

Purification and identification of globulin-1 S allele as a novel allergen with N-glycans in wheat (*Triticum aestivum*)

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ARTICLE INFO

Keywords:

Allergens

Globulin-1 S allele

Glycoprotein

Wheat soluble protein

ABSTRACT

Wheat (*Triticum aestivum*) contains various allergenic proteins and induces diverse allergic reactions. Although many allergens are found to be glycoproteins, research on glycosylated allergens in wheat is rare. In this study, the main glycoprotein in wheat soluble protein was purified by sequential multi-column chromatography including affinity chromatography, gel filtration chromatography, and ion-exchange chromatography. The purified proteins were identified as globulin-1 S allele by liquid chromatography-tandem mass spectrometry and characterized by circular dichroism spectra. The strong IgE-binding capacity of the purified fractions was further demonstrated, suggesting globulin-1 S allele to be a novel allergen in wheat. Further de-N-glycosylated treatment by N-Glycosidase F showed that N-glycosylation enhanced the IgE-binding of globulin-1 S allele. These results identified globulin-1 S allele as a novel allergen in wheat, demonstrated the role of glycosylation in IgE-binding, and provided new insights into the prevention and treatment of wheat allergy.

1. Introduction

As one of the most widely grown cereals, wheat (*Triticum aestivum*) provides humans with rich nutrients such as starch, protein, minerals, B vitamins, and biological activity ingredients with high nutritional value and palatability (Fu et al., 2020; Gómez, González, & Oliete, 2012; Ma et al., 2014). Moreover, due to the specific contribution of water-soluble protein and gluten protein in dough stickiness, mixing properties, and gas retaining properties, the wheat flour showed unique rheological properties of elasticity and viscoelasticity, resulting in the specific texture and flavor, making wheat to be an irreplaceable food material (Dhaliwal & MacRitchie, 1990; Hosney, 1991). However, wheat is commonly identified as one of the “big eight” categories of food allergens, which can initiate both immunoglobulin E (IgE) and non-IgE mediated food hypersensitivity towards sensitized individuals (Zuidmeer et al., 2008). The proportion of wheat allergies is about 0.4% in the world (Kucek, Veenstra, Amnuaycheewa, & Sorrells, 2015). According to epidemiological studies, wheat allergy affects 0.4% of American adults (Vierk, Koehler, Fein, & Street, 2007), up to 3% of the general

American pediatric population (Nwaru, Hickstein, Panesar, Roberts, Muraro, & Sheikh, 2014), and as many as 3.6% children in northern China (Wang, Zhuang, Ma, Zhang, & Wang, 2018). Depending on the different routes of allergen exposure, wheat allergy induces various adverse reactions and clinical symptoms such as baker's asthma, wheat-dependent exercise-induced anaphylaxis (WDEIA), allergic rhinitis, and atopic dermatitis (Inomata, 2009).

Wheat contains 10%–15% of proteins, which can be classified into four classes: water-soluble albumin, salt-soluble globulin, ethanol-soluble gliadin, and urea, detergent, or KOH soluble glutenin (Maruyama et al., 1998; T. B. Osborne, 1924; T. J. I. Osborne, Washington DC, USA, 1907). Albumin and globulin are also called soluble protein. To date, 28 proteins have been registered as allergens by the World Health Organization and International Union of Immunological Societies (WHO/IUIS, <https://www.foodallergy.org>). In general, these allergens cover all four classes of wheat proteins.

Glycans play an important role in the formation of protein structure and function, and participate in the processes of molecular recognition and signaling (Johnson, Jones, Ryan, & Cobb, 2013). Many allergens

Abbreviations: IgE, immunoglobulin E; WHO/IUIS, World Health Organization and International Union of Immunological Societies; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; TBST, Tris-buffered saline tween-20; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; LC-MS/MS, liquid chromatography-tandem mass spectrometry; CD, circular dichroism; PNGase F, N-glycosidase F.

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<https://doi.org/10.1016/j.foodchem.2022.133189>

Received 4 September 2020; Received in revised form 5 May 2022; Accepted 6 May 2022

Available online 10 May 2022

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have been identified as glycoproteins with N-glycan or O-glycan, forming the carbohydrate-specific IgE epitopes that affect the allergenicity. Song (Song et al., 2015) reported that wheat proteins with glycan were highly overlapped with gliadin, and N-glycan in gliadin is a possible carbohydrate epitope in wheat allergy. Besides, Tilley (Tilley, Lookhart, Hosney, & Mawhinney, 1993) confirmed that the high molecular weight glutenin subunits (HMW-GS) 2, 7, 8, and 12 from Chinese spring and TAM 105 wheats were glycosylated with mannose. In addition, Zhu (Zhu & Zhou, 2005) purified and characterized a novel glycoprotein with O-glycosidic linkage from wheat germ water-soluble extracts.

Based on the wide variety of protein insoluble fractions, whether there are potential glycoprotein allergens, of which glycans may affect allergenicity, has not been fully elucidated. In the present study, the main allergen glycoproteins in wheat soluble protein were isolated, purified, and identified. In addition, the IgE-binding capacity and the correlation with glycosylation were investigated.

2. Materials and methods

2.1. Wheat, sera, and cells

Chinese spring wheat grown in the northeast of China was purchased from an online market. Human sera samples from volunteer individuals with or without wheat allergy (Table 1 and Supplemental Fig. 1A) were provided by PlasmaLab International (USA), an FDA-licensed blood collection company. The patients were westerners aged from 16 to 50 with allergic rhinitis and they had wheat-specific IgE levels within 7.43 to 49.1 kU/L, as tested by ImmunoCAP. This procedure as well as the whole study were approved by Zhejiang Gongshang University Ethics Review Committee, as it was part of a routine procedure where no additional consent was required by law. The human mast cells (LAD2) were originally from ATCC (USA).

2.2. Preparation of wheat partial soluble protein

Wheat partial soluble protein was prepared according to a previous report (Xian, Wu, Jian, & Liu, 2008). In brief, wheat flour was degreased with acetone (1:10, g/v) at 4 °C for three times, then the solid product was transferred to 10 mM phosphate-buffered saline (PBS, 136.89 mM NaCl, 2.67 mM KCl, 8.1 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4) and soaked at 4 °C for 24 h. After centrifugation (12,000g, 15 min, 4 °C), the supernatant was collected as the wheat partial soluble protein and stored at 4 °C for further investigations.

2.3. Purification of glycoprotein

The wheat partial soluble protein was filtered at 0.45 µm, and the crude glycoprotein was enriched by Hitrap ConA 4B affinity column (1 mL) and the ÄKTA protein purification system (pure 25, General Electric Co., USA) with the following procedures. The column was first washed

and equilibrated with Buffer A (20 mM Tris-HCl, 0.3 M NaCl, 1 mM MnCl₂, 1 mM CaCl₂, pH 7.4), then the purified protein was loaded to the column. After washing by Buffer A for additional 20 column volumes, the bound proteins were eluted by Buffer B (20 mM Tris-HCl, 0.3 M NaCl, 0.3 M methyl- α -D-glucopyranoside, pH 7.4) and collected as the crude glycoprotein. For further purification, gel filtration chromatography was performed with Superdex 200 Increase 10/300 GL column (General Electric Co., USA) in Buffer C (20 mM Tris-HCl, 0.1 M NaCl, pH 8.0), the injection volume was 0.5 mL, the flow rate was 0.5 mL/min, and each 0.5 mL eluent were collected as a fraction. The purified proteins were detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and the fractions with the same kinds of proteins were combined for further purification. Finally, the Hitrap Q HP ion exchange column (1 mL) was used for the third step of purification. The proteins were loaded in Buffer C, and eluted with a linear gradient (20 column volume) from Buffer C to Buffer D (20 mM Tris-HCl, 0.5 M NaCl, pH 8.0), each 1 mL eluent was collected as a fraction. The purified proteins were detected by SDS-PAGE, and the fractions with the purest proteins were collected and desalted using a centrifugal filter unit (Amicon Ultra-4, 10 kDa, Meck Millipore Ltd., Ireland), and then lyophilized and stored at –20 °C for further analysis.

2.4. IgE-binding capacity analysis

Western-blot. The samples were dissolved by PBS and SDS-PAGE loading buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 10% Glycerol, 0.01% bromophenol blue dye, and 2% β -mercaptoethanol), heated to 95 °C for 5 min and separated by conventional SDS-PAGE with 15% Gel. After that, the gel was transferred to a polyvinylidene fluoride (PVDF) membrane (Amersham Hybond P, 0.45 µm, General Electric Co., USA), and blocked with Blocking buffer (5% BSA (bovine serum albumin), diluted in TBST (Tris-Buffered Saline Tween-20, 0.1% Tween-20 in Tris-buffered saline)) at room temperature for 1 h. After washing three times with 200 µL TBST, the membrane was incubated with human sera (1:10 dilution in Blocking buffer) for 1.5 h at 37 °C. After washing three times with TBST, horseradish peroxidase (HRP) conjugated goat anti-human IgE antibody (Abcam, mouse anti-human IgE-HRP, 1:5000) was added and incubated for 1 h at 37 °C. Then the membrane was washed three times with TBST and developed using electrochemiluminescence (ECL) luminous liquid for 1–2 min. The membrane was then scanned using a gel imager (ChemIDoc XRS+, Bio-Rad, USA) and analyzed by the AlphaView software.

Enzyme-linked immunosorbent assay (ELISA). The samples were diluted to 15 µg/mL with PBS and then was immobilized in 96 well microtiter plates (Corning, 96-well EIA/RIA Clear Flat Bottom Polystyrene High Bind Microplate) overnight at 4 °C, followed by washing three times with 200 µL of PBS and blocking with 200 µL of 5% BSA for 2 h at 25 °C. After that, the plates were washed three times and incubated with 100 µL of pooled sera (1:30 dilution) from ten allergic individuals for 1.5 h at 37 °C, and a non-allergic serum was used as the negative control. After washing three times, 100 µL of HRP conjugated goat anti-human IgE (1:5000) was added into each well and incubated for 1.5 h at 37 °C. Then 100 µL tetramethylbenzidine solution was added into each well and incubated for 15 min at 37 °C. The color development reaction was stopped by 50 µL 2 M H₂SO₄ solution. The optical density of each well was measured at 450 nm with a microplate reader (Molecular Devices, USA).

2.5. Human mast cell degranulation assay

LAD2 (1 × 10⁵ cells/well) were seeded on a 96-well plate in RPMI 1640 medium including 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37 °C/5% CO₂. Then cells were sensitized by human sera (1:20 dilution) for 24 h. Cells were centrifuged (900 g, 25 °C, 5 min) and resuspended in N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) buffer (10 mM HEPES, 137 mM NaCl, 2.7 mM

Table 1
Characterization of positive sera from patients with wheat allergy.

No.	Gender	Age	Clinical Symptoms	Wheat specific IgE* (kU/L)
1	Male	34	allergic rhinitis	13.2
2	Male	30	allergic rhinitis	8.448
3	Female	22	allergic rhinitis	9.16
4	Male	27	allergic rhinitis	14.2
5	Female	29	allergic rhinitis	7.43
6	Male	20	allergic rhinitis	49.1
7	Female	50	allergic rhinitis	35.7
8	Male	16	allergic rhinitis	3.68
9	Female	41	allergic rhinitis	8
10	Female	38	allergic rhinitis	9.91

*Wheat specific IgE was tested by ImmunoCAP against the wheat allergens.

KCl, 0.38 mM Na₂HPO₄, 5.6 mM glucose, 1.3 mM MgSO₄, 1.8 mM CaCl₂, and 0.5% BSA, pH 7.4). Degranulation was induced by incubating with different protein samples or PBS for 60 min at 37 °C/5% CO₂. The sensitized LAD2 was centrifuged (900 g, 25 °C, 5 min) and the supernatant (30 µL/well) was added to a 96-well plate, together with 50 µL p-NAG (1 mM in 0.1 M citric acid buffer, pH 4.5). After incubation for 90 min at 37 °C, 100 µL glycine solution (0.2 M, pH 10.7) was added to each well and the absorbance of the hydrolyzed substrate was quantified using a microplate reader (Molecular Devices, USA) at 450 nm. The cell pellet was lysed with Triton 0.1% X-100 before removing the supernatant, and the total β -hexosaminidase was determined as described above. The results were displayed as the percentage of β -hexosaminidase activity measured in supernatant samples compared with the total values in the corresponding cell lysates.

2.6. Identification and structural analysis

Liquid chromatography-tandem mass spectrometry (LC-MS/MS). The SDS-PAGE band of target protein was excised, destained, and digested with trypsin, and analyzed by LC-MS/MS using a mass spectrometer (TripleTOF® 6600, AB Sciex, USA). The mass spectrometry parameters were set as follows: spray voltage, 2.20 kV; capillary temperature, 320 °C; normalized collision energy, 50%. And the search parameters were set as follows: software, Maxquant 1.5.3; database, UniProt-wheat, enzyme, trypsin; allowance, up to two missed cleavage.

Circular dichroism (CD). The secondary structure of the sample was investigated in the spectral range of 190–260 nm using a CD spectrometer (MOS-450, Bio-Logic, USA). In brief, the sample was diluted to 0.2 mg/mL with PBS and collected in a rectangular quartz cuvette with a path length of 1 cm. PBS was used as the blank solution. The operation

indexes were as follows: bandwidth, 1.0 nm; scan speed, 100 nm/min; response time, 2 s. This process was performed at least twice and the average value of ellipticities of the molar residues was expressed in the unit of mdeg. The contents of different secondary structures were calculated by DichroWeb (University of London, UK).

Protein modeling. Modeling of the 3D structure of globulin-1 S allele was performed using SWISS-MODEL (<https://www.swissmodel.expasy.org>) (Biasini et al., 2014). The globulin-1 S allele was searched by BLAST against the amino acid sequence contained in the Protein Data Bank (PDB). The crystal structure with the highest scoring was then selected as the template for model building.

2.7. De-N-glycosylation

To release N-glycan, 10 µL of N-Glycosidase F (PNGase F, 10,000 u/mL, Promega, USA) was added to 1 mL 1 mg/mL (dissolved in PBS) sample and incubated at 37 °C for 24 h. Then, the deglycosylated sample was purified by ultrafiltration (Amicon Ultra-4, 10 kDa, Meck Millipore Ltd., Ireland), and then lyophilized and stored at −20 °C.

2.8. Statistical analysis

All the quantitative results were analyzed with three replicates according to a completely randomized design. All data were performed by analysis of variance (ANOVA), and Duncan's Multiple Range Test was used for pair-wise comparison of the data. Data were statistically analyzed using SPSS 17.0 (International Business Machines Co., USA) and $p < 0.05$ was considered statistically significant.

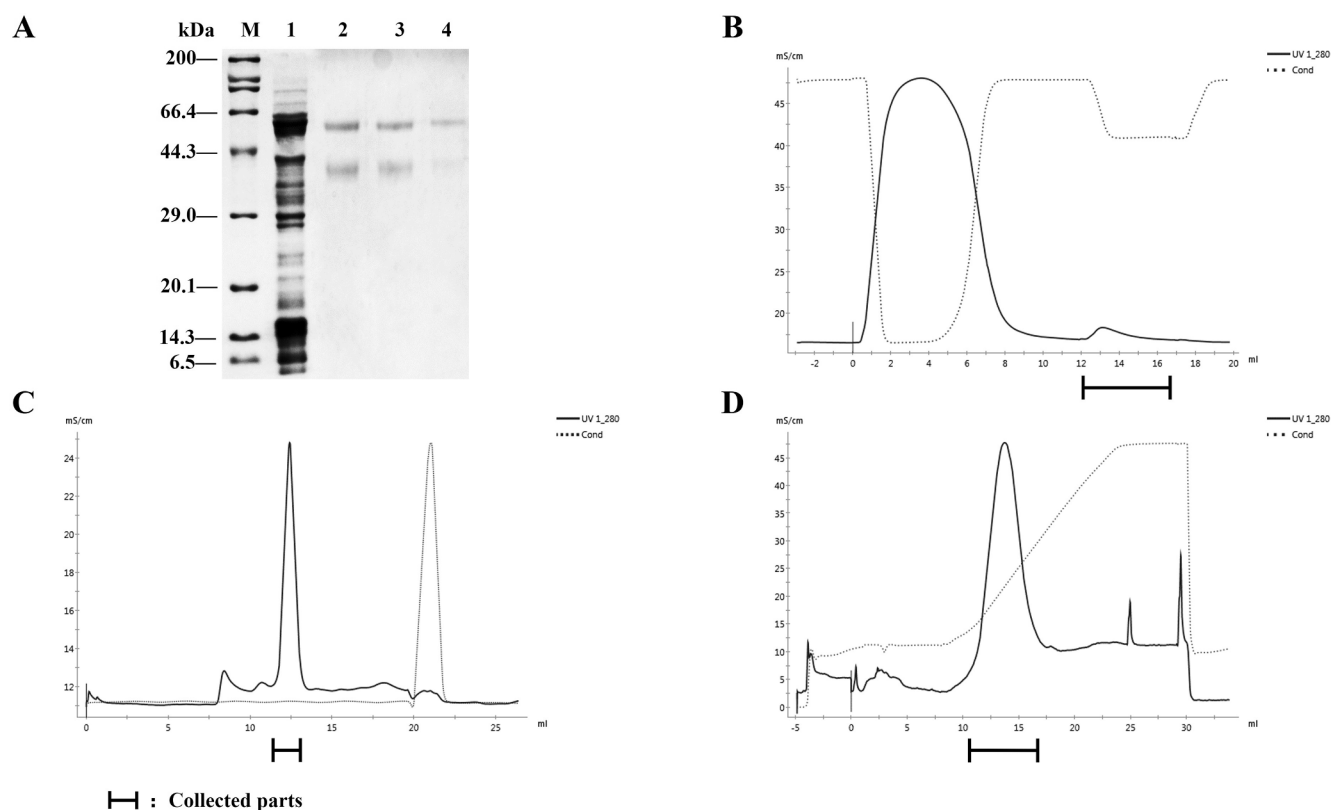


Fig. 1. Chromatographic purification of the main glycoprotein from wheat soluble protein. (A) SDS-PAGE analysis of wheat soluble protein and purified proteins. Lane M, marker; lane 1, soluble protein; lane 2, purified protein by affinity chromatography; lane 3, purified protein by affinity chromatography and gel filtration chromatography; lane 4, purified protein by affinity chromatography, gel filtration chromatography, and ion-exchange chromatography. (B) Chromatogram of affinity chromatography. (C) Chromatogram of gel filtration chromatography. (D) Chromatogram of ion-exchange chromatography. The collected fractions were marked.

3. Results

3.1. Purification of the main glycoprotein from wheat

To investigate the main glycoprotein from wheat (*Triticum aestivum*), the whole soluble proteins were extracted as the starting material (Fig. 1A), and the glycoproteins were enriched by a ConA affinity chromatographic column, which immobilized soluble protein revealed multiple protein bands ranging from 6.5 to 66.4 kDa, the enriched glycoproteins contained only two major bands, one was a single band with a molecular weight of 55 kDa and another was a smeared band with the molecular weight near 40 kDa (Fig. 1A). To further purify the glycosylated proteins, the ConA eluents were further separated by gel filtration (Fig. 1C) and anion-exchange chromatography (Fig. 1D). Both chromatograms showed only one main peak with high symmetry, implying the obtained eluent was homogeneous and not degraded. However, the SDS-PAGE result still showed two bands with similar chemometrics as the ConA eluent, indicating that the two protein parts form a stable complex. Noticeably, according to the chromatograms of gel filtration (Fig. 1C), the molecular weight of this glycoprotein complex was about 150 kDa.

3.2. IgE-binding capacity of the main wheat glycoprotein

The IgE-binding capacity of the purified main glycoprotein was first analyzed by Western blot (Fig. 2A) and ELISA (Fig. 2B) using pooled sera from ten wheat allergic patients. IgE binding to the multiple protein bands in the whole soluble protein extract with the molecular masses ranging from 20.1 to 66.4 kDa. These protein bands could be identified as wheat allergens (Fig. 2A, lane 2). Furthermore, with the evidence of strong reactivity with patient sera, both bands of the purified main glycoprotein were considered allergens (Fig. 2A, lane 3). Meanwhile,

when quantifying the IgE-binding by ELISA (Fig. 2B), the results showed that the IgE-binding capacity of the purified main glycoprotein was extremely higher than the whole soluble protein with the same protein amount (1.5 μ g), indicating that glycoprotein showed stronger allergenic potential in wheat.

To further investigate the detailed IgE-binding capacity of the main wheat glycosylated protein, sera from ten wheat allergic patients were used separately. Western blot results showed that compared with negative control, the purified main glycoprotein showed a significant binding ability to seven allergic sera (No.2, 4, 5, 6, 7, 9, and 10) (Fig. 2C) but not to sera from healthy individuals or food allergy individuals without wheat allergy (Supplemental Fig. 1B). Similarly, in ELISA, while all the ten sera reacted with purified glycosylated proteins with statistically significant difference compared with negative control, most allergic sera (No.2, 3, 4, 5, 6, 7, 9, 10 and W1, W3, W4, W5, W6, W8, W9, W10, W11) showed higher activity than the negative control (Fig. 2D and Supplemental Fig. 1C), which was roughly consistent with the Western blot results. Moreover, two sera (No.6 and No.7) showed an especially high binding capacity to the purified main glycoprotein. These results demonstrated that most wheat allergy patients are sensitized to the purified glycoprotein complex, implying it to be one of the major allergens in wheat.

3.3. Identification of the purified glycoprotein

To identify the protein nature of the allergenic glycoprotein, the two major bands of the purified glycoprotein fraction in SDS-PAGE (Fig. 1A, lane 4) were excised separately and subjected to LC-MS/MS, and the identified peptides were searched in the UniProt database (Fig. 3 and Supplemental Dataset 1). Interestingly, the results revealed both bands to be the uncharacterized protein A0A3B6KNF4, with a total of 25 peptides that matched the protein sequence, which covered 48.4% of the

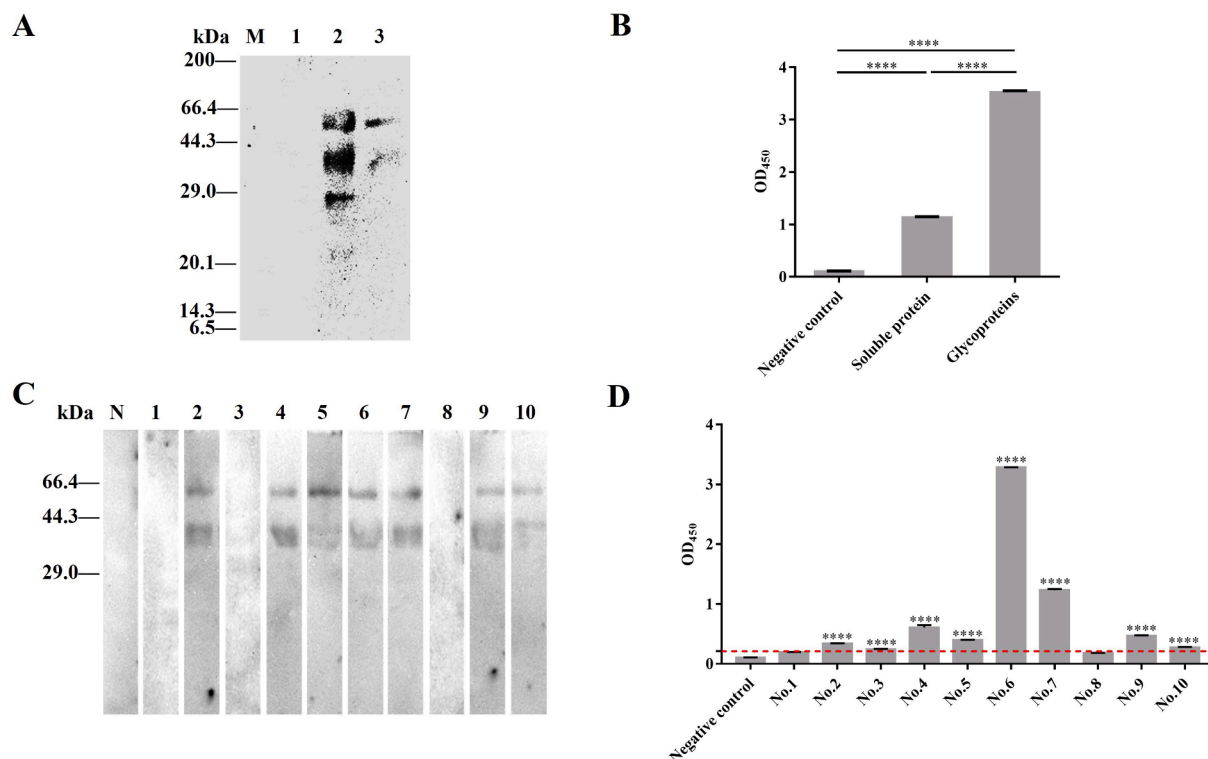


Fig. 2. The IgE-binding capacity of the main glycoprotein from wheat soluble protein. (A) Western blot of soluble protein and the purified main glycoprotein with pooled sera. Lane M, marker; lane 1, negative control (BSA); lane 2, soluble protein; lane 3, the purified main glycoprotein. (B) ELISA of soluble protein and the purified main glycoprotein with pooled sera. (C) Western blot of the purified main glycoprotein with ten separate sera. Lane N, negative control; lane 1 to 10, experimental groups with different sera. (D) ELISA of the purified main glycoprotein with ten separate sera. The dashed line showed twice the value of negative control.

A0A3B6KNF4	1	MKSAVRSPWL	VLAIVLSLCL	SLSFASWDAE	DVGRGSRRWQ	EGGDEGRSGG	50
Globulin-1 S allele (<i>Triticum urartu</i>)	1	MKSAVRSPWL	VLAIVLSLCL	SLSFASWDAE	DVGRGSRRWQ	EGGDEGRSGG	50
A0A3B6KNF4	51	SGRPYHFGQE	SYREWAKSRH	GHFKVLERFD	HELLRGSIGD	YRVAYLDAAP	100
Globulin-1 S allele (<i>Triticum urartu</i>)	51	SGRPYHFGQE	SYREWAKSRH	GHFKVLERFD	HELLRGSIGD	YRVAYLDAAP	100
A0A3B6KNF4	101	RAFLQPSHHD	ADEIAFVREG	EGVLVLLRNG	KRESFCIREG	DVIVIPAGSI	150
Globulin-1 S allele (<i>Triticum urartu</i>)	101	RAFLQPSHHD	ADEIAFVREG	EGVLVLLRNG	KRESFCIREG	DVIVIPAGSI	150
A0A3B6KNF4	151	VYSANTHRSK	WLRVVMFINP	VSTPGRFQEF	FLIGSGDERP	QSFLSVFSDE	200
Globulin-1 S allele (<i>Triticum urartu</i>)	151	VYSANTHRSK	WLRVVMFINP	VSTPGRFQEF	FLIGSGDERP	QSFLSVFSDE	200
A0A3B6KNF4	201	VIQAALNTRR	EDVDRVFESK	SKGEGEIEYA	SEEQIRELSR	SCSRGGRGGG	250
Globulin-1 S allele (<i>Triticum urartu</i>)	201	VIQAALNTRR	EDVDRVFESK	SKGEGEIEYA	SEEQIRELSR	SCSRGGRGGG	250
A0A3B6KNF4	251	GSGSEKEDIQ	PRSLTGEKPR	YSNKHGRFHQ	ITGDQCHHLR	KLDMDVTLVN	300
Globulin-1 S allele (<i>Triticum urartu</i>)	251	GSGSEKEDIQ	PRSLTGEKPR	YSNKHGRFHQ	ITGDQCHHLR	KLDMDVTLVN	300
A0A3B6KNF4	301	ITRGSMTALK	YTTRSTRIVY	VVEGRDGYFE	MACPHISSSG	RSERREHEQE	350
Globulin-1 S allele (<i>Triticum urartu</i>)	301	ITRGSMTALK	YTTRSTRIVY	VVEGRDGYFE	MACPHISSSG	RSERREHEQE	350
A0A3B6KNF4	351	REREHGQGR	SEEREREQGR	GRRSEEREQE	QGRQEEEQGH	GREQEKSARGY	400
Globulin-1 S allele (<i>Triticum urartu</i>)	351	REREHGQGR	SEEREREQGR	GRRSEEREQE	EQGH	GREQEKSARGY	393
A0A3B6KNF4	401	RQVRAQIKVG	SVIVLPAGHP	ATFVAGNDGN	LALLSFGVGA	NNDEEVFTVG	450
Globulin-1 S allele (<i>Triticum urartu</i>)	394	RQVRAQIKVG	SVIVLPAGHP	ATFVAGNDGN	LALLSFGVGA	NNDEEVFTVG	443
A0A3B6KNF4	451	GNSVLKQLDE	AAKALSFQQ	ARELADRVIR	AQPESVFVAG	PQQQRRVADM	500
Globulin-1 S allele (<i>Triticum urartu</i>)	444	GNSVLKQLDE	AAKALAFQQ	ARELADRVIR	AQPESVFVAG	PQQQRRVADM	493



The peptides identified by MS

The differential sequences in A0A3B6KNF4 and Globulin-1 S allele (*Triticum urartu*)

Fig. 3. LC-MS/MS of the main allergen glycoprotein from wheat soluble protein. The uncharacterized protein A0A3B6KNF4 sequence was aligned with globulin-1 S allele from *Triticum urartu* (GenBank: EMS62417.1), and the different sequences were circled by the dotted line. The peptides detected by LC-MS/MS in the purified glycoprotein were labeled by the grey background.

whole protein. Further analysis by BLAST showed that this protein was highly homologous to globulin-1 S allele in *Triticum urartu* (GenBank: EMS62417.1), with 98% identity. Overall, these results demonstrated that the purified allergic glycoprotein was globulin-1 S allele in wheat (*Triticum aestivum*).

3.4. Structural analysis of globulin-1 S allele

Protein structure is the basis of allergenicity. To investigate the structure of glycosylated globulin-1 S allele, the purified protein complex was assessed by CD. The CD results displayed at 190–260 nm implied the secondary structure of the protein (Fig. 4A). Further calculation based on the CD results by DICHROWEB analysis webserver (Whitmore & Wallace, 2008) revealed that the secondary structure of globulin-1 S allele consisted of 51.8% α -helix, 10.8% β -sheet, 26.4% β -turn, and 11% unordered structure. Furthermore, the 3D structure of globulin-1 S allele monomer was simulated using SWISS-MODEL, with adzuki bean 7S globulin-1 (ID: 2ea7.1.A) as the template. As shown in Fig. 4C, the predicted structure of globulin-1 S allele was roughly spherical containing a large portion of α -helix and β -sheet, which was consistent with the common characteristic of globulin and the CD result of the globulin-1 S allele protein complex. Noticeably, the value of GMQE was in the range of 0 to 1 and the value of QMEAN was in the range of −4 to 0, which suggested that the modeling result was credible (Fig. 4D).

3.5. N-glycosylation enhanced the IgE-binding capacity of globulin-1 S allele

A great deal of research has demonstrated the effect of glycosylation on protein allergenicity (Do, Yang, Yao, Hamilton, Schroeder, & Gao, 2017; Park, Yoon, Kim, Han, & Choi, 2017; Zhang, Xiao, Zhang, & Zhou, 2019). To investigate whether glycosylation is involved in the IgE-binding capacity of globulin-1 S allele, the purified protein fraction was treated with PNGase F to specifically remove N-glycans (Bodnar et al., 2016). The molecular weight of the protein decreased obviously after PNGase F treatment (Fig. 5A, lane 2), indicating the N-glycans were removed. Thereafter the IgE binding capacity of the protein was detected by Western blot (Fig. 5B) and ELISA (Fig. 5C) with allergic sera. The protein treated by PNGase F showed relatively weaker IgE binding capacity than the intact one (Fig. 5B). This result was also verified by ELISA. Furthermore, to investigate the effect of PNGase F treatment on the IgE-binding capacity of globulin-1 S allele in vitro, the protein treated by PNGase F was applied in the LAD2 degranulation assay. The result showed that the removal of N-glycans reduced β -hexosaminidase release (Fig. 5D), indicating that the removal of N-glycans significantly reduced the IgE-binding capacity of globulin-1 S allele in wheat. Overall, all these results indicated that N-glycans have an important effect on the IgE-binding capacity of globulin-1 S allele and the removal of N-glycans can reduce the effect.

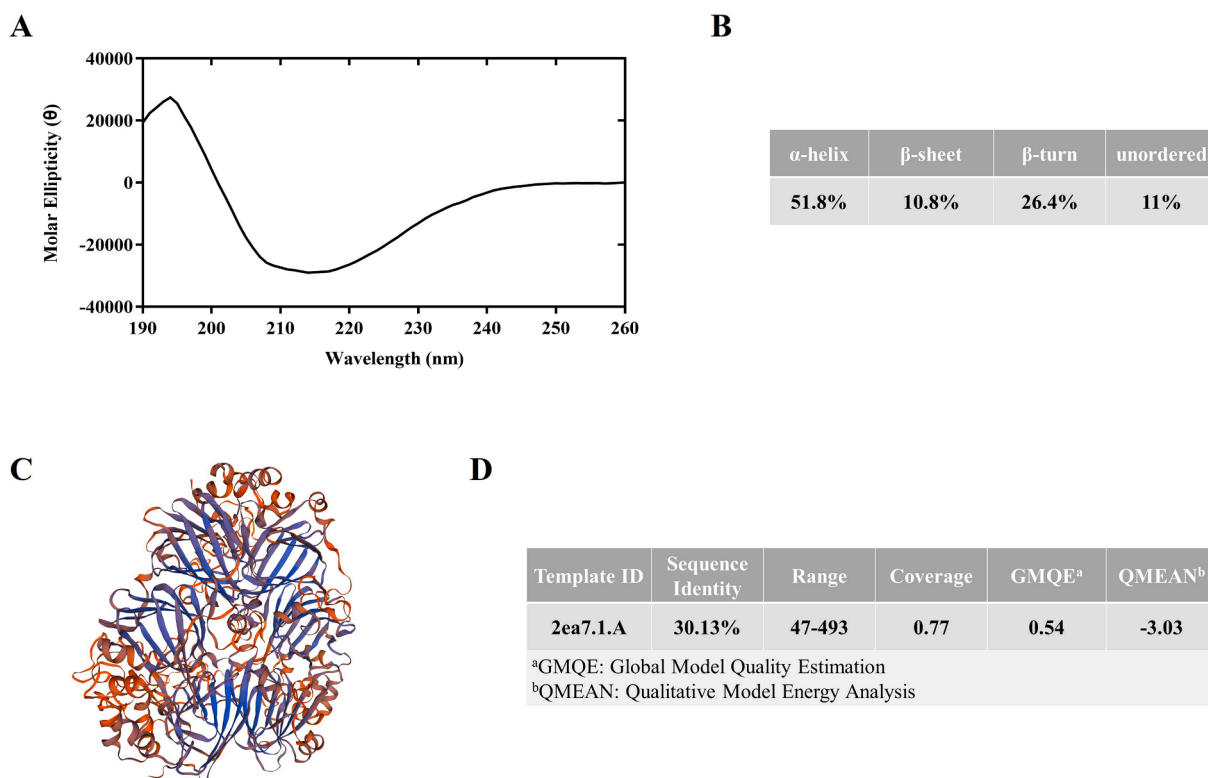


Fig. 4. Structural analysis of the purified globulin-1 S allele from wheat soluble protein. (A) CD analysis of the purified globulin-1 S allele. (B) The percentage of secondary structure calculation based on the CD results. (C) Protein structure model for globulin-1 S allele using SWISS-MODEL. (D) Parameters and results of SWISS-MODEL modeling.

4. Discussion

As one of the “big eight” categories of food allergens, wheat (*Triticum aestivum*) contains various allergens and induces significant allergic reactions with different routes of exposure such as inhalation, ingestion, and skin contact (Palacin et al., 2007; Salcedo, Quirce, & Diazperales, 2011; Wieser, Koehler, & Scherf, 2020). Identification and analysis of wheat allergens can facilitate the prevention and therapy of wheat allergy. Although previous studies have identified several glycoproteins in wheat (Fausch, Kundig, & Neukom, 1963; Yun, Wang, Wu, Li, & Zhang, 2018; Yun, Wu, Li, & Zhang, 2018), globulin-1 S allele identified in this study was novel. This protein is the most abundant fraction enriched by the ConA affinity column. Using concanavalin A lectin as the ligand, this column specifically binds to certain carbohydrate structures. Therefore, globulin-1 S allele could be one of the most abundant glycoproteins in wheat. However, it should be noted that concanavalin A (the ligand of ConA column) showed binding preference rather than unbiasedly binding to all the glycoproteins (Halina & Nathan, 1986; Sumner, Gralen, & Erikssonquensel, 1938). Moreover, although with lower abundance, the MS results also identified other proteins in the purified samples, such as the uncharacterized protein A0A3B6J76. Therefore, this study did not exclude the existence of other glycoproteins in wheat with allergenic potential, and the investigation of other allergenic glycoproteins with higher-resolution methods (such as 2D PAGE) would be an interesting problem for further studies.

According to previous reports (Jia, Zhang, Chen, Wang, & Qi, 2012), there are many multimeric proteins in wheat such as α -amylase inhibitor, gliadin, and glutenin. In the present study, the results of SDS-PAGE (Fig. 1A) showed that the purified globulin-1 S allele contains multiple subunits. While the major subunits showed molecular weights of 55 and 40 kDa by SDS-PAGE, the gel filtration revealed the molecular weight of the whole complex to be 150 kDa, indicating the formation of a protein complex. Interestingly, both protein subunits were identified as

globulin-1 S allele. One possible reason for the phenomenon is the different post-translational modification patterns of the protein. Specifically, because globulin-1 S allele is a glycoprotein, the glycosylation may result in different molecular weights in SDS-PAGE. Besides, the regulated degradation of the protein, and the existence of two isoforms of the same protein, can also be the possible reasons, but an uncontrolled degradation can be excluded because both SDS-PAGE and chromatogram results proved that the protein complex was homogeneous. Noticeably, the CD analysis and 3D structure modeling results revealed that the globulin-1 S allele protein complex and monomer had similar secondary structures, implying that the structures of different forms of globulin-1 S allele are probably not significantly different. However, the current data cannot elucidate the exact mechanism of the different molecular weights of globulin-1 S allele, and the architecture of the protein complex was not clear, so further experiments are required to investigate this interesting problem.

So far, 28 allergens from *Triticum aestivum* have been identified and registered in WHO/IUIS (<https://www.allergen.org>), but globulin-1 S allele was not included. Moreover, there were no related reports about globulin-1 S allele in *Triticum aestivum*, and the allergenicity of globulin-1 S allele of other organisms has not been assessed in foods. In this study, we identified globulin-1 S allele as a glycoprotein, and both ELISA and Western blot results demonstrated the significant IgE-binding capacity of globulin-1 S allele in vitro. Hence, globulin-1 S allele was proved to be a novel allergen in wheat. It should be noted that this work only measured the IgE-binding capacity of globulin-1 S allele by in vitro experiments, but did not assess the in vivo allergenicity. To further verify globulin-1 S allele as a real food allergen and investigate the specific allergic responses induced by this allergen, more detailed investigations are required. On the one hand, more solid quantitative evidence, such as the investigations with recombinant globulin-1 S allele, or the comparison between globulin-1 S allele and other wheat allergens, would validate the IgE-binding capacity of globulin-1 S allele

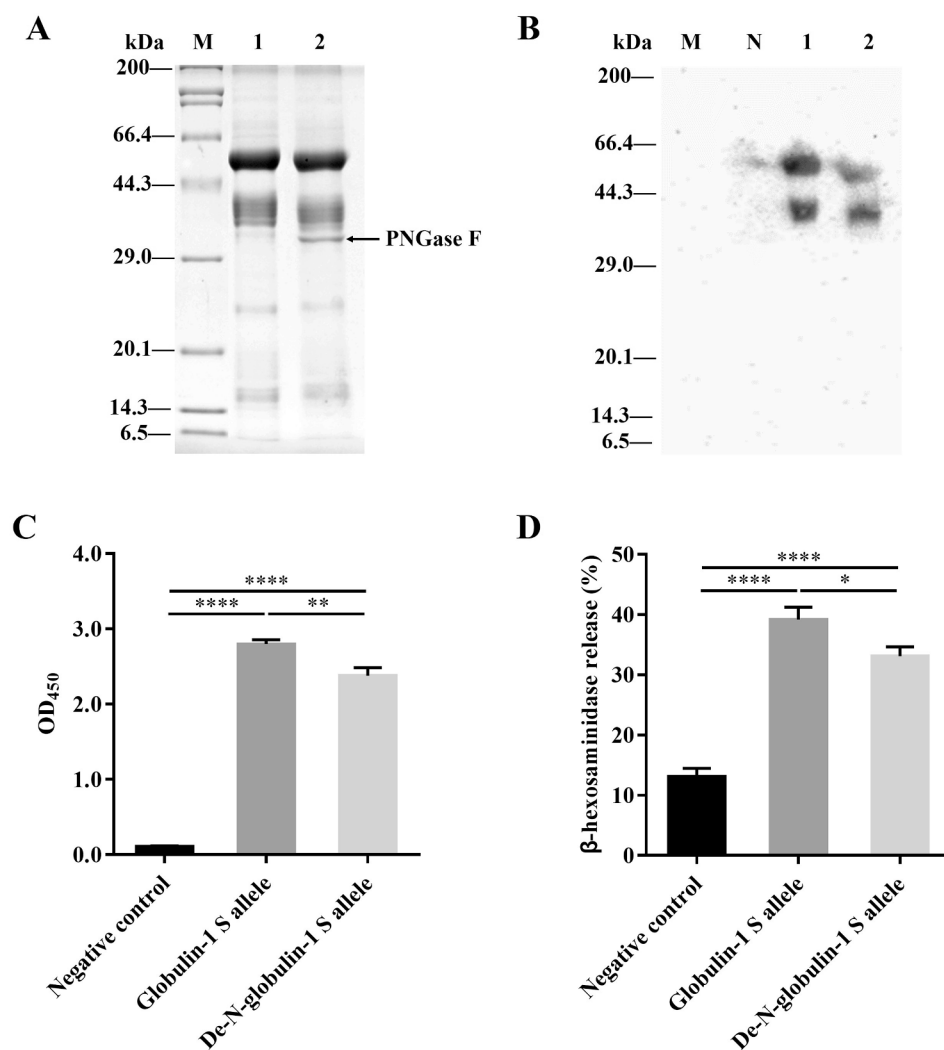


Fig. 5. The IgE-binding capacity of the purified globulin-1 S allele from wheat soluble protein and the de-N-glycosylated globulin-1 S allele. (A) SDS-PAGE analysis of the purified globulin-1 S allele. Lane M, marker; lane 1, purified globulin-1 S allele; lane 2, de-N-glycosylated globulin-1 S allele. (B) Western blot of the purified globulin-1 S allele and the de-N-glycosylated globulin-1 S allele. Lane M, marker; lane N, negative control; lane 1, purified globulin-1 S allele; lane 2, de-N-glycosylated globulin-1 S allele. (C) ELISA of the purified globulin-1 S allele and the de-N-glycosylated globulin-1 S allele with pooled allergic sera. (D) β-hexosaminidase release from LAD2 treated by the intact and de-N-glycosylated globulin-1 S allele.

and reveal the underlying molecular mechanisms. On the other hand, in vivo experiments, such as allergic mouse models and clinical studies including ImmunoCAP measurement and skin prick testing, would confirm the allergenicity of globulin-1 S allele and provide insight to practical applications.

Many previous studies have demonstrated that glycosylation is crucial for the allergenicity of specific allergens, which can produce additional epitopes and trigger essential immune signals (Homann, Schramm, & Jappe, 2017). Whereas some other studies have shown that glycosylation can reduce the allergenicity of specific allergens by blocking epitopes (Tian, Liu, Zhang, Tao, & Xue, 2020; Yuan et al., 2018). In the present study, the ELISA results showed that removing N-glycans significantly reduced the IgE-binding capacity of globulin-1 S allele.

Because de-glycosylation may also change the binding capacity to the ELISA plate, Western blot and degranulation assay were also performed to validate the finding. As the IgE-binding capacity was measured by direct interaction with IgE, it can deduce that glycosylation of globulin-1 S allele directly affects the IgE-binding epitopes, but whether glycans can further intensify allergenicity by other indirect approaches, such as activating immune signals by specific glycan receptors, requires further investigations. It should be noted that the de-N-glycosylated globulin-1 S allele still had a strong IgE-binding capacity, indicating that glycosylation is an enhancer rather than the only source of the allergenicity. Based on these results, deglycosylation treatment could be an effective approach to reducing wheat allergenicity, but the

practical value should be validated by further application studies.

5. Conclusion

In summary, this study identified globulin-1 S allele as a novel glycoprotein in *Triticum aestivum*, and demonstrated it to be one of the major allergens in wheat. The results also showed that the glycans were responsible for the IgE-binding capacity of globulin-1 S allele. These findings expanded the knowledge of wheat allergens and provided deglycosylation as a promising processing method for reducing wheat allergenicity.

Funding

This study was financially supported by the National Natural Science Foundation of China [grant number 31871735 and 31801452], and the State Key Research and Development Plan [grant number 2019YFC1605002].

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Chengbo Zhu: Investigation, Writing – original draft. **Chong Wang:** Investigation, Writing – review & editing, Project administration. **Jinru Zhou:** Methodology. **Yanbo Wang:** Conceptualization, Funding acquisition. **Qing Chen:** Resources. **Linglin Fu:** Conceptualization, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2022.133189>.

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