

Mass Spectrometric Analysis of *N*-Glycoforms of Soybean Allergenic Glycoproteins Separated by SDS-PAGE

Lingmei Li,^{†,§} Chengjian Wang,^{†,§} Shan Qiang,[†] Jixiang Zhao,[†] Shuang Song,[‡] Wanjun Jin,[†] Bo Wang,[†] Ying Zhang,[†] Linjuan Huang,^{*,†} and Zhongfu Wang^{*,†}

[†]Key Laboratory of Resource Biology and Biotechnology in Western China, Ministry of Education and Provincial Key Laboratory of Biotechnology, College of Life Sciences, Northwest University, Xi'an 710069, China

[‡]School of Food Science and Technology, National Engineering Research Center of Seafood, Dalian Polytechnic University, Dalian 116034, China

S Supporting Information

ABSTRACT: Glycosylation of many proteins has been revealed to be closely related with food allergies, and screening and structural analysis of related glycoproteins and glycoallergens are essential for studies in this field. Herein, we describe detailed *N*-glycoform analysis of all glycoprotein fractions of soybean protein isolate (SPI) separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to disclose structural features of the glycan moieties of more soybean glycoproteins. SPI was fractionated by SDS-PAGE, and the generated protein bands were recovered and subjected to in-gel *N*-glycan release and labeling using a one-pot method newly developed by our group, followed by detailed analysis by electrospray ionization mass spectrometry (ESI-MS) and online hydrophilic interaction liquid chromatography coupled with electrospray ionization tandem mass spectrometry (HILIC-ESI-MS/MS). As a result, we found seven bands mainly containing oligomannose-type glycans; two mainly contain core α 1,3-fucosylated glycans, and six have no glycans. This study is the first report that discovers core α 1,3-fucosylated *N*-glycans in bands 1, 2, and 6 and discloses bands 3, 4, 5, and 7 as glycoproteins and their *N*-glycoforms. Therefore, it can expand our knowledge about soybean protein glycosylation and provide significant structural reference for research of soybean allergens.

KEYWORDS: soybean allergy, glycoprotein, *N*-glycans, ESI-MS, HILIC-ESI-MS/MS

1. INTRODUCTION

As an important nutrient source from plants, soybean is widely used in the food industry.¹ With a protein content of 35–40%,² it has an amino acid composition similar to that of milk and is equated with animal proteins in terms of nutritional value. However, soybean is known as one of the common allergenic foods.^{3–6} Food allergy is mediated by IgE, and the epitopes reside in certain regions of the allergenic proteins.^{7–9} When an antigen enters the body, plasma cells in the intestine lamina propria can be activated and produce large amounts of IgE antibody, which rapidly combines with mast cells. As the same antigen gets into the body again, it combines with the IgE antibody located on the surface of mast cells and causes the degranulation of mast cells to release a series of inflammatory mediators, which can increase the vascular permeability and induce serious allergic inflammation.¹⁰ Soybean allergy affects approximately 0.4% of children¹¹ and 0.25% of adults.¹² Atopic dermatitis, eczema, and asthma are common symptoms in foods allergies. Sometimes these reactions can threaten people's lives.

Glycosylation is one of the most common post-translational modifications of proteins¹³ and usually plays an important role in food allergy. Many foods rich in glycoprotein can cause allergic reactions, such as olives,¹⁴ peaches,¹⁵ kiwi fruit,¹⁶ peanuts,¹⁷ barley,¹⁸ nuts,¹⁹ and potatoes.²⁰ It is worth noting that the glycan moieties of glycoproteins in plants are generally different from those in mammals. Modifications of *N*-glycans such as α 1,3-fucosylation and β 1,2-xylosylation are common in plants

but absent in mammals.²¹ For this reason, plant glycoproteins usually have antigenic activities against mammalian cells. It has been found that the human IgE antibody can crossreact with plant foods, pollen, and honeybee venom, and glycoproteins *N*-glycans with α 1,3-fucose and β 1,2-xylose are the most important epitopes of IgE in allergic patients.^{22–25} Therefore, analysis of glycoprotein *N*-glycans with α 1,3-fucosylation and β 1,2-xylosylation is essential for studies of food allergies.

Soybean also has many allergenic glycoproteins, the relative molecular weights (MWs) of which range from 7.0 to 71 kDa.^{26–28} Gly m Bd 60K (7S α), Gly m Bd 30K,^{29,30} and Gly m Bd 28K^{31,32} are the main soybean allergens and can be recognized by a serum of 25% of soybean allergic patients.^{4,33,34} It has been found that all three subunits of β -conglycinin (7S α , 7S α' , and 7S β) have *N*-glycans and are potential food proallergens.³⁵ The *N*-glycans of Gly m Bd 30K and Gly m Bd 28K have also been proven to be important epitopes with α 1,3-fucose and β 1,2-xylose that can cause allergic reactions.^{36,37} Except for these several major glycoproteins reported, however, little is known about the glycosylation of the other soybean proteins, restricting further in-depth and systematical investigations of soybean allergies.

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In this article, we report detailed *N*-glycoform analysis of all glycoprotein fractions of soybean protein isolate (SPI) separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), to disclose structural features of the glycan moieties of more soybean glycoproteins that may react in the human body as food allergens.

2. MATERIALS AND METHODS

2.1. Reagents and Materials. 1-Phenyl-3-methyl-5-pyrazolone (PMP) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Analytical-grade glacial acetic acid, aqueous ammonia (26–28%, v/v), petroleum ether (boiling range of 30–60 °C), methylene dichloride, and ammonium acetate were from Tianli Chemical Reagent Co. Ltd. (Tianjin, China). Peptide:*N*-glycosidase F (PNGase F) was the product of New England BioLabs (Ipswich, MA, USA). Nonporous graphitized carbon (Carbograph) solid-phase extraction (SPE) columns (150 mg/4 mL) were purchased from Alltech Associates (Deerfield, IL, USA), and SepPak C18 SPE columns (100 mg/1 mL) were from Waters (Milford, MA, USA). HPLC-grade methanol and acetonitrile were obtained from Fisher Scientific (Fairlawn, NJ, USA). MW markers of proteins were from Fermentas (Burlington, Canada). The MD34 (retention MW: 8000–14000) dialysis membrane was from Union Carbide Co. (Danbury, CT, USA). Sodium dodecyl sulfate (SDS), DL-dithiothreitol (DTT), and Nonidet P-40 (NP-40) were purchased from Aladdin Industrial Inc. (Shanghai, China). Other chemical reagents used were of analytical-grade. Water was purified via a Milli-Q ultrapure water purification system from Millipore (Burlington, MA, USA). Nontransgenic soybean (*Glycine max* L.) was purchased from an agricultural product market in Xi'an.

2.2. Preparation of SPI. Soybean seeds were screened through a 60-mesh sieve, followed by comminution using a pulverizer and defatting with petroleum ether for 3 h. The petroleum ether was removed after centrifugation (6000g, 10 min), and the defatting operation was repeated three times. Defatted soybean powder was mixed with 15-fold Milli-Q water, and the pH value was adjusted to 7.0 with 2 M aqueous NaOH solution, prior to centrifugation at 4 °C (9000g, 20 min). The obtained supernatant was recovered, and its pH value was adjusted to 4.8 with 2 M aqueous HCl solution. After centrifugation at 4 °C (9000g, 20 min), the obtained precipitate was collected and suspended in water again. After the pH value was adjusted to 7.0 with 2 M NaOH, the solution was lyophilized, and then SPI was successfully prepared.³⁸

2.3. Separation of SPI by SDS-PAGE and Recovery of Protein Bands. SDS-PAGE was conducted with the method reported by Laemmli,³⁹ using 5% stacking gel and 12% separating gel. SPI (2 mg) was dissolved in 500 μ L of loading buffer (10 mM Tris-HCl, pH 8.0, 1% SDS, 40% glycerol, 0.1% DTT, and 0.05% bromophenol blue) and denatured at 100 °C for 10 min. Then, 10 μ L of SPI solution was loaded onto the SDS-PAGE gel. The MW markers of proteins were used. Separation was performed at 90 V in the stacking gel and 120 V in the separating gel for about 3 h. Subsequently, the gel was stained using Coomassie Blue R250 (0.1%) in a mixture containing methanol, water, and glacial acetic acid (9:9:2), prior to decolorization by heating at 90 °C in water for 1 h. Protein bands were recovered by excision and decolorized by repeated washing with 50% aqueous acetonitrile (v/v). Finally, the recovered gel fragments were ground to powder and dried by vacuum centrifugal concentration.

2.4. Release of *N*-Glycans from Glycoproteins by PNGase F. SPI (2–5 mg) was dissolved in protein denaturing solution (500 μ L) containing 5% SDS and 0.4 M DTT and incubated at 100 °C for 10 min. When the sample was cooled to room temperature, 50 μ L of sodium phosphate buffer (0.5 M, pH 7.5), 50 μ L of 10% aqueous NP-40, and 1 μ L of PNGase F (500 units) solution were sequentially added, followed by incubation at 37 °C for 24 h. Subsequently, the sample was boiled for 5 min to terminate the enzymatic reaction and then loaded onto a Sep-pak C18 SPE column. Elution of *N*-glycans was performed with 15 mL of water. Desalting of *N*-glycans was achieved using a Carbograph SPE column. The column was washed with 3 mL of water to remove salts, and the *N*-glycans were eluted

with 25% aqueous acetonitrile solution. The eluates were concentrated to dryness under reduced pressure for further use.

2.5. Nonreductive Chemical Release and Simultaneous Labeling of Glycoprotein *N*-Glycans. The operation was according to a method developed in our laboratory.⁴⁰ Briefly, 2–5 mg of SPI or the SDS-PAGE gel powder containing protein bands derived from 2–5 mg of SPI was suspended in 4 mL of 0.5 M aqueous NaOH solution containing 0.7 M PMP prior to the addition of 2 mL of methanol to the sample. The obtained mixture was incubated at 75 °C for 32 h. When the reactions were completed, the sample was neutralized with glacial acetic acid and washed three times with 4 mL of dichloromethane to remove excess PMP. The obtained sample solution was concentrated to dryness to remove methanol and excess acetic acid. Finally, the sample was redissolved in 1 mL of water and loaded onto a Sep-Pak C18 SPE column for purification. The column was washed with 6 mL of water to remove salts, and the PMP derivatives of *N*-glycans were eluted with 3 mL of 25% acetonitrile. The eluates were concentrated to dryness for further analysis.

2.6. ESI-MS and MS/MS Analysis. The MS analysis of glycans was performed on an LTQ XL linear ion trap mass spectrometer coupled with an electrospray ion source and a HPLC system (Thermo Scientific, USA). The glycan samples were dissolved in water, and the sample solutions were directly infused via a 2 μ L Rheodyne loop and then brought into the electrospray ion source by a stream of 50% methanol at a flow rate of 20 μ L/min. The spray voltage was set at 4 kV, with a sheath gas (N_2) flow rate of 20 arb., an auxiliary gas (nitrogen gas) flow rate of 10.0 arb., a capillary voltage of 37 V, a tube lens voltage of 250 V, and a capillary temperature of 300 °C. For MS/MS analysis, *N*-glycans were subjected to fragmentation by collision induced decomposition (CID), with helium (He) as the collision gas. Collision parameters were left at default values with a normalized collision energy degree of 30 and an isotope width of m/z 3.00. The activation Q was set at 0.25 and the activation time at 30 ms. The MS and MS/MS data were recorded using the LTQ Tune software. The structure of each glycan was assigned according to MS/MS data combined with computational analysis using the GlycoWorkbench software⁴¹ and knowledge of *N*-glycan biosynthesis.

2.7. Online HILIC-MS/MS Analysis. Online HILIC-MS/MS analysis was also performed on the HPLC-ESI-MS system (Thermo scientific, USA), using a TSK-GEL amide-80 column (4.6 mm $\times$ 250 mm, 5 μ m) (Tosoh Corporation, Tokyo, Japan). The glycan sample was dissolved in 20 μ L of deionized water, and 10 μ L of sample solution was injected by an autosampler. The elution gradient was as follows: solvent A, ACN; solvent B, 100 mM aqueous ammonium

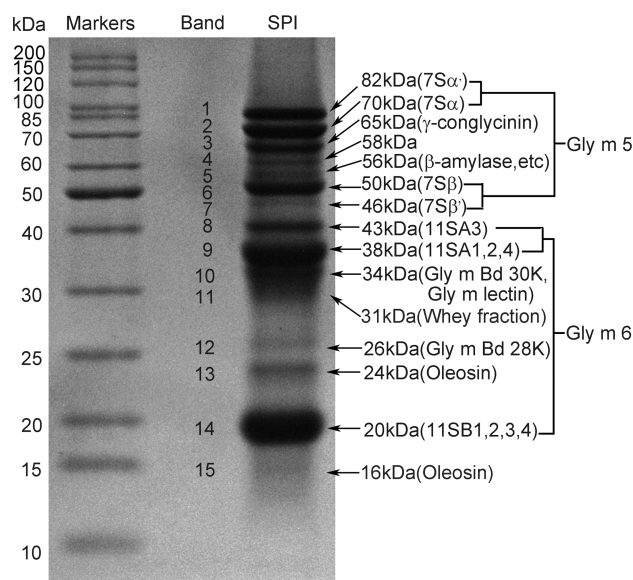


Figure 1. SDS-PAGE profile of the SPI.

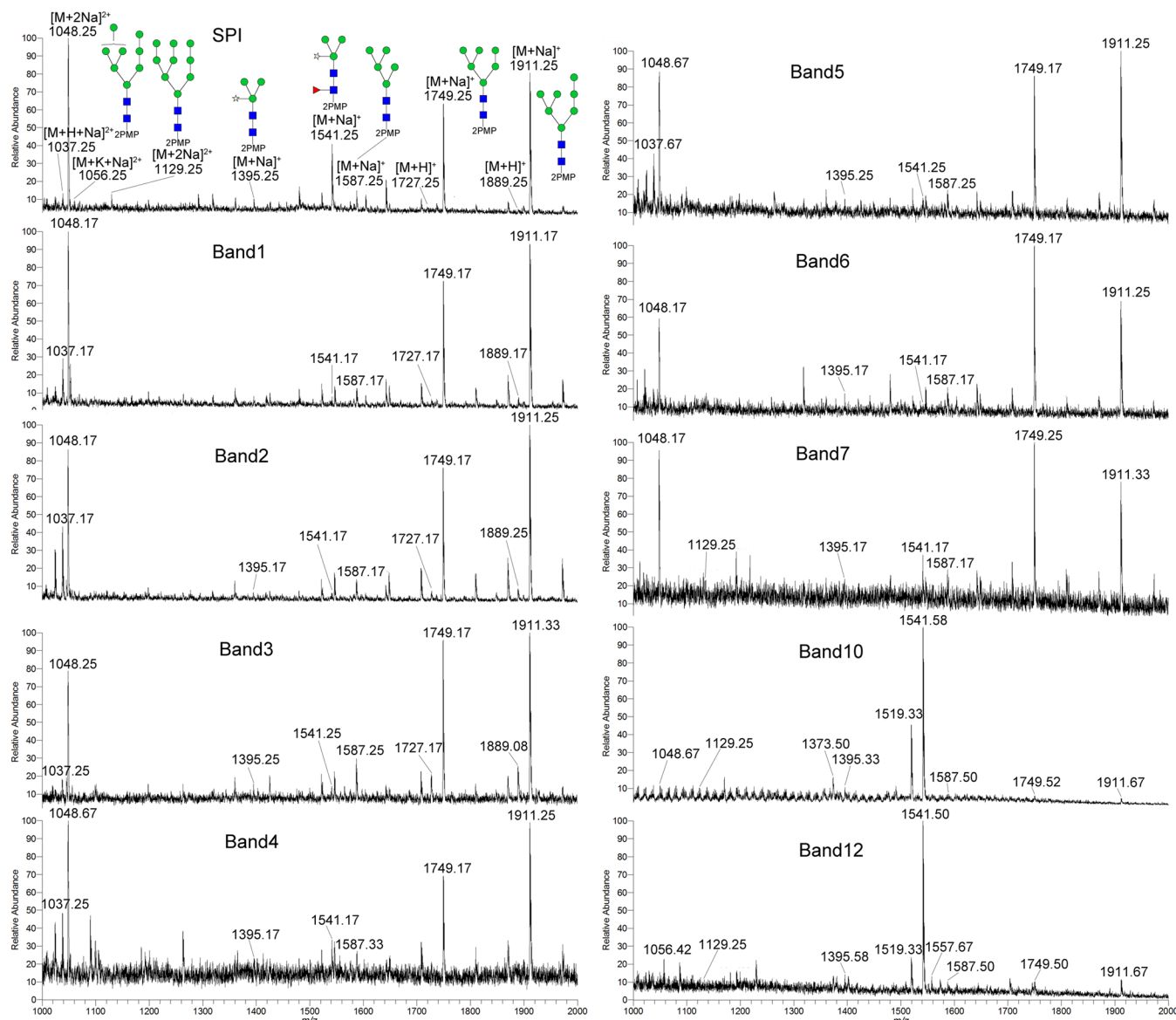


Figure 2. ESI-MS spectra of PMP derivatives of the *N*-glycans released from SPI and its SDS-PAGE bands by the one-pot chemical method. The glycan sequences were obtained according to MS/MS analysis. Structural formulas: blue square, *N*-acetylglucosamine; red triangles, fucose; orange stars, xylose; green circle, mannose.

acetate (pH 6.0); time = 0 min ($t = 0$ min), 80% A, 20% B, 1 mL/min; $t = 120$ min, 60% A, 40% B, 1 mL/min. The fractions eluted from the chromatographic column were directly imported into the ESI-MS system for detection. For the ESI-MS system, a rapid alteration mode between the segments of positive form MS and MS-dependent MS/MS was adopted. For the MS-dependent MS/MS, the normalized collision energy was set at 30, and the lowest signal intensity was set at 500. The other parameters used for MS and MS-dependent MS/MS were the same as those described above. Data acquisition was performed using Xcalibur software (Thermo). The obtained data were manually interpreted, and the proposed *N*-glycan compositions and sequences were checked using GlycoWorkbench software.⁴¹

3. RESULTS AND DISCUSSION

3.1. SDS-PAGE Separation of Soybean Proteins. For the glycoform analysis of diverse glycoproteins, their purification from complex biological samples needs to be carried out at the first step. Soybean has many kinds of proteins, the preparation of which with high purity is rather complex work. Compared with various tedious chromatographic methods, SDS-PAGE

represents a rapid, efficient protein-separating strategy, which is very suitable for the recovery of microamounts of proteins from complex biological mixtures. Thus, here we exploit SDS-PAGE for the separation of soybean proteins. In this experiment, the appropriate concentration of separating gel was proven to be about 12% after repeated tries. The obtained SDS-PAGE separation profile of SPI is shown in Figure 1.

In total, 15 protein bands were observed in the SDS-PAGE gel, including 7 major proteins and 8 minor ones. With reference to the ladder of MW makers, the MWs of band 1 to band 15 are estimated at approximately 82, 71, 65, 60, 56, 50, 46, 43, 36, 34, 31, 28, 24, 20, and 16 kDa, respectively. These gel bands are assigned to a series of soybean proteins, according to their MW values with combination of many closely related literature reports. Bands 1, 2, and 6 are assigned respectively to α' -, α -, and β -subunits of β -conglycinin, which is a trimer molecule in the 7S fraction.^{3,42–45} Band 7 is assigned to β' -subunits of the 7S fraction.^{46,47} Glycinin is a hexamer molecule in the 11S fraction that contains six subunits, and each

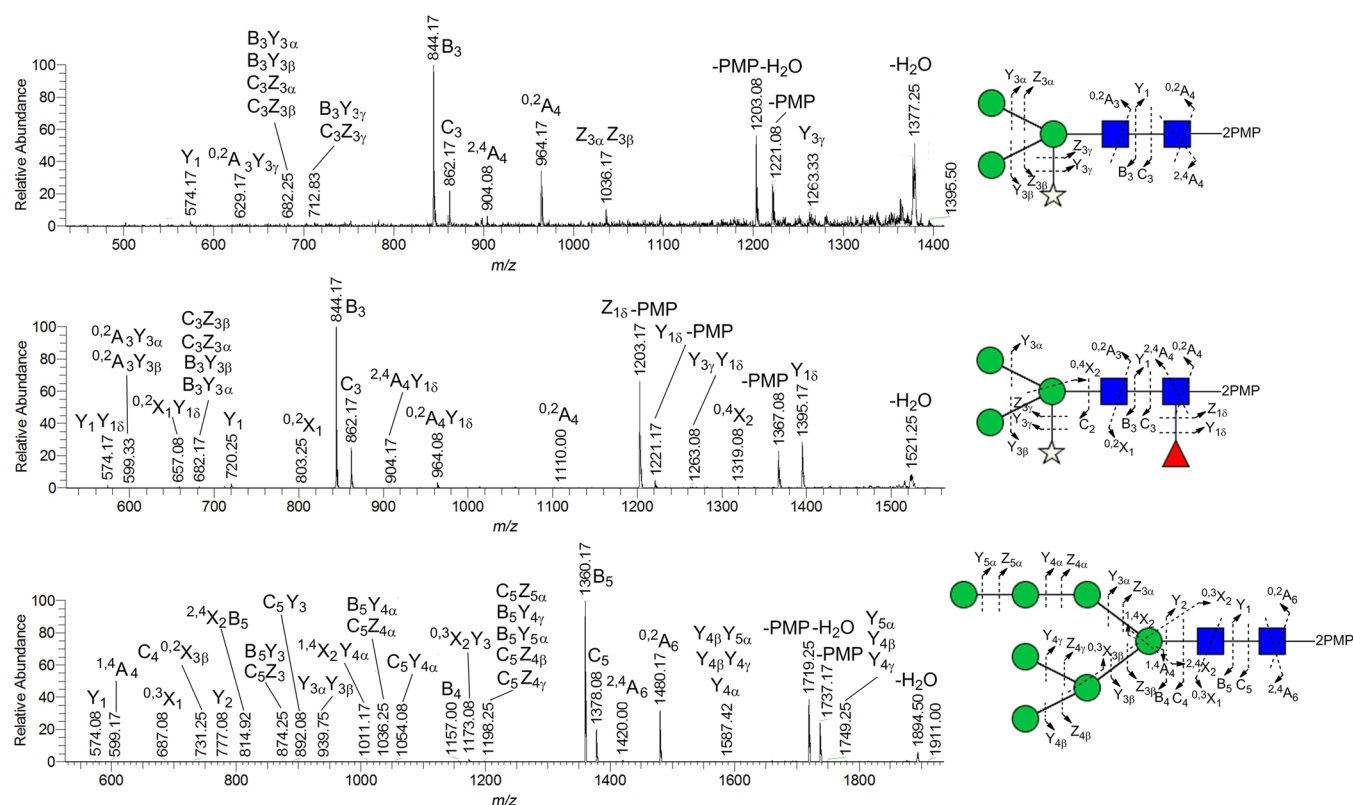


Figure 3. MS/MS analysis of soybean *N*-glycans. Structural formulas: blue square, *N*-acetylglucosamine; red triangles, fucose; orange stars, xylose; green circle, mannose.

subunit has an acidic A polypeptide and a basic B polypeptide with connection via a disulfide bond. Band 8 is assigned to the acidic A3 polypeptide, band 9 to a mixture of the acidic A1, A2, and A4 polypeptides, and band 14 to the basic B1, B2, B3, and B4 components of glycinin.^{43,44,48} Band 3 is proposed as γ -conglycinin,⁴⁹ band 4 as the sucrose binding protein,⁴⁶ band 5 as β -amylase,⁵⁰ and band 11 as whey fraction.⁵¹ Gly m lectin is very close to Gly m Bd 30K in terms of MW, and both of them are located in band 10.^{49,52,53} Band 12 is Gly m Bd 28 K.^{31,32} Bands 13 and 15 may be oleosin and some proglycinin A2B1.^{46,49} It is worth noting that most of these bands contain some known major allergenic proteins, including Gly m Bd 30K, Gly m lectin, Gly m Bd 28K, Gly m 5 consisting of α , α' , β , and β' subunits of the 7S proteins, and Gly m 6 consisting of A1, A2, A3, A4, B1, B2, B3, and B4 subunits of 11S proteins. These soybean allergens can be retrieved in some food allergen databases, such as the database of the IUIS Allergen Nomenclature Sub-Committee, the Structural Database of Allergenic Proteins (SDAP), and Allergome. Although SDS-PAGE has a limited protein-separating resolution and some of these obtained bands may contain more than one protein, the glycoform analysis of these soybean protein bands can be insusceptibly performed.

3.2. ESI-MS Profiling of the *N*-Glycans Released from SDS-PAGE Bands of Soybean Proteins. To identify glycoproteins from SPI and analyze their glycoforms, the 15 protein bands obtained were separately excised from the SDS-PAGE gel and then subjected to *N*-glycan release and labeling. PNGase F and PNGase A have been widely used for the cleavage of *N*-glycans from glycoproteins, but PNGase F cannot release core α 1,3-fucosylated *N*-glycans⁵⁴ and PNGase A is approximately inefficient to intact glycoproteins.^{55,56} Moreover,

Table 1. Proteins in the SDS-PAGE Gel Bands and Their Glycosylation Status

band	homologous proteins	MW(kDa)	glycoprotein
1	β -conglycinin α' subunit	82	yes
2	β -conglycinin α subunit	70	yes
3	γ -conglycinin	65	yes
4	sucrose binding protein	58	yes
5	β -amylase	56	yes
6	β -conglycinin β subunit	50	yes
7	β -conglycinin subunit fragment	46	yes
8	glycinin A3	43	no
9	glycinin A1, A2, A4	38	no
10	Gly m Bd 30K, lectin	34	yes
11	whey fraction	31	no
12	Gly m Bd 28K, β -conglycinin α subunit, etc.	26	yes
13	oleosin, proglycininA2B1, etc.	24	no
14	glycininB1, B2, B3, B4, etc.	20	no
15	oleosin, etc.	16	no

they can hardly achieve in-gel release of *N*-glycans due to their large MWs. However, our laboratory recently developed a new chemical method for nonreductive *N*-glycan release and simultaneous labeling with PMP in one pot, which has no selectivity to different types of *N*-glycans.⁴⁰ Therefore, here we utilized this new method to release and label *N*-glycans from SPI and its SDS-PAGE gel bands, and the obtained *N*-glycan samples were finally analyzed by ESI-MS and MS/MS.

First, the *N*-glycans of SPI were profiled by ESI-MS. As shown in Figure 2, a series of conspicuous MS peaks were observed in the mass spectrum of chemically released *N*-glycans of SPI. They were assigned to different types of pseudo-molecular ions of seven *N*-glycan compositions, including $H_3N_2X_1$ at *m/z*

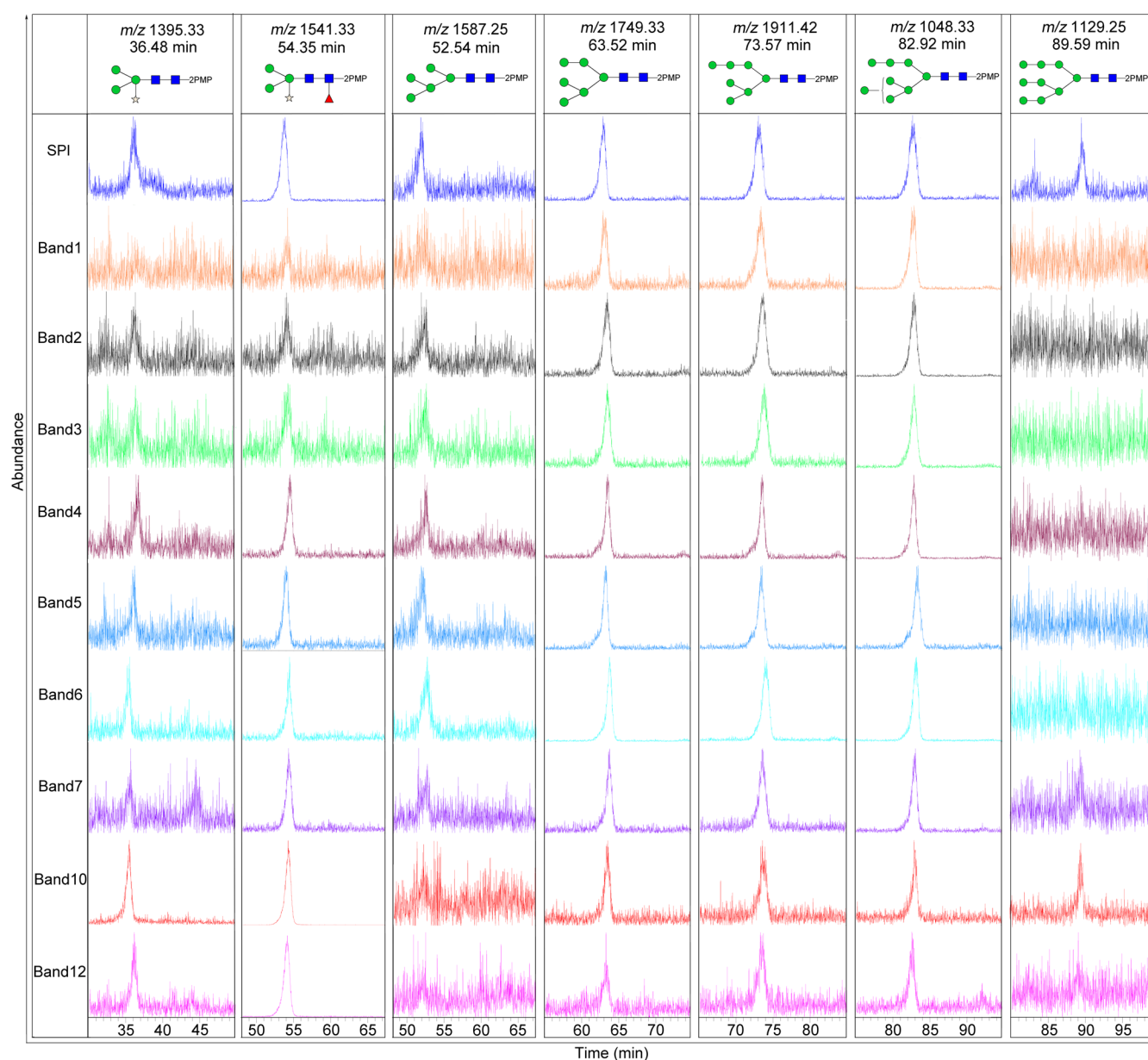


Figure 4. EICs of the *N*-glycans released from SPI and its SDS-PAGE bands. Structural formulas: blue square, *N*-acetylglucosamine; red triangles, fucose; orange stars, xylose; green circle, mannose.

1395 ($[M + Na]^+$), $H_3N_2X_1F_1$ at m/z 1541 ($[M + Na]^+$), H_5N_2 at m/z 1587 ($[M + Na]^+$), H_6N_2 at m/z 1727 ($[M + H]^+$) and 1749 ($[M + Na]^+$), H_7N_2 at m/z 1889 ($[M + H]^+$) and 1911 ($[M + Na]^+$), H_8N_2 at m/z 1037 ($[M + H + Na]^{2+}$), 1048 ($[M + 2Na]^{2+}$) and 1056 ($[M + Na + K]^{2+}$), and H_9N_2 at m/z 1129 ($[M + 2Na]^{2+}$). All of these *N*-glycans were sequenced in detail by MS/MS, as presented in Figures 3 and S1. Of these *N*-glycans, $H_3N_2X_1F_1$ at m/z 1541 theoretically has two possible isomers, namely, core α 1,3- and core α 1,6-fucosylated *N*-glycan structures. It is already known that PNGase F is ineffective to core α 1,3-fucosylated *N*-glycans, while the one-pot chemical method has no selectivity to the both isomers.^{40,54} Therefore, the two methods were employed here to differentiate α 1,3- and core α 1,6-fucosylated *N*-glycans. As shown in Figure S2, the *N*-glycan at m/z 1541 was not observed in the SPI *N*-glycans released by PNGaseF but in those obtained by the one-pot chemical method, demonstrating that this glycan has only core α 1,3-fucosylation modification. Besides, this also suggests

that the one-pot chemical method has excellent reliability and applicability to different types of *N*-glycans. These *N*-glycan structures are very consistent with those previously reported,^{57,58} confirming the good reliability of this study.

Second, the *N*-glycoforms of each SDS-PAGE band of SPI were also profiled by ESI-MS. As shown in Figure 2, nine of the SPI protein bands were identified to have *N*-glycoproteins. Obviously, seven of these glycoprotein bands, bands 1–7, have similar glycoforms, which are rich in oligomannose-type *N*-glycans but poor in *N*-glycan structures with xylose or fucose modification. In contrast, the other two glycoprotein bands, bands 10 and 12, have similar *N*-glycoforms that are conspicuously different from those of SPI as well as the above seven bands. They are rather rich in core α 1,3-fucosylated *N*-glycans but poor in the other types. However, there were not any *N*-glycan signals found in the samples of the other six protein bands, including bands 8, 9, 11, 13, 14, and 15 (Figure S3). According to these results, the glycosylation status

Table 2. Summary of the Information of Soybean *N*-Glycans Obtained from Comprehensive Analysis by ESI-MS and Online HILIC-MS/MS

<i>m/z</i>	Ion Type	Monosaccharide composition ^a	Proposed Structure ^b	Retention Time(min)	Release Method	Protein Source (Band number)
1373.50	[M+H] ⁺	H ₃ N ₂ X ₁ (PMP) ₂		36.48	Chemical PNGaseF	2-7, 10, 12
1395.33	[M+Na] ⁺	H ₃ N ₂ X ₁ F ₁ (PMP) ₂		54.35	Chemical	1-7, 10, 12
1519.33	[M+H] ⁺	H ₃ N ₂ X ₁ F ₁ (PMP) ₂		54.35	Chemical	1-7, 10, 12
1541.33	[M+Na] ⁺	H ₃ N ₂ X ₁ F ₁ (PMP) ₂		54.35	Chemical	1-7, 10, 12
1587.25	[M+Na] ⁺	H ₅ N ₂ (PMP) ₂		52.54	Chemical PNGaseF	1-7, 10, 12
1727.25	[M+H] ⁺	H ₆ N ₂ (PMP) ₂		63.52	Chemical PNGaseF	1-7, 10, 12
1749.33	[M+Na] ⁺	H ₆ N ₂ (PMP) ₂		63.52	Chemical PNGaseF	1-7, 10, 12
1889.25	[M+H] ⁺	H ₇ N ₂ (PMP) ₂		73.75	Chemical PNGaseF	1-7, 10, 12
1911.42	[M+Na] ⁺	H ₇ N ₂ (PMP) ₂		73.75	Chemical PNGaseF	1-7, 10, 12
1037.33	[M+H+Na] ²⁺	H ₈ N ₂ (PMP) ₂		82.92	Chemical PNGaseF	1-7, 10, 12
1048.33	[M+2Na] ²⁺	H ₈ N ₂ (PMP) ₂		82.92	Chemical PNGaseF	1-7, 10, 12
1056.42	[M+K+Na] ²⁺	H ₈ N ₂ (PMP) ₂		82.92	Chemical PNGaseF	1-7, 10, 12
1129.25	[M+2Na] ²⁺	H ₉ N ₂ (PMP) ₂		89.59	Chemical PNGaseF	7, 10, 12

^aH, hexose; N, *N*-acetylhexosamine; X, xylose; F, fucose. ^bStructural formulas: blue square, *N*-acetylglucosamine; green circle, mannose; red triangles, fucose; orange stars, xylose.

of the soybean proteins as SDS-PAGE bands is summarized in Table 1.

In this study, bands 3, 4, 5, and 7 are newly found glycoproteins, which contain *N*-glycans with β 1,2-xylose and core α 1,3-fucose and might be potential soybean allergens. It is reported that bands 1 (7S α'), 2 (7S α), and 6 (7S β) have only oligomannose-type *N*-glycans,⁵⁹ but here we found that band 1 contains the *N*-glycan at *m/z* 1541 (H₃N₂X₁F₁) and bands 2 and 6 contain the *N*-glycans at *m/z* 1541 (H₃N₂X₁F₁) and *m/z* 1395 (H₃N₂X₁). This study also first found that the 7S protein contains *N*-glycans with β 1,2-xylose and α 1,3-fucose, which can provide a new explanation for its potential allergenicity.³⁵ In addition, previous studies reported that Gly m Bd 30K and Gly m Bd 28K only contain the *N*-glycan at *m/z* 1541 (H₃N₂X₁F₁) and Gly m lectin only has the *N*-glycan at *m/z* 1229 (H₉N₂).^{36,37,60} However, here we found a β 1,2-xylose-modified *N*-glycan and five oligomannose-type *N*-glycans besides the glycan H₃N₂X₁F₁ in both band 10 (Gly m Bd 30K, Gly m lectin) and band 12 (Gly m Bd 28K). Thus, this study newly discovered five *N*-glycans in band 10 and six in band 12.

3.3. Separation and Quantitative Analysis of Soybean *N*-Glycans by Online HILIC-ESI-MS/MS. Online HILIC-ESI-MS/MS analysis of the *N*-glycans derived from SPI and its SDS-PAGE protein bands was performed to separate possible *N*-glycan isomers and obtain information about their distributions in abundance. The obtained extracted ion chromatograms (EICs) of these *N*-glycans are shown in Figures 4 and S4. Their qualitative information is summarized in Table 2, and quantitative information is in Figures 5 and 6.

As presented in Figure 4, the online LC-MS EICs give good profiling for the *N*-glycoforms of SPI and its SDS-PAGE protein bands, especially for the possibly existing isomers of these *N*-glycans. Briefly, seven peaks (*m/z* 1395.33, 1541.33, 1587.25, 1749.33, 1911.42, 1048.33, 1129.25) were found in SPI bands 7, 10, and 12, six peaks (*m/z* 1395.33, 1541.33, 1587.25, 1749.33, 1911.42, 1048.33) in bands 2–6, and five

peaks (*m/z* 1541.33, 1587.25, 1749.33, 1911.42, 1048.33) in band 1. The observed peaks of the seven *N*-glycans at *m/z* 1395.33, 1541.33, 1587.25, 1749.33, 1911.42, 1048.33, and 1129.25 occur at 36.48, 54.35, 52.54, 63.52, 73.57, 82.92, and 89.59 min, respectively. Obviously, no glycan isomers were found in all of these soybean protein samples. In addition, to confirm the structure of the *N*-glycan at *m/z* 1541.33, comparative analysis of the *N*-glycan samples of SPI obtained by PNGase F and the one-pot chemical method was performed. As shown in Figure S4, all of the EIC profiles of the SPI *N*-glycans obtained by PNGase F are similar to those obtained by the chemical method except the one at *m/z* 1541.33, suggesting the occurrence of core α 1,3-fucosylation in it. The structure of these glycans was identified by online MS/MS, the results of which were the same as those presented in Figures 3 and S1.

In order to characterize the quantitative distribution of different types of *N*-glycans for each soybean protein sample, their occupancy rates in the total *N*-glycans of each sample are summarized in Figure 5, based on the integral area of their UV peaks of HPLC. Obviously, the SPI contains oligomannose-type (75.9%) and complex-type (24.1%) *N*-glycans, and the four most abundant *N*-glycans are H₃N₂X₁F₁ (22.6%), H₈N₂ (37.0%), H₇N₂ (21.3%), and H₆N₂ (13.7%). However, its SDS-PAGE fractions have different *N*-glycan compositions. Briefly, the soybean protein bands can be divided into two groups, according to their *N*-glycan composition character. The first group contains bands 1–7. These SPI fractions feature quantitatively predominant oligo mannose-type *N*-glycans, the total occupancy rates of which remain over 88%. The three most abundant oligomannose-type *N*-glycans are H₈N₂, H₇N₂, and H₆N₂. In contrast, the other two complex-type *N*-glycans, H₃N₂X₁F₁ and H₃N₂X₁, have occupancy rates below 10% only, except the glycan H₃N₂X₁F₁ of band 7 (11%). The second group contains bands 10 and 12. Both bands have quantitatively predominant complex-type *N*-glycans, the total occupancy rates of which are over 84%. It is worth noting that the occupancy

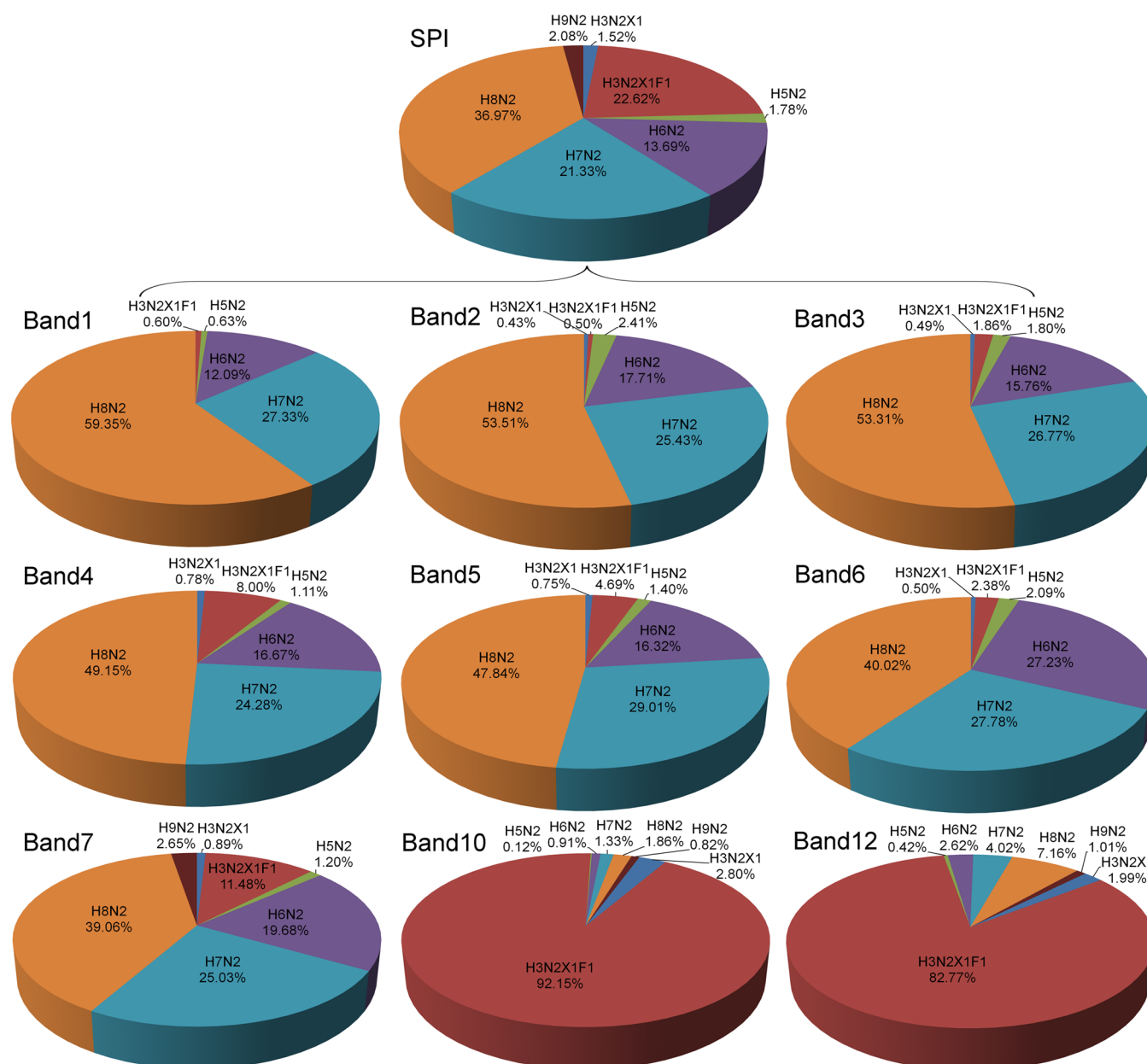


Figure 5. Charts showing the quantitative distribution of the *N*-glycans of SPI and its SDS-PAGE bands. Key symbols: H, hexose; N, *N*-acetylglucosamine; X, xylose; F, fucose.

rate of the core α 1,3-fucosylated *N*-glycan H₃N₂X₁F₁ remains over 92 and 83% in bands 10 and 12, respectively. In contrast, the oligomannose-type *N*-glycans exist in very low abundance in the two bands. Interestingly, previous studies reported that the occupancy of H₃N₂X₁F₁ in total *N*-glycans of SPI is as low as 1.3%,⁴¹ while this data reaches 23% in our study. This difference might arise from the fact that the one-pot chemical method that we used in this study has a higher *N*-glycan yield than that of the hydrazinolysis method utilized in the literature.⁴¹ In order to elucidate more clearly the difference among the occupancy rates of each glycan in different soybean protein samples, their occupancy rate data are also comparatively summarized in Figure 6. Obviously, each *N*-glycan structure has different occupancy values in different samples. Briefly, the two complex-type *N*-glycans, H₃N₂X₁ and H₃N₂X₁F₁, have lower occupancy ratios in bands 1–7 but much higher occupancy ratios in bands 10 and 12, compared with those in SPI. In contrast, four of the

oligomannose-type *N*-glycans, H₅N₂, H₆N₂, H₇N₂, and H₈N₂, generally have slightly higher occupancy ratios in bands 1–7 but lower occupancy ratios in bands 10 and 12 than those in SPI. The remaining oligomannose-type *N*-glycan, H₉N₂, has lower occupancy ratios in bands 1–6 as well as in bands 10 and 12 but a higher occupancy ratio in band 7. Therefore, considering the occurrence of xylosylated or core α 1,3-fucosylated *N*-glycans, which have been proved to be closely related with food allergies, all of the SPI bands that have *N*-glycans contain glycoproteins that might be potential soybean allergens, and band 10 (Gly m Bd 30K) and band 12 (Gly m Bd 28K) may be the two major allergens in soybeans.

This is the first study for systematic screening of soybean allergic glycoproteins and identification and quantification of their *N*-glycoforms, based on in-gel chemical release and labeling of *N*-glycans using the one-pot method followed by analysis by ESI-MS, MS/MS, and online HILIC-MS/MS. In total,

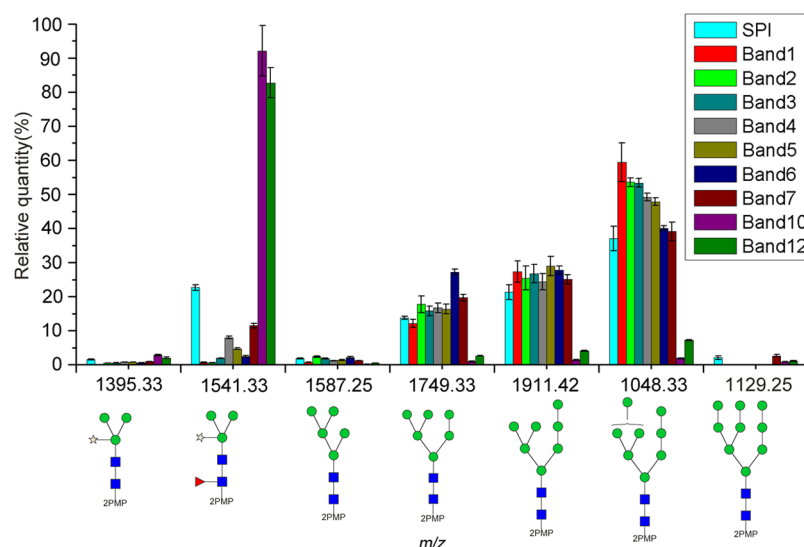


Figure 6. Histogram showing the occupancy rates of each *N*-glycan in different soybean protein samples. Structural formulas: blue square, *N*-acetylglucosamine; red triangles, fucose; orange stars, xylose; green circle, mannose.

15 protein bands (bands 1–15) were obtained from the SDS-PAGE gel, including nine bands containing glycoproteins and six without any glycoproteins. Of these glycoprotein bands, seven mainly contain oligomannose-type glycans, and two mainly contain core α 1,3-fucosylated glycans. The core α 1,3-fucosylated *N*-glycans were first discovered in bands 1 (7S α'), 2 (7S α), and 6 (7S β), and bands 3 (γ -conglycinin etc.), 4 (sucrose binding protein etc.), 5 (β -amylase etc.), and 7 (β -conglycinin subunit fragment) were first proved to be glycoproteins, and their *N*-glycoforms were first analyzed qualitatively and quantitatively. This study provides a foundation for further research on glycosylation sites and allergic bioactivities of more soybean glycoproteins.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b02773.

MS/MS analysis of *N*-glycans released from SPI, representative ESI-MS profiles of SPI *N*-glycans released by the one-pot chemical method and PNGaseF combined with separated PMP labeling, and representative ESI-MS profiles of the *N*-glycans of some SDS-PAGE bands of SPI obtained with the one-pot chemical method, extracted ion chromatograms from online HILIC-MS analysis of the *N*-glycans released from SPI using the one-pot chemical method and PNGaseF (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: wangzhf@nwu.edu.cn. Tel: +86 29 88305853. Fax: +86 29 88303534 (Z.W.).

*E-mail: huanglj@nwu.edu.cn. Tel: +86 29 88305853. Fax: +86 29 88303534 (L.H.).

Author Contributions

[§]L.L. and C.W. contributed equally to this work.

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Notes

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■ ABBREVIATIONS USED

SPI, soybean protein isolate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ESI-MS, electrospray ionization mass spectrometry; MS/MS, tandem mass spectrometry; HILIC-ESI-MS/MS, online hydrophilic interaction liquid chromatography coupled with electrospray ionization tandem mass spectrometry; PMP, 1-phenyl-3-methyl-5-pyrazolone; PNGase F, peptide:*N*-glycosidase F; SPE, solid-phase extraction; MW, molecular weight; SDS, solid-phase extraction; DTT, dithiothreitol; NP-40, Nonidet P-40; CID, collision-induced decomposition; He, helium; EICs, extracted ion chromatograms

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Supporting Information

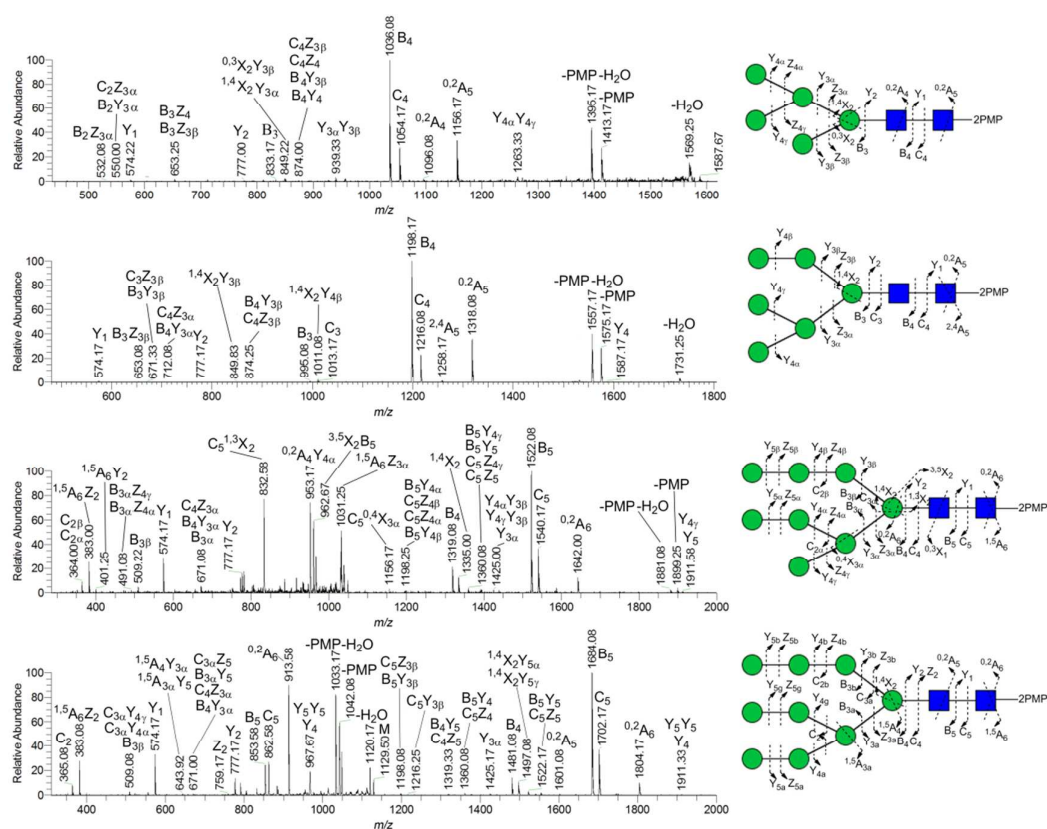


Figure S1. MS/MS analysis of *N*-glycans released from SPI. Structural formulas: blue square, *N*-acetylglucosamine; green circle, mannose.

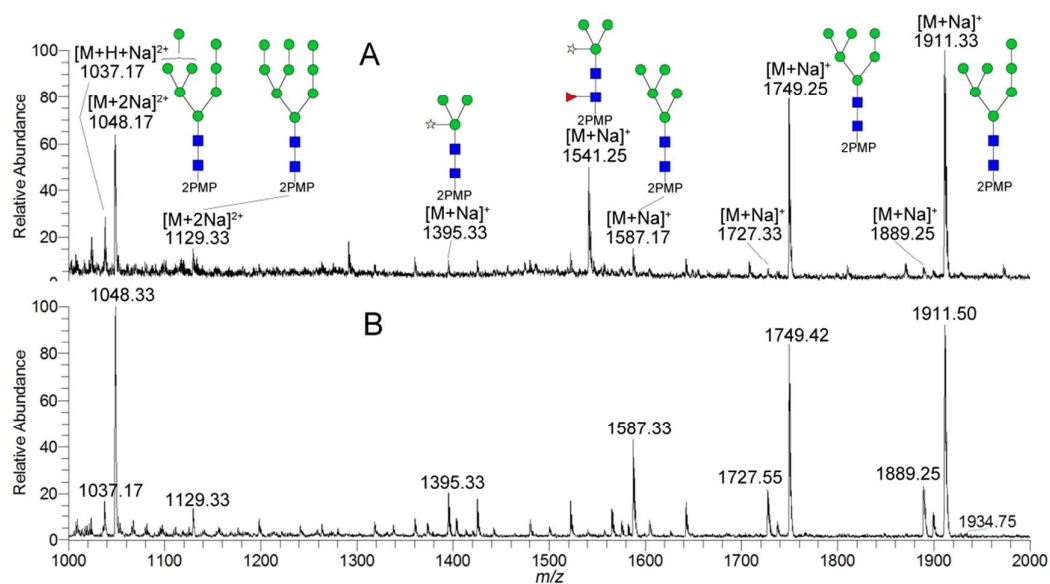


Figure S2. Representative ESI-MS profiles of SPI *N*-glycans released by the one-pot chemical method (A) and PNGaseF combined with separated PMP labeling (B). Structural formulas: blue square, *N*-acetylglucosamine; red triangles, fucose; orange stars, xylose; green circle, mannose.

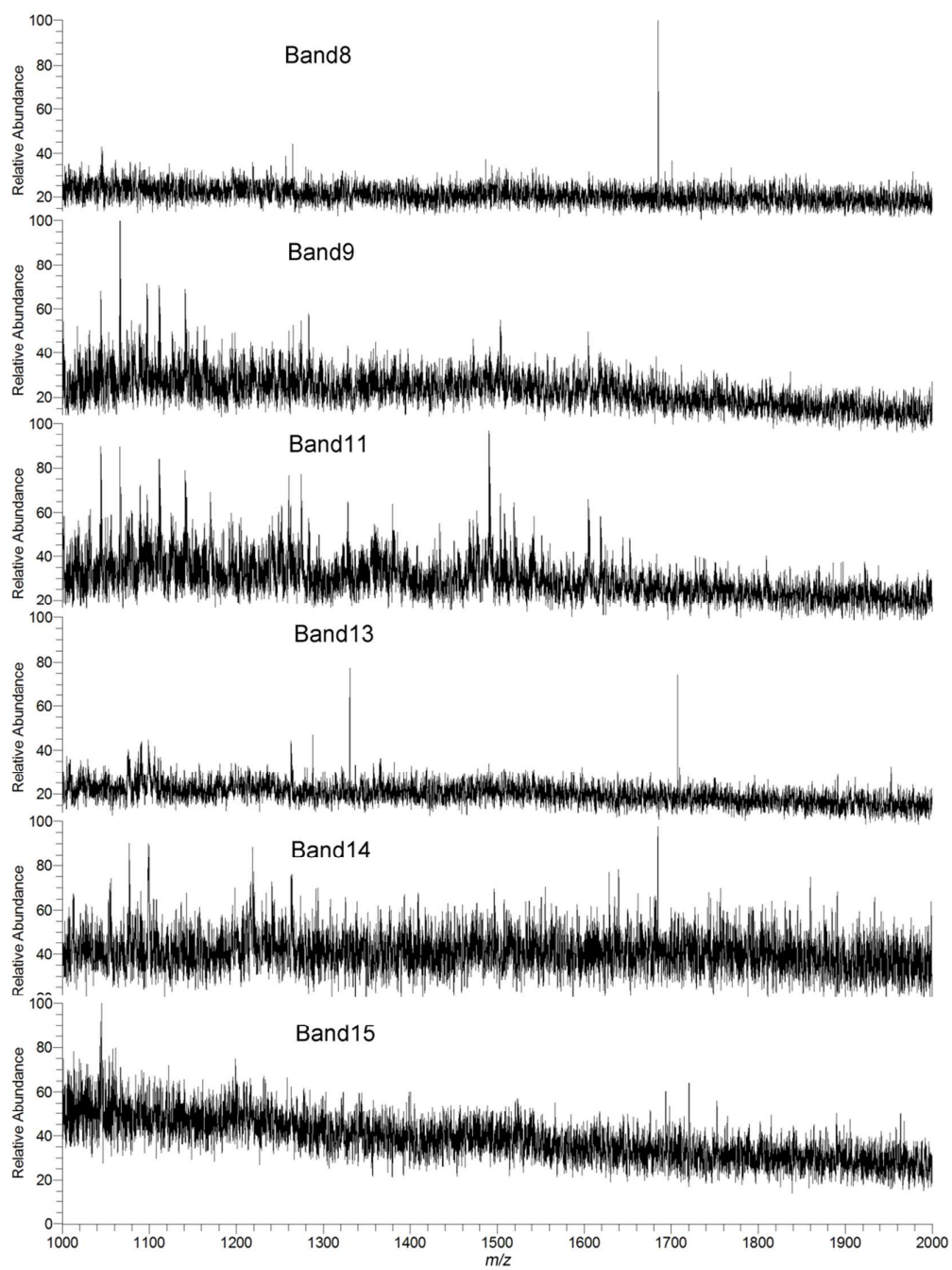


Figure S3. Representative ESI-MS profiles of the *N*-glycans of some SDS-PAGE bands of SPI obtained with the one-pot chemical method.

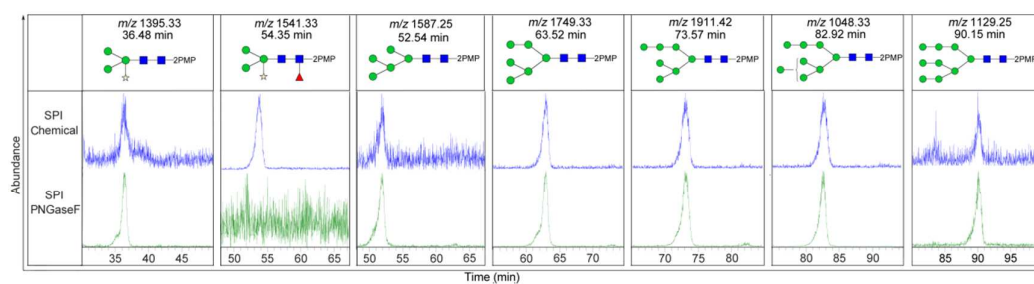


Figure S4. Extracted ion chromatograms (EICs) from online HILIC-MS analysis of the *N*-glycans released from SPI using the one-pot chemical method and PNGaseF. Structural formulas: blue square, *N*-acetylglucosamine; red triangles, fucose; orange stars, xylose; green circle, mannose.