal standard compared to that of the intact protein, e.g., due to *in vitro* modification of the proteins,[19] and (d) assumption of complete tryptic digestion of the protein, which is often not the case.[20]

Quantification at the intact protein level can circumvent the above-mentioned limitations as the intact protein itself is utilized as a reference standard for quantification. Intact proteins have been chromatographically resolved and quantified by either LC−UV or LC−MS approaches.[21−23] The major challenges are isolating the protein of interest from a complex biological matrix in order to develop a reference standard and chromatographically resolving the protein of interest from other coeluting components for accurate quantification. The former is less of an issue for relatively abundant proteins but can be nontrivial for isolation of very low abundant proteins. The latter challenge can be overcome by increasing the resolving power by utilizing multidimensional chromatographic approaches.[24−26] The enhanced resolving power of a multi-dimensional chromatographic approach allows the possibility of mitigating the complexity of these samples.[27] A few multi-dimensional approaches have been applied to identify, characterize, and quantify intact proteins.[28−30]

In this study, endogenous Gly m 4 was isolated based on the purification scheme for r-Gly m 4.[31,32] To the best of our knowledge, this is the first time that a native endogenous Gly m 4 protein has been isolated from soybean seeds. Following isolation and characterization, a 2DLC−UV/MS based quantitative method was developed. The method was evaluated for assay selectivity, linearity, sensitivity, and recovery and applied to determine the concentrations of Gly m 4 in 10 commercial lines. This report describes, for the first time, the use of a 2DLC−UV/MS based assay to accurately quantify Gly m 4 in soybean seed samples for the assessment of a food allergen.

EXPERIMENTAL SECTION

Materials. The soybean seeds were purchased from the following: H387 (lot no. JK1700) from Hoffman (Hoffmann, IL); HS38C60 (lot no. SZ090151) from Hi Soy (Bloomington, IL); C3884N (lot no. 1208294) from LG Seeds (Elmwood, IL); William 82 (lot no. 9S-10-107) from Missouri Seed Foundation (Columbia, MO); 3930 (lot no. S080184 GR01-596) from Croplan LC (St. Paul, MN); 99915 (lot no. DSR-3590 029301K) from Dairyland/Precision Soya (West Bend, WI); XP843-252 (lot no. 9E394N) from Brown Seed (Neoga, IL); Maverick (lot no. 8S-18-07) from Missouri Foundation (Columbia, MO); 363 (lot no. PS9-3633) from Phillips (Hope, KS); and pH-4396 (lot no. 01A (75148)) from Porter Hybrid (Wilmington, OH). Dithiothrietol (DTT), iodoacetamide (IAM), bis-Tris buffer, sodium sulfate, and ammonium bicarbonate buffer were purchased from Sigma (St. Louis, MO). Sodium hydroxide, sodium chloride, glycerol, LC−MS grade trifluoroacetic acid (TFA), β-mercaptoethanol, HPLC-grade isopropanol (IPA), tris buffer, monosodium phosphate, hydrochloric acid, acetone, hexanes, and acetonitrile (ACN) were purchased from Thermo Fisher Scientific (Waltham, MA). Steriflip disposable vacuum filtration system with a 0.22 μm membrane filter was purchased from Millipore (Billerica, MA). Molecular weight standards, Tween-20, Criterion 4−20% Tris-Tricine gels were purchased from Bio-Rad (Hercules, CA). Trypsin was obtained from Roche Applied Sciences (Indianapolis, IN). For all analyses, Milli-Q (Millipore, Billerica, MA) deionized water was used. Sodium chloride and hydrochloric acid solutions were used to adjust the pH as needed.

Reference Gly m 4 Preparation: Purification of Gly m 4 from Nontransgenic Soybean Seed. Extraction and isolation of the soybean derived Gly m 4 was performed as follows. Conventional soybean kernels were ground to a fine powder with a Robot Coupe grinder (model no. RSI 2Y-1, Robot Coupe USA, Inc.) containing an equal amount of dry ice. The dry ice was allowed to vent off overnight at −20 °C.

The milled soybean seeds were defatted with hexanes (milled soybean seeds/*n*-hexanes; 1:5 w/v) for 1 h at room temperature. This was followed by centrifugation and removal of the oil containing supernatant. The procedure was repeated, and defatted soybean seeds were subsequently extracted with 40% v/v IPA/water for 1 h at room temperature based on the extraction method described previously.[33] The supernatant, containing residual fine flour particles, was filtered using SteriFlip disposable vacuum filtration units (0.22 μm) and subjected to protein precipitation by adding 4× excess (by volume) of cold acetone (−20 °C), followed by vortexing and placing the tubes at −20 °C overnight. The tubes with precipitated proteins were centrifuged at 7200*g* for 15 min at 4 °C, and the supernatant was discarded. The protein precipitate

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was dried under nitrogen to remove traces of acetone. The protein precipitate was resolubilized in the solubilization buffer (0.1 M Na_2SO_4/0.2 M NaH_2PO_4/5 mM DTT/0.05% Tween-20/5% glycerol, pH 8) followed by filtration (0.22 μm) and dialysis (3500 kDa MWCO) against 25 mM bis-Tris buffer at pH 6.0. The resultant solubilized protein extract was then separated by reversed phase chromatography using a linear gradient of A (0.1% TFA in water) and B (0.1% TFA in ACN) mobile phases. A Waters XBridge300 C4 250 mm × 4.6 mm column (Milford, MA) held at 30 °C was equilibrated at 35.5% B prior to loading 250 μL of the extract at a flow rate of 1.4 mL/min. The column was held at initial conditions for 4.5 min. Subsequently, gradient elution from 35.5% B to 45.5% B was applied over 50 min at 0.48 mL/min. The peak eluting at ∼40 min was collected. Hydrophobic components were eluted by increasing the gradient to 55.5% B over 10 min at 1.439 mL/min. The column was re-equilibrated to initial conditions for 15 min. The collected RP-HPLC fractions were examined by LC−MS analyses. The fractions containing Gly m 4 protein were combined, dried under nitrogen, resolubilized in 100 mM Tris buffer/5% glycerol/10 mM dithiothrietol, pH 8.5 buffer, and the protein concentration was determined by quantitative amino acid analysis. The pooled fractions were aliquoted into vials and stored at −80 °C.

SDS-PAGE Analysis and Protein Digestion. To facilitate identification and characterization of Gly m 4, SDS-PAGE was run with 10−20% Tris-Tricine gels. In brief, protein fractions were dried in a centrifugal evaporator, and resolubilized in 20 μL of Tris-Tricine sample buffer containing 2% β-mercaptoethanol. The solubilized samples were heated at 95 °C for 10 min, then cooled to room temperature, and centrifuged at 16 000*g* for 1 min. The protein samples, along with molecular-weight standards, were separated by Tris-Tricine SDS-PAGE and stained with Coomassie brilliant blue G-250 dye. The protein bands of interest along with a blank gel section were excised, macerated, destained, and reduced with DTT and alkylated with iodoacetamide. The processed gel bands were dried in a centrifuge evaporator and incubated with trypsin at 37 °C overnight. The resulting tryptic peptides were sequentially extracted from the gel with 50% ACN/0.05% TFA and 70% ACN/5% FA in 25 mM ammonium bicarbonate buffer. The peptide extracts were pooled, dried, and reconstituted in 5% ACN/0.1% TFA and subsequently analyzed by mass spectrometry.

Chromatographic and Mass Spectrometric Conditions for Characterization of Gly m 4 and Variant. Complete peptide mass fingerprinting and intact mass determination were carried out on Gly m 4 reference standard and variants. All mass spectra was acquired in the positive ion mode on Agilent 6538 Q-TOF mass spectrometer equipped with a dual ESI ion source and coupled to an Agilent 1290 liquid chromatography system (Santa Clara, CA). Unless otherwise mentioned, UV data were acquired using Agilent 1290 diode array detector. Instrumental parameters for mass spectral acquisition were as follows: VCap was set at 3500 V, fragmentor at 175 V, skimmer1 at 65 V, gas temperature at 350 °C, gas flow at 10 mL/min and nebulizer at 20 psi.

For intact molecular weight determination, purified Gly m 4 reference standard was diluted 1:3 v/v in 100 mM Tris/5% glycerol/10 mM DTT/pH 8.5 solution. Chromatography was performed by gradient elution from Acquity BEH300 C4 column (Waters, Milford, MA) at 70 °C with column dimensions of 100 mm × 2.1 mm and 1.7 μm particle size. The column was equilibrated using 95% mobile phase A (0.1% (v/v) (FA in water; MPA) and 5% mobile phase B (0.1% (v/v) FA in IPA; MPB) at a flow rate of 100 μL/min. A constant 8 μL injection volume was utilized. A linear gradient was employed from 5% MPB to 40% MPB over 55 min at 100 μL/min. This was followed by increasing the flow rate to 300 μL/min to re-equilibrate the column. Both UV (215 nm) and MS (50−2500 amu, 0.5 Hz) data were acquired. The maximum entropy algorithm (MAXENT) included with the BioConfirm module of Agilent MassHunter Qualitative Analysis software was used to transform the spectra to a mass axis. The maximum entropy algorithm was set to optimize the spectra in the mass range of 16 100−17 100 Da with a resolution (mass step) of 1 Da, S/N threshold of 5, and automatic isotope width selection.

For detailed characterization of tryptic digest of Gly m 4, chromatography was performed by gradient elution from a Acquity BEH130 C18 column (Waters, Milford, MA) at 50 °C with column dimensions of 100 mm × 2.1 mm and 1.7 μm particle size. The column was equilibrated using 95% mobile phase A (0.1% (v/v) formic acid (FA) in water; MPA) and 5% mobile phase B (0.1% (v/v) FA in acetonitrile; MPB) at a flow rate of 350 μL/min. A constant 20 μL injection volume of tryptic digest of Gly m 4 was utilized. A linear gradient was employed from 5% MPB to 40% MPB over 44 min at 100 μL/min. This was followed by increasing the flow rate to 350 μL/min from 40% MPB to 60% MPB over 5.0 min. The column was then re-equilibrated to initial conditions for 6 min. Both UV (210−600 nm) and MS (100−2200 amu, 1 Hz) data were acquired. During tandem MS experiments, data dependent auto MS/MS mode with active exclusion was utilized. Peaks were isolated for tandem MS with a 9 amu isolation width, and a ramped collision energy based on charge state was applied. All acquired data (MS and MS/MS) were processed manually and using the BioConfirm software from Agilent.

Extraction of Gly m 4 from Soybean Seeds for 2DLC−UV/MS Assay. Milled ground soybean seeds, stored at −20 °C, were thawed at room temperature in a drybox containing Dry-Rite. Approximately 250 mg of milled soybean seeds were weighed, defatted using 1 mL of hexanes, and mixed at 1200 rpm for 1 h at 25 °C on a Thermomixer. The sample was clarified by centrifugation at 20 817*g* for 5 min at 4 °C. The supernatant was discarded, and 1 mL of hexanes was added to the sample and the procedure was repeated. The sample was clarified by centrifugation at 20 817*g* for 10 min at 4 °C. The supernatant was discarded, and residual hexanes was removed by evaporation for approximately 10 min. The resulting sample was resuspended in 1 mL of protein extraction buffer (40% v/v IPA/water). This was followed by vortexing and extraction at 1200 rpm for 2 h at 25 °C using a Thermomixer. Following extraction, the sample was centrifuged at 20 817*g* for 30 min at 4 °C. The supernatant was transferred to a microfuge tube and subjected to additional centrifugation at 20 817*g* for 5 min at 4 °C to remove residual particles. The extracts were then aliquoted for subsequent analyses by 2DLC−UV/MS analyses.

Chromatographic and Mass Spectrometric Conditions for 2DLC−UV/MS Assay. Two-dimensional liquid chromatography with ultraviolet and mass spectrometric detection (2DLC−UV/MS) was performed on a 2DLC system combined by coupling Agilent's 1260 and 1290 LC systems as described previously.[30] Strong anion exchange chromatography (SAX) was used in the first dimension, and reversed phase chromatography (RPC) was used in the second dimension. The samples were injected using a partial loop fill injection

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mode and 10 μL injection volumes. This corresponded to approximately 12 μg of reference Gly m 4 protein and approximately 30 μg of extracted soybean protein (from control soybean sample). Propac SAX Column (Thermo Scientific, Waltham, MA) with dimensions of 250 mm × 4 mm and 5 μm particle size was equilibrated at 30 °C with 95% mobile phase A1 (10 mM Tris buffer, pH 7.6; MP A1) and 5% B1 (10 mM Tris buffer, 1 M NaCl, pH 7.6; MP B1) at a flow rate of 500 μL/min for 5 min. A linear gradient was employed from 5% B1 to 25% B1 over 20 min. To accommodate for minor retention time changes in the first dimension, reference Gly m 4 standard was analyzed before each set of analyses. The trapping window determined for Gly m 4 reference standard was maintained for the rest of analyses. The proteins of interest eluted between 9.2 and 13.5 min from the first dimension SAX column were diverted toward a 10 port valve containing two 5 mm × 2.1 mm VanGuard BEH300 C4 reversed phase (RP) trap cartridges (Waters, Milford, MA). The proteins eluting between 9.2 and 11.2 min were trapped on the first RP trap cartridge, and the proteins eluting between 11.2 and 13.5 min were trapped on the second RP trap cartridge. In the second dimension, a reversed phase Acquity BEH300 C4 column (Waters) at 70 °C with column dimensions of 50 mm × 2.1 mm and 1.7 μm particle size was used. The column was equilibrated using 74% mobile phase A2 (0.1% (v/v) TFA in water; MP A2) and 26% mobile phase B2 (0.1% (v/v) TFA in 90/10 v/v IPA/ACN; MP B2) at a flow rate of 300 μL/min. A linear gradient was employed from 26% MP B2 to 35% MP B2 over 17 min for the first reversed phase cut and 26% MP B2 to 35% MP B2 over 9 min for the second reversed phase cut.

Both UV (215 nm; 0.6 Hz; bandwidth 4 nm; slit width 4 nm) and MS (500−2500 amu, 1.32 spectra/s) data were acquired. UV data was acquired using a variable wavelength detector (Agilent) for the first dimension and a diode array detector (Agilent) for the second dimension. Positive-ion electrospray ionization (ESI) was performed on Agilent's 6538 mass spectrometer. A solution containing 7/25/68 v/v % of glycerol/acetonitrile/water was introduced as a postreversed-phase column additive to improve ionization efficiency of eluting proteins in the presence of TFA.[21] The mass spectrometer was calibrated prior to use in the mass range 100−3200 amu.

Validation Experiments. Quantification of Gly m 4 in soybean seeds was based on the UV response factor calculated from the purified reference standard of Gly m 4. The reference standard was prepared in 100 mM Tris buffer/5% glycerol/10 mM DTT, pH 8.5 and stored at −80 °C in 10 μL aliquots. On the basis of amino acid analyses, the concentration of reference Gly m 4 was found to be 1210 μg/mL. For calculation of Gly m 4 level in soybean seeds, chromatographic response obtained for the monomeric form of Gly m 4 (purity ∼95% by second Dimension RPC; 215 nm) was utilized. The 2DLC based analyses of Gly m 4 with UV detection was coupled to a mass spectrometer to ensure method selectivity and determine if there are any coeluting components within the chromatographic peaks for Gly m 4 in the respective second dimensions.

The basal level of Gly m 4 in the control soybean sample was first determined by carrying out 2DLC−UV/MS analyses in triplicate. The recovery and linearity of the 2DLC−UV/MS assay were determined by analyzing soybean seed extracts with Gly m 4 at different concentration levels. A total of nine points were utilized for the recovery/linearity analysis including the control soybean sample. This was achieved by diluting control soybean sample to three different concentration levels with the extraction buffer. An additional five points were achieved by spiking Gly m 4 reference standard to the control sample. This analysis covered the range from 12.5 μg/mL to 531.7 μg/mL. A single point calibration was performed using reference Gly m 4 calibration standard. Assay recovery (percent relative error, % RE) was calculated and is presented in Table 1. Linearity of the method was also evaluated using the same data. A linearity curve was obtained by plotting the peak area of Gly m4 versus concentration. A linear regression was used to obtain a linear equation over the range of 12.5 μg/mL to 531.7 μg/mL. The equation was not forced through the origin for the linearity curve calculations. Analyte carryover was evaluated by analyzing solvent blank immediately following the highest standard sample. The percent absolute carryover was calculated as a percent relative to the lowest recovery level.

Table 1. Multipoint Recovery Analyses for Determination of Gly m 4 at Different Levels in Soybean Seed Extracts[a]

expected Gly m 4 (μg/g)	expected Gly m 4 (μg/mL)	observed Gly m 4 (μg/mL)	recovery (% RE)
48.0	12.5	13.6	9.2
98.8	25.7	25.3	−1.5
151.5	39.4	42.2	7.1
198.3	51.5	51.5	0.0
550.9	143.2	156.2	9.1
880.0	228.7	253.4	10.8
1286.0	334.3	364.4	9.0
1676.1	435.6	468	7.4
2045.6	531.7	570.3	7.3
		average	6.5

[a]Gly m 4 concentrations are reported both in terms of concentration in solution and corresponding concentration in seed.

The precision of the assay to measure Gly m 4 levels was evaluated on each of 4 days by analyzing three replicates at three concentration levels (low, medium, and high) by two different analysts. The Gly m 4 levels were calculated by utilizing a single point reference Gly m 4 calibration standard which was analyzed in triplicate each day to obtain an average response factor. Assay intraday and interday precision (percent coefficient of variation, % CV) was calculated and is presented in Table 2.

Robustness of the method was evaluated by investigating Gly m 4 extraction efficiency, the influence of column temperature on Gly m 4 response in the two chromatographic dimensions, and stability of Gly m 4 in the protein mixture comprised of proteins extracted from soybean seeds at 4 °C and room temperature. Extraction efficiency of Gly m 4 was evaluated by carrying out three successive 2 h extractions with 40% v/v IPA solution at 25 °C. The level of Gly m 4 was determined by 2DLC−UV/MS analysis. In addition, to evaluate the effect of column temperature, both the first and second dimension column temperatures were separately varied. Initially, the first dimension column temperature was maintained at 30 °C, and the second dimension column temperature was evaluated at three different temperatures, i.e., 65, 70, and 75 °C. Similarly, the second dimension column temperature was kept constant at 70 °C, and the first dimension temperatures were changed to 27, 30, and 33 °C. The stability of the Gly m 4 in soybean seed extracts was evaluated at room temperature and 4 °C over a period of 24 and 45 h. An aliquot of soybean seed extract and

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Table 2. Intra- and Interday Precision for Determination of Gly m 4 Present in Three Different Soybean Lines Using Single Point Reference Standard Calibration[a]

day/analyst		concentration of Gly m 4		
		low	medium	high
	μg/g	190.4	324.2	485.9
	% CV	1.4	2	7.1
I/A	no. of replicates	3	3	3
	μg/g	180.4	317.7	464
	% CV	1.4	4.2	4.6
2/A	no. of replicates	3	3	3
	μg/g	177.8	314.8	463.6
	% CV	3.2	5.5	4.7
3/B	no. of replicates	3	3	3
	μg/g	176.7	285.2	487.8
	% CV	3.6	6.1	11.2
4/B	no. of replicates	3	3	3
	avg μg/g	181.3	310.5	475.3
	% CV	3.4	5.6	2.8
	no. of replicates	12	12	12

[a]Values have been rounded to show significant digits; statistical calculations have been done with full precision.

reference Gly m 4 were stored at 4 °C and room temperature for 24 and 45 h. Response of both reference Gly m 4 and soybean seed extract was measured immediately after sample preparation. After storage, the stability samples were analyzed by 2DLC–UV/MS. Stability was evaluated by comparing the stored samples to freshly prepared reference Gly m 4.

Analysis of Gly m 4 in Soybean Lines. In total, 10 different soybean seed lines were prepared in duplicate as described. Single injections of each preparation were subjected to 2DLC–UV/MS analyses. The level of Gly m 4 was determined using a single point Gly m 4 reference standard as the calibrant.

RESULTS AND DISCUSSION

Gly m 4 Isolation and Characterization. Although Gly m 4 has been identified as a major allergen in soybean, methods to isolate and quantify the native endogeneous protein have not yet been established. On the basis of the SAM22 cDNA sequence, Gly m 4 has been recombinantly expressed in *Escherichia coli* and purified using a combination of ion exchange and reversed phase chromatography.[31,34] Recently, endogenous Gly m 4 was identified in soybean seeds by extracting soybean proteins.[33] A systematic approach was carried out to isolate endogenous Gly m 4 from soybean seeds.

SDS-PAGE analysis was carried out on the soybean seed extract and purified Gly m 4 protein. Approximately 15 μg and 6 μg of extracted protein and purified protein were loaded on Tris-Tricine gel (Figure 1A). A protein band at approximately 16 kDa was observed (Figure 1A, lane 4). Gly m 4 reference standard was further characterized by reversed phase liquid chromatographic analysis with UV (215 nm) and ESI-MS detection as depicted in Figure 1B,C. A single chromatographic peak was observed with a retention time of 24.4 min. The transformed maximum entropy spectra revealed the presence of a principal mass component at mass 16 629.3 Da. In addition, deconvoluted masses representing multiple water adductions were observed (16 683.6 Da, 16 737.9 Da, 16 791.7 Da). The observed mass was within 0.002% of the theoretical average molecular mass of Gly m 4. To further characterize the Gly m 4 reference standard, peptide mass fingerprinting using trypsin was carried out for the determination of a N- and C-termini and sequence coverage map. A summary of mass spectral data plus assignments from ESI/LC–MS analyses are presented in Table 3. The peptide sequences were further confirmed by tandem ESI/LC–MS/MS analyses. Manual interpretation of the full scan MS and MS/MS spectra of all observed precursor charge states revealed the sequence. Every amino acid residue was confirmed at a minimum, by either a y or a b ion series generated by fragment ions. On the basis of ESI/LC–MS and ESI/LC–MS/MS data, complete sequence coverage was obtained for the isolated protein. A BLAST search was conducted on the experimentally observed Gly m 4 sequence.[35,36] A complete sequence match was observed with NCBI reference sequence: NP_001236562.1 (uncharacterized protein LOC100527731). The endogenous Gly m 4 (*cv* Maverick) has 88.0% sequence homology and 94% positives (residues that are very similar to each) with des-Met[1] SAM22 protein (*cv* Mandarin). Similarly, endogenous Gly m 4 has 92% sequence homology and 97% positives with H4 protein (*cv* Williams) (Figure 2). This result is further confirmation of the earlier observations that multiple sequences related to SAM22 exist in the soybean genome.[10] On the basis of the above data, it can be concluded that the isolated endogenous Gly m 4, SAM22, and H4 proteins belong to the same stress-induced gene family.

Method Development. A two-dimensional liquid chromatographic assay was developed to quantify Gly m 4 at the intact protein level. Figure 3 depicts the schematic for the analytical setup utilized for Gly m 4 quantification. The analytical method was based on a recently published configuration with minor modifications.[30] Briefly, the configuration consists of two orthogonal separation dimensions with UV detection after each dimension. The two dimensions are coupled using a 10 port valve equipped with two trap columns for simultaneous trapping of more than one component from the first dimension in a single 2DLC analysis. The second dimension is further coupled to a mass spectrometer to enable detection of any coeluting components, thereby providing high method selectivity.

For Gly m 4 quantification, a strong anion exchange column and reversed phase column were utilized as the first and second dimensions, respectively. The two dimensions were selected owing to good chromatographic response of Gly m 4 in each of the dimensions (Figure 4). In the first dimension, Gly m 4 elutes between 9.3 and 13.5 min during the linear salt gradient (Figure 4A). This region of the gradient was introduced to the 10 port valve where the protein was trapped using two reversed phase trap columns. The first dimension regions between 9.3 and 11.2 and 11.2–13.5 min were trapped in first and second trap columns, respectively. To further resolve each Gly m 4 from other proteins, reversed phase chromatography was performed as observed in Figure 4B. The deconvoluted mass spectra for the components eluting at 26.5 and 52.3 min in the second dimension are illustrated in Figure 4C. While the peak with the retention time of 26.5 min corresponds to Gly m 4, the peak at 52.3 min corresponds to another isoform of Gly m 4 isoform A. For the two peaks, identical m/z profiles were observed for the deconvoluted mass spectra. Another minor peak at retention time 24.0 min was also observed with similar m/z profile and deconvoluted mass spectra to Gly m 4. However, the intensity of the component was very low for further analyses. The peaks eluting at 26.5 and 52.3 min were

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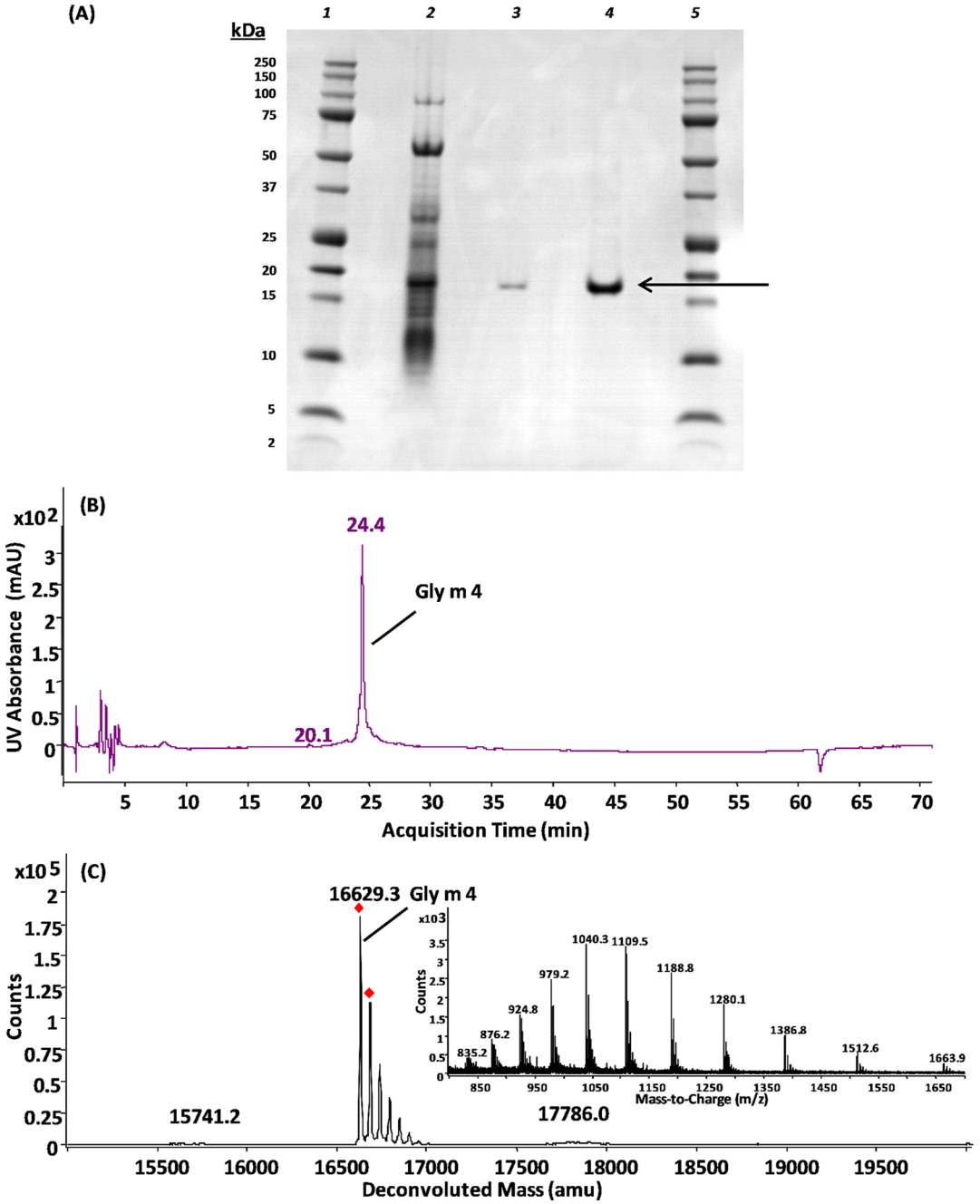

Figure 1. Characterization of reference Gly m 4 standard. (A) Isolation of endogenous Gly m 4 from soybean seed. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified protein. Lane 1 and 5: molecular weight standards; lane 2, soybean seed extract; lane 3, Gly m 4 isolated from 1st RPC cut of 2DLC−UV analysis of soybean seed extract; lane 4, reference Gly m 4 standard. The arrow indicates the Gly m 4 band. (B) A blank subtracted LC−UV (215 nm) chromatogram of intact reference Gly m 4 standard. (C) Deconvoluted mass spectrum of reference Gly m 4 standard (inset: multiple charge envelope mass spectrum).

further confirmed for their identical sequence during method validation for selectivity.

A number of extraction conditions were evaluated in order to optimize the Gly m 4 extraction protocol for 2DLC−UV/MS analyses of Gly m 4. These include extraction time, ratio of seed and extraction volume, and extraction solvent. On the basis of the different conditions evaluated, extraction with 40% IPA for 2 h in 1:4 v/v seed/extraction volume was observed to provide the best results (data not shown). The protein content in the 40% IPA extraction was observed to be approximately 12−16 mg/g of seed.

Extractions from soybean seeds of certain lines contained Gly m 4 variants that were similar in mass and retention characteristics in the two dimensions. The selectivity of the method to resolve Gly m 4 from other proteins with similar reversed-phase retention characteristics can be inferred in Figure 5. It was observed that 90/10 v/v% IPA/ACN as the strong second dimension eluant was needed due to selectivity constraints in the second dimension. The second dimension temperature was maintained at 70 °C. Use of high temperature and IPA mitigated any carryover concerns between runs as also demonstrated previously.[36] A strong anion exchange profile for

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Table 3. Summary of Peptide Mass Fingerprinting of Gly m 4 Reference Standard from *Glycine max* (*cv.* Maverick)

fragment	sequence	charge state	theoretical m/z (mi)[a], amu	experimental m/z (mi)[a], amu
Tl	(−) GVFTFEDETTSPVAPATLYK(A)	1+	2173.070	
		2+	1087.039	1087.038
		3+	725.028	725.028
T2	(K) ALVTDADNVIPK(A)	1+	1255.689	1255.69
		2+	628.348	628.349
		3+	419.235	419.236
T3	(K)AVDAFR(S)	1+	678.357	678.358
		2+	339.682	339.683
T4	(R) SVENVEGN→DGGPGTIK(K)	1+	1458.707	1458.710
		2+	729.857	729.858
T5−6	(K)KITFLEDGETK(F)	1+	1280.673	1280.673
		2+	640.840	640.841
		3+	427.563	427.563
T6	(K)ITFLEDGETK(F)	1+	1152.578	1152.578
		2+	576.793	576.793
T7	(K)FVLHK(I)	1+	643.393	643.394
		2+	322.200	322.201
T8	(K) IEAIDEANLGYSYSVVGGDGLPDTVEK(I)	1+	2811.368	
		2+	1406.188	1406.182
		3+	937.794	937.790
		4+	703.598	703.595
T9	(K) ITFEC(carbamidomethyl)K(L)	1+	797.386	797.387
		2+	399.197	399.197
T10	(K) LAAGANGGSAGK(L)	1+	973.506	973.507
		2+	487.257	487.257
T10	(K) LAAGAN → DGGSAGK(L)	1 +	974.490	974.492
		2+	487.749	487.749
Til	(K)LTVK(Y)	1+	460.313	460.314
T12	(K) YQTK(G)	1+	539.282	539.283
T13	(K) GDAQPNQDDLK(I)	1+	1200.549	1200.549
		2+	600.778	600.779
T14	(K)IGK(A)	1 +	317.218	317.219
T15-T16	(K)AKSDALFK(S)	1 +	293.836	293.837
		2+	440.250	440.251
		3+	879.493	879.493
T16	(K) SDALFK(A)	1+	680.361	680.362
		2+	340.684	340.685
T17	(K) AVEAYLLAHPDYN(−)	1 +	1475.717	1475.716
		2+	738.362	738.362
		3+	492.577	492.577

[a]mi: monoisotopic mass.

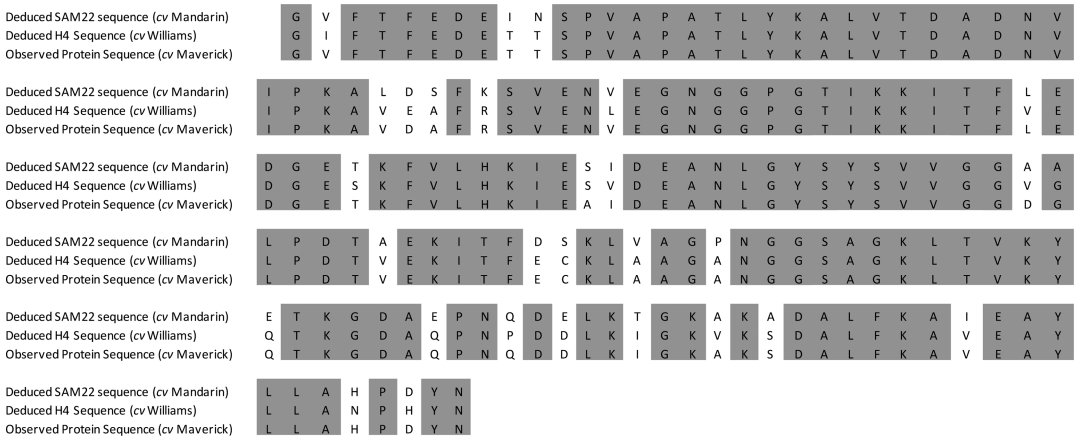

Figure 2. Characterization of reference Gly m 4 standard. Alignment of deduced amino acid sequence for stress related genes, SAM22 and H4, with experimentally observed protein sequence of isolated protein. The shaded background denotes identical amino acids within the sequences.

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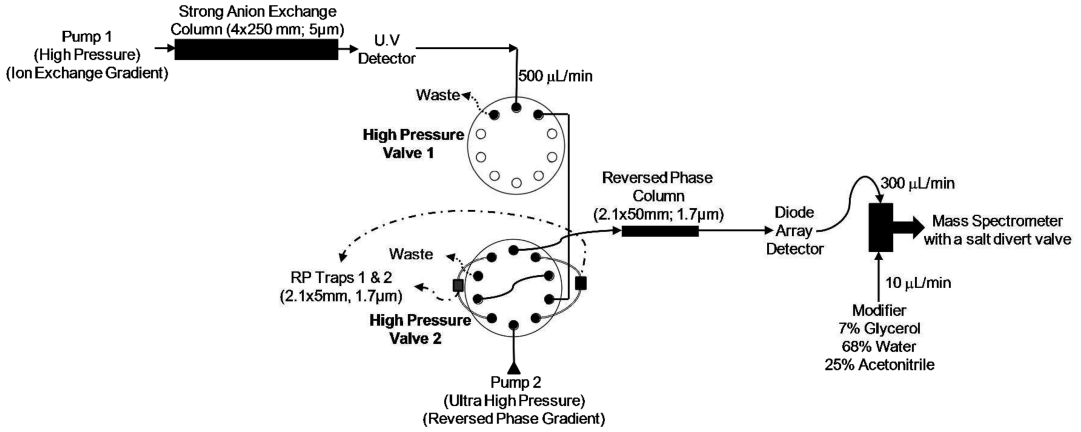

Figure 3. Schematic of analytical configuration for 2DLC–UV/MS quantification of Gly m 4.

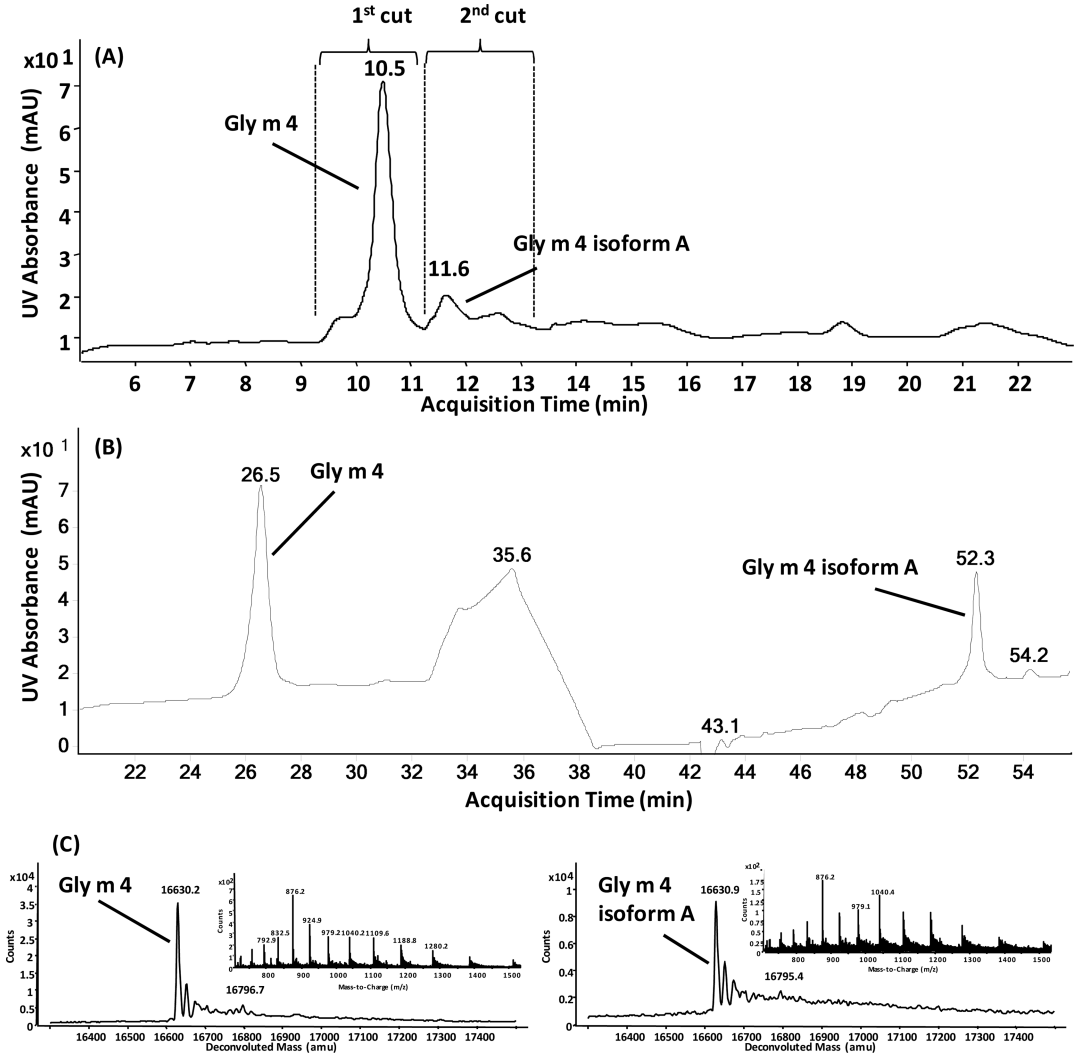

Figure 4. Representative 2DLC–UV/MS analyses of reference Gly m 4 standard. (A) First dimension strong anion exchange profile (UV, 215 nm) of Gly m 4, (B) second dimension reversed phase chromatograms of trapped first dimension cuts, and (C) deconvoluted mass spectra of respective second dimension peaks (inset: multiple charge envelope mass spectrum).

elution of the proteins extracted from milled soybean seeds can be observed from two different soybean lines (Figure 5A,D). For quantification of both Gly m 4 variants in the protein mixture, the identical window was selected between 9.3 and 11.2 and 11.2–13.5 min. The two time segments were trapped and successively eluted. In both soybean lines, two isoforms were observed for Gly m 4 in the second dimension (Figure 5B,E). The mass spectrometric confirmation of selective elution of Gly m 4 among other intact proteins was found to be critical during the method development for detecting any coelution

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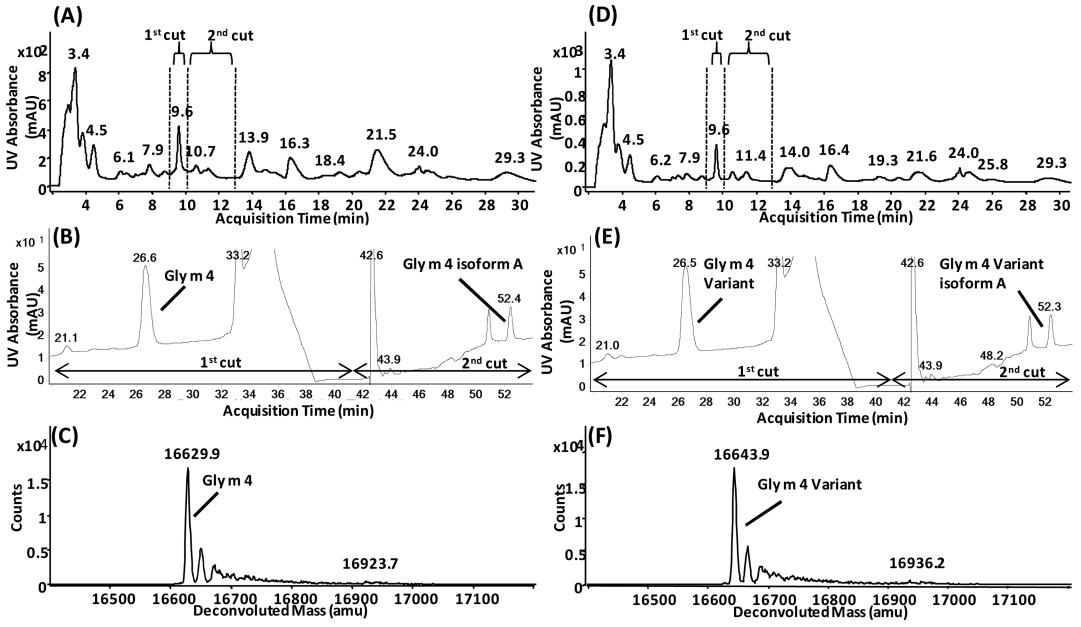

Figure 5. Representative 2DLC−UV/MS analyses of soybean extracts from lines 9 and 10. UV chromatograms (215 nm) from the (A) first and (B) second dimension separations for line 9 and (C) deconvoluted mass spectrum of the component at retention time 26.6 min. UV chromatograms (215 nm) from the (D) first and (E) second dimension separations of line 10 and (F) deconvoluted mass spectrum of the component at retention time 26.5 min are shown.

from other components in the complex matrix. The intact mass of Gly m 4 was observed to be either 16 629.5 ± 0.5 or 16 643.8 ± 0.2 Da in different soybean lines (Figure 5C,F). Further characterization of the Gly m 4 variants were conducted by collecting the LC fractions and subjecting them to in-solution proteolysis with trypsin followed by LC−ESI/MS/MS analyses. Peptide mass fingerprinting and tandem MS data revealed that a single amino acid polymorphism was observed at position 140 (aspartic acid to glutamic acid). Full sequence coverage was achieved by a combination of LC−MS and LC−MS/MS for the Gly m 4 variant. This was also corroborated by the 14 amu difference observed at the intact protein level (Figure 5). The change in the amino acid was attributed to the differences in the sequence between soybean cultivars.

Assay Validation. Apart from detection by UV (215 nm), additional mass spectrometric detection was utilized to address the issue of method selectivity. Gly m 4 containing fractions from the respective second dimensions were manually collected and pooled. The Gly m 4 fraction from the first RPC cut was analyzed by SDS-PAGE to further ensure absence of any coeluting components (Figure 1). The SDS-PAGE band of interest was digested with trypsin and confirmed for the presence of Gly m 4 protein. Gly m 4 isoform A was not analyzed by SDS-PAGE due to insufficient amounts of the isolated protein. The pooled fraction was in-solution digested and confirmed for Gly m 4 peptides based on LC−MS analyses.

For evaluating linearity and recovery of the 2DLC−UV/MS assay, the basal level of endogenous Gly m 4 in soybean seeds was determined. On the basis of the analyses, 51.5 μg/mL of endogenous Gly m 4 is present in the control soybean sample. Representative chromatograms from the second dimension separation of protein extract at different Gly m 4 levels are presented in Figure 6A,B. The developed 2DLC−UV/MS assay was observed to be linear with an R^2 value of 0.99 over the range of 12.5−531.7 μg/mL in extracted soybean seeds (Figure 6C). This corresponds to 48.0−2045.6 μg/g of Gly m 4 in soybean seeds. The recovery of the assay was also calculated for the same linear range. The assay recovery range (% RE) was observed to be −1.5% to 10.8% with average recovery of 6.5% (Table 1). The LOQ for the method was found to be 12.5 μg/mL using the ASTM signal-to-noise approach. Carryover present in a blank sample that followed the highest Gly m 4 spiked sample was found to be approximately 0.8%. The carryover after highest Gly m 4 spiked sample corresponded to 35.7% of the area of the lowest concentration Gly m 4 sample analyzed. In order to eliminate any carryover effects, blank runs were carried out after each spiked sample.

The validation results for interday and intraday precision were determined at three different Gly m 4 levels (Table 2). Precision was measured by the use of three different soybean seed lines that were previously determined to have low, medium, and high levels of Gly m 4. The precision range (% CV) at the three different levels for the method during the four day validation study was found to be 1.4 to 11.2%. The average precision over all the samples was found to be 3.9%.

The stability of Gly m 4 analyte in seed extracts was evaluated at 4 °C and room temperature at different time intervals. At 4 °C, a loss of 6.3 and 7.1% was observed for Gly m 4 in seed extracts after approximately 24 and 45 h, respectively. At room temperature, a loss of 17.2 and 12.5% was observed for Gly m 4 in seed extracts after approximately 24 and 45 h, respectively. The losses observed at 4 °C are within the precision of the method. On the basis of the above data, the analyses need to be performed using an autosampler maintained at 4 °C.

The column temperature in both the dimensions was systematically varied to understand the effect of temperature on the assay. Initially, the first dimension column temperature was fixed at 30 °C, and the concentration of Gly m 4 at second dimension temperatures of 65, 70, and 75 °C were determined to be 51.4, 54.2, and 54.3 μg/mL, respectively. Similarly with second dimension temperature fixed at 70 °C, the concen-

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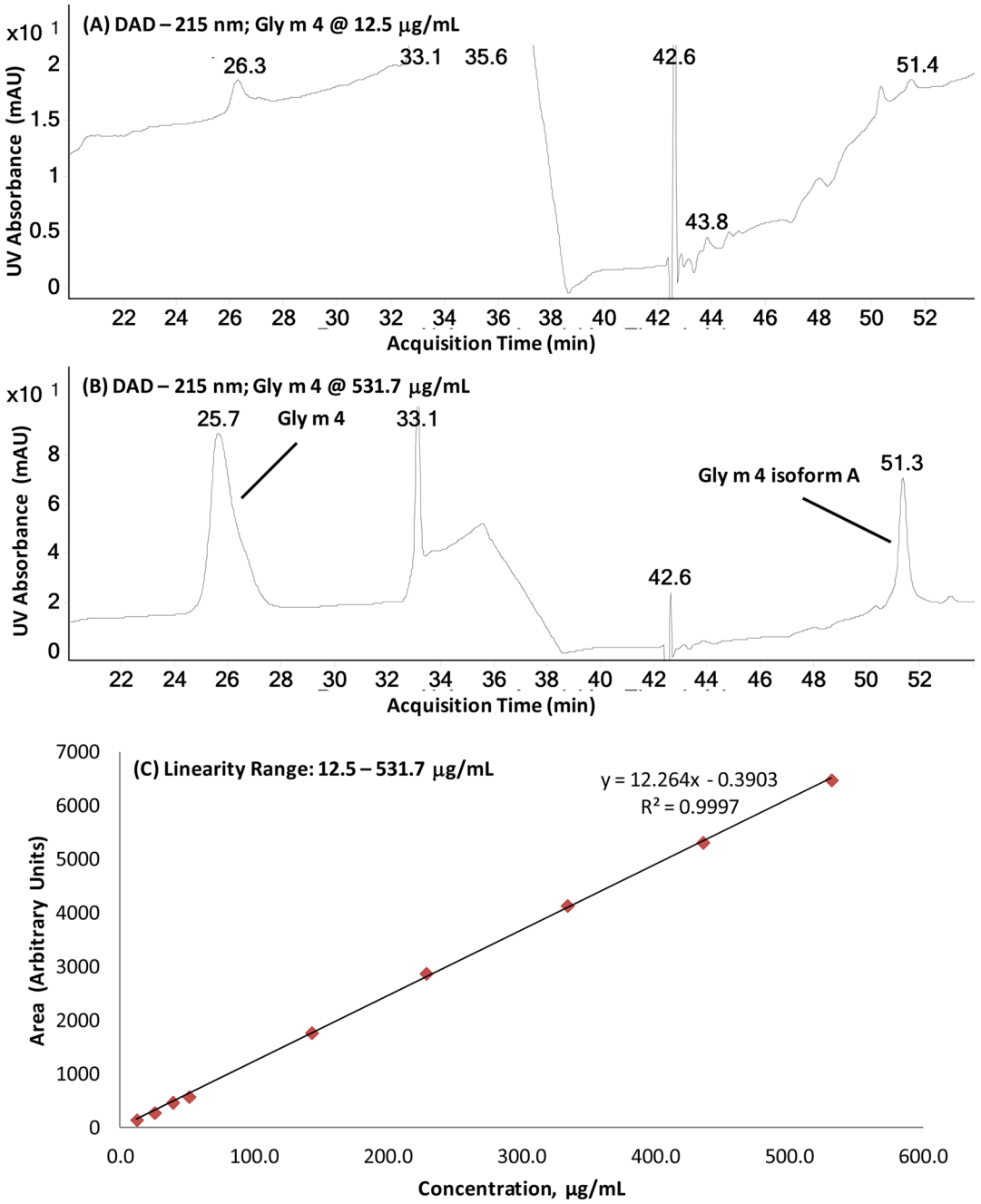

Figure 6. Second dimension UV (215 nm) chromatograms obtained from the soybean seed kernel extracts at Gly m 4 concentrations of (A) 12.5 μg/mL and (B) 435.6 μg/mL and (C) a standard linearity curve for Gly m 4 over the range 12.5−531.7 μg/mL.

tration of Gly m 4 at first dimension temperatures of 27, 30, and 33 °C were found to be 55.6, 54.2, and 51.3 μg/mL, respectively. The change in Gly m 4 levels observed is within the precision of the developed method. To evaluate Gly m 4 extraction efficiency, three successive extractions were carried out. On the basis of the 2DLC−UV/MS analyses, the second and third extractions did not yield additional Gly m 4 protein. This suggests that no additional extractions are necessary for quantitating Gly m 4 levels in soybean seeds utilizing this approach.

Analyses of Gly m 4 in Soybean Lines. The level of Gly m 4 and its variants and isoforms were quantified using the assay developed method in the 10 commercial lines. The concentration of Gly m 4 and its variant were observed to be in the range 364.5 and 608.8 μg/g (Table 4). The precision (% CV) obtained for the analysis of the 10 soybean lines is consistent with the precision of the assay determined during the validation studies. On the basis of the data, varying levels of Gly m 4 were observed in the different lines. The changes in the Gly m 4 levels could be due to differences in geographical locations among others. On the basis of the data, it can be concluded that the Gly m 4 protein can be reproducibly measured using the developed method.

■ CONCLUSIONS

An endogenous Gly m 4 protein was successfully isolated, purified, and characterized from soybean seed extracts. A 2DLC−UV/MS assay was developed for quantification of Gly

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Table 4. Determination of Gly m 4 and Variant from Different Soybean Lines[a]

line no.	line	Gly m 4 intact mass (Da)	Gly m 4 concentration, (μg/g) 1st cut	2nd cut	total	precision (% CV)
1	H387	16629.9	315.4	49.2	364.5	6.5
2	3930	16629.9	386.4	59	378.5	4.4
3	8S-18-07	16629.9	400.8	61.9	415.9	2.3
4	HS38C60	16643.9	435.1	78.2	445.4	2.4
5	XP843-252	16629.9	319.9	58.6	460.5	10.4
6	C3884N	16644.0	401.6	69.2	462.7	0.2
7	99915	16644.0	384.9	75.5	470.7	8.9
8	William 82	16629.9	345	71	513.3	8.1
9	363PS9-3633	16629.9	481.7	94.5	576.2	3.8
10	pH-4396	16643.9	521.9	86.8	608.8	7.1

[a]Values have been rounded to show significant digits; statistical calculations have been done with full precision. % CV reported is based on μg/g.

m 4 protein from soybean seed extracts. The ability to utilize two orthogonal dimensions enhanced the chromatographic peak capacity, thereby enabling resolution of Gly m 4 protein from other components in the soybean seed extracts. The assay allows for separation and detection of Gly m 4 and isoforms, thereby enabling successful quantification of Gly m 4 at the intact protein level. The assay was successfully applied to compare levels of Gly m 4 in 10 commercial soybean lines. On the basis of the data, it can be concluded that the assay can successfully quantify Gly m 4 protein at physiologically relevant levels. However, unlike other allergens, for example, Bet v 1 with approximately 50 cloned and sequenced isoforms,[37] limited literature is available regarding the heterogeneity of Gly m 4. In future, additional studies need to be carried out to understand Gly m 4 heterogeneity, and methods need to be developed for detection and quantification of at least major Gly m 4 proteins. Although this method is suitable for quantification of Gly m 4 in the soybean lines evaluated within this study, further method development and validation will be required for expanding the detection and quantification of different isoforms or variants of Gly m 4 in other commercial lines. In addition, this approach could be expanded to other soybean allergens.

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Notes

The authors declare no competing financial interest.

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