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Production of omega-5 gliadin monoclonal antibodies for allergenic evaluation of WDEIA-causing wheat varieties

Jae-Ryeong Sim[1†], Jong-Yeol Lee[1†] and Sewon Kim[1*]

Abstract

In allergic individuals, ingestion of wheat can lead to wheat-dependent exercise-induced anaphylaxis (WDEIA). Many studies have been conducted to find WDEIA allergen–deficient wheat, including by generating omega-5 gliadin antibodies. However, the reported antibodies have not been specific enough to detect omega-5 gliadins encoded on the 1B chromosome. In this study, we generated monoclonal antibodies against the major allergens causing WDEIA, omega-5 gliadins. Using these antibodies (mono-O5B-1C10), we assessed accumulation of omega-5 gliadins in wild-type and nullisomic-tetrasomic (NT) lines of the wheat (*Triticum aestivum*) varieties Chinese Spring (CS) by one- and two-dimensional gel electrophoresis, followed by Coomassie blue staining or immunoblotting with mono-O5B-1C10. We also tested mono-O5B-1C10 for major omega-5 gliadins in various wheat germplasms. Our results thus demonstrate the specificity of mono-O5B-1C10 for major omega-5 gliadins and potentially useful for identifying of omega-5 gliadin–deficient wheat varieties that should not cause WDEIA.

Keywords Immunoblot analysis, Monoclonal antibody, Standard operating procedures (SOP), Omega-5 gliadins, Wheat-dependent exercise-induced anaphylaxis (WDEIA), Wheat

Introduction

Wheat is one of the most important food crops worldwide, accounting for more than 20% of human protein and calorie intake. Approximately 778.2 million tonnes of wheat were produced in 2021 [1, 2]. Unlike other grains, wheat contains gluten proteins that exhibit unique viscoelastic properties that confer a wide range of desirable end-use qualities after milling. Glutens account for 70–80% of flour proteins and are divided into glutenins and gliadins depending on their solubility in aqueous alcohol. Glutenins confer elasticity to dough and represent 50–60% of gluten proteins, whereas gliadins confer extensibility to dough and account for 40–50% of gluten proteins. Gliadins are further categorized into four groups depending on their mobility during polyacrylamide gel electrophoresis (PAGE): alpha/beta (28–33% of gliadins), gamma (23–31% of gliadins), omega-5 (3–6% of gliadins), and omega-1,2 (4–7% of gliadins) [3].

Although gluten proteins are useful because of the end-use qualities they convey, they are also causative agents of several diseases. In addition to non-celiac gluten hypersensitivity and autoimmune disease (such as celiac disease), wheat consumption can lead to wheat-dependent exercise-induced anaphylaxis (WDEIA) [4–7], a severe allergy whereby ingestion of wheat products followed by physical exercise induces anaphylactic symptoms such as hypotension, dyspnea, and generalized urticaria [8]. Typically seen in adults [9], WDEIA is diagnosed by an exercise challenge following consumption of wheat. However, testing is time consuming and unsafe due to the amount of food and the degree of movement involved, and it

† Jae-Ryeong Sim and Jong-Yeol Lee contributed equally.

*Correspondence:
Sewon Kim
sewonk@korea.kr
[1] National Institute of Agricultural Sciences, RDA, Jeonju 54874, South Korea

frequently results in anaphylactic shock. Therefore, an alternative, in vitro diagnostic test is needed [10]. Skin-prick and specific immunoglobulin E (IgE) tests are also used to diagnose WDEIA, but they provide unreliable results [11]. An additional approach to diagnose WDEIA in vitro is to measure immune response to wheat by analyzing the patient's serum [12, 13]. However, this approach is not ideal both because obtaining a patient's serum is costly and time consuming and because some patients' immune responses may be too low to detect.

Omega-5 gliadins represent the major class of gluten antigens that cause WDEIA. Omega-5 gliadins are encoded by *Gli-B1* and *Gli-D1* genes on the short arm of chromosome 1 [12, 14]. Their primary structure consists of an N-terminus starting with SRL or TRQ residues, followed by repeat sequences of FPQQQ and QQIPQQ and then a C-terminus [12, 15–17]. Omega-5 gliadins do not contain any cysteine (Cys) residues and therefore do not form disulfide bonds [18, 19]. They also contain multiple glutamine residues in the repeat domain [18]. The primary IgE-binding sites on omega-5 gliadins are QQIPQQQ and QQFPQQ [4, 5, 15, 17, 20, 21].

Research on ways to generate wheat that does not causes disease is ongoing. For example, omega-gliadin-reduced materials have been developed through traditional breeding methods [22]. Moreover, advanced genetic technologies such as RNA interference (RNAi) have enabled the development of transgenic wheat lines that contain lower amounts of omega-5 gliadin [23]. Despite these advances, it remains difficult to accurately identify disease-avoiding cultivars. Thus, we sought to develop an additional approach.

In this study, we describe a monoclonal antibody (denoted mono-O5B-1C10) that specifically recognizes omega-5 gliadins. First, mono-O5B-1C10 was validated in standard wheat cultivar Chinese Spring (CS) and its nullisomic-tetrasomic (NT) lines via 2-DE, 1-D SDS-PAGE and immunoblot analysis. CS is cultivated bread wheat in which various aneuploid lines were developed. One of the aneuploid wheat lines called nullisomic-tetrasomic (NT) lines have two of their six homoeologous chromosomes lacking and two of their six homoeologous chromosomes added. These features are useful to study in wheat genetics. For examples, 1B chromosomes short arms are encoding major omega-5 gliadins. Among those NT lines 'nullisomic 1B tetrasomic 1A' (N1BT1A) and 'nullisomic 1B tetrasomic 1D' (N1BT1D) lines are each lacking 1B chromosomes and added 1 A and 1D chromosomes respectively. In these NT lines, we could observe no omega-5 gliadins because they do not have 1B chromosomes which are encoding omega-5 gliadins. We also validated the specificity of mono-O5B-1C10 via PAGE and subsequent Coomassie blue staining and immunoblot analysis using wheat cultivars in which we assessed the presence of omega-5 gliadins through PCR and RP-ULPC analyses. Our development of an omega-5-gliadin-specific antibody is beneficial for both research and industrial purposes. Mono-O5B-1C10 can be utilized to efficiently screen for wheat varieties deficient in omega-5 gliadin and can be used during food production to ensure processed foods will not cause WDEIA.

Materials and methods

Production of omega-5 gliadin monoclonal antibody

To generate monoclonal antibodies against omega-5 gliadins, a custom-synthesized peptide, C-SRLLSPRGKELG (derived from Altenbach et al. [12]), representative of the N-terminal fragment of omega-5 gliadins was mixed with adjuvant and injected into four BALB/c mice over 6 weeks. After fusion and culture of mouse spleen and myeloma cells, 17 single-cell clones out of 33 single- cell clones that specifically reacted with the Cys-SRLLSPRGKELG peptide antigen (yielding an $OD_{450nm} > 0.5$) were selected through ELISA (PerkinElmer VictoX3). Among the selected cell clones, the 1C10 clone was chosen which as to be mono-O5B-1C10. The chosen cell clone was cultivated followed by intraperitoneal injection into BALB/c mice. After 1–2 weeks, 10 ml of ascites was extracted from the abdominal cavity of mice, filtered, and the supernatant was slowly poured into Protein A resin to be combined, and washed with 1x PBS. Antibody was eluted with 0.1 M Glycine buffer (pH 3.0) and neutralized with pH 8.8 Tris buffer. The purified antibody was quantified by Bradford protein assay, and the antibody status was confirmed through SDS-PAGE. Purified antibody was aliquoted and stored at − 20 °C until use.

Plant materials

Control and omega-5 gliadin–deficient wheat (*Triticum aestivum* L.) varieties were planted in 50% clay loam soil at the National Institute of Crop Science, Jeonju, South Korea. Each plot consisted of three 4-m rows planted 25 cm apart. Before sowing, fertilizer was applied at 5:7:5 (N:P:K) kg/10 acres. Harvested grain was dried to 14% moisture using forced air at 22 °C. The control wheat varieties were kindly provided by the United States National Plant Germplasm System (https://www.ars-grin.gov/npgs/). Omega-5 gliadin–deficient germplasms were selected from 665 wheat germplasms of the National Institute of Crop Science, South Korea.

Total flour protein extraction and 2-DE analysis

CS and NT (N1AT1B, N1AT1D, N1BT1A, N1BT1D, N1DT1A, and N1DT1B) lines of CS were ground into fine powder. Total proteins were extracted from the resulting flour using SDS buffer (2% SDS, 10% glycerol,

50 mM DTT, 40 mM Tris-Cl, pH 6.8) for 1 h at room temperature with intermittent mixing. Insoluble material was removed by centrifugation at 16,000×*g* for 10 min as previously described [24]. Proteins were precipitated with acetone, and protein concentrations were determined via the Lowry protein assay [25]. Proteins were separated by 2-DE using capillary tube gels with an isoelectric point (pI) range of 3–10 in the first dimension and NuPAGE 4–12% Bis-Tris protein gels, 2D wells (ThermoFisher, Waltham, USA) in the second dimension. Gels were stained with Coomassie blue G-250 (Sigma-Aldrich, St. Louis, USA) and imaged with a calibrated scanner.

Gliadin extraction and SDS-PAGE analysis

50 mg of flour was dissolved in 0.5 ml of 60% ethanol/water and shaken for 30 min at room temperature. Gliadins were extracted with 60% ethanol/water. and centrifuged at 16,000×*g* for 10 min. This step was repeated twice. The supernatant (1 ml total) was transferred to a new 1.5-ml tube, and the gliadins were freeze-dried overnight and stored at −80℃ until use. To confirm protein extraction, 10 µl of the extracted gliadins was loaded into a NuPAGE Bis-Tris protein gel (4–12%, ThermoFisher, Waltham, USA).

Immunoblot analysis

Following 2-DE as described above, proteins were transferred onto nitrocellulose membranes using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, USA). The membranes were incubated in blocking solution (4% [w/v] skim milk in a solution of phosphate-buffered saline with 0.05% [v/v] Tween-20 [PBST]) for 1 h at room temperature. The membranes were then incubated with mono-O5B-1C10 (1:5,000 in PBST) for 1 h and 30 min at room temperature, washed three times for 10 min with PBST, and incubated with HRP-conjugated goat anti-mouse IgG (AbClon, Seoul, Korea) (1:20,000 in PBST) for 1 h and 30 min at room temperature. Proteins on the membrane were visualized by addition of West-Q Femto Clean ECL solution (GenDEPOT, Katy, USA) and subsequent imaging with the FluorChem M imaging system (ProteinSimple, San Jose, USA).

RP-ULPC analysis of gliadins

Dried gliadin fractions were analyzed by reverse-phase ultraperformance liquid chromatography (RP-UPLC) using a Waters ACQUITY UPLC H-class system equipped with an ACQUITY UPLC Peptide BEH C_{18} column (Waters Corp, USA, 2.1×100 mm, particle size 1.7 µm, pore size 300 Å). Mobile phase A comprised 0.06% (v/v) trifluoroacetic acid (TFA) in water, and mobile phase B comprised 0.06% (v/v) TFA in acetonitrile (ACN). Dried gliadin pellets were dissolved in 200 µl of 0.06% TFA and 20% ACN and filtered using a 0.22-µm PVDF filter syringe (Whatman, Maidstone, UK). 2 µl of each sample was injected for RP-UPLC analysis, and the analysis was caried out with a flow rate of 0.3 ml/min, a column temperature of 65℃, and a wavelength of 210 nm. Samples were eluted using a linear gradient of 25–45% of mobile phase B. Triplicate determinations were made.

Genomic DNA extraction from wheat and PCR analysis

Genomic DNA was extracted from 100 mg of wheat flour using a DNeasy® Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA was quantified on a NanoDrop spectrophotometer (Thermo Scientific, Waltham, USA) and diluted to 30 ng/µl. PCR was performed in reaction volumes of 20 µl using 60 ng of genomic DNA, 1.25 U of GoTaq® DNA Polymerase (Promega, Madison, USA), 5× Green GoTaq® Reaction Buffer (containing 1.5 mM $MgCl_2$), 200 µM dNTP mix, and 10 pmol of each primer (ω-5B-F, 5′-AGTAGGCTGCTAAGCCCTAGA-3′; ω-5B-R, 5′-ATATTGTTGGTATGGGGAAGG-3′). The primers for *omega-B6* were based on the sequence of 'AB181300' and expected amplified fragment size were 1212 bp [12]. PCR products were separated by electrophoresis on 1.5% (w/v) agarose gels in 0.5× TBE buffer, stained with ethidium bromide, and visualized under ultraviolet light.

Results

Preparation of monoclonal antibody specific for omega-5 gliadins in mice

To enable screening of wheat germplasms for those with reduced or no omega-5 gliadins, we generated monoclonal antibodies against omega-5 gliadins. We conjugated Cys to the N-terminus of the peptide sequence SRLLSPRGKELG, which was used to identify omega-5 gliadins in a previous study [12]. The Cys-conjugated peptide represents the N-terminal fragment of omega-5 gliadins. We injected this peptide into BALB/c mice and ultimately isolated single-cell clones via ELISA screening. In the primary screening, 33 clones were obtained using the ELISA and 17 clones out of 33 clones were identified that specifically reacted with omega-5 gliadins (Table 1). Among the 17 clones, the most specific from which a monoclonal antibody of the 1C10 cell line was selected and purified into in vivo IgG (mono-O5B-1C10). The mono-O5B-1C10 mass-purified and concentrated from the 1C10 clone showed a final concentration of 5.565 mg ml^{-1}.

2-DE Coomassie and immunoblot analysis of omega-5 gliadins in standard wheat varieties

Gliadins can be analyzed in CS wheat via 2-DE [26]. In addition, analysis of NT lines of CS defined a correlation

Table 1 The results of enzyme-linked immunosorbent assay (ELISA) test for selecting first fusion of omega-5 gliadins

Clone No.	$O.D_{450\ nm}$[a]		Clone No.	$O.D_{450\ nm}$	
	Omega-5 gliadin (Ag[b])	SMCC[c] control		Omega-5 gliadin (Ag)	SMCC control
1C10[d]	**2.572**	0.064	6G4	0.317	0.047
1D1	0.287	0.055	**6H4**	**2.686**	0.046
2B5	**1.43**	0.049	**7F3**	**2.304**	0.049
3E10	0.145	0.053	8B3	0.047	0.05
3F12	0.046	0.05	**8D1**	**2.36**	0.046
3G8	0.157	0.05	**8D8**	**2.468**	0.048
3H3	0.406	0.052	8H7	0.25	0.056
3H5	0.096	0.13	**8H11**	**2.565**	0.057
4B10	**1.245**	0.052	9A7	0.491	0.045
4C6	**2.192**	0.048	9A9	0.477	0.044
4C9	**1.372**	0.049	9D8	0.155	0.044
4H6	**0.543**	0.05	9E5	0.048	0.047
4H11	0.045	0.22	**9F9**	**0.803**	0.053
5H1	0.19	0.046	**10B5**	**2.282**	0.046
6C9	**2.401**	0.047	**10D6**	**2.31**	0.055
6E11	**2.475**	0.058	10F8	0.269	0.055
6F2	**1.133**	0.053	Positive	2.677	0.454
			Negative	0.045	0.045

[a] Optical density

[b] Antigen

[c] Sulfo-SMCC (sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate)

[d] Bold means 17 clones that specifically reacted with omega-5 gliadins

between prolamin proteins and their encoding chromosomes. To validate the functionality of mono-O5B-1C10, we extracted proteins from CS and CS NT wheat lines and separated them by molecular weight and pI via 2-DE. We stained the resulting gels with Coomassie to detect the proteins and performed immunoblot analysis of the gels with mono-O5B-1C10. In the Coomassie blue-stained gels, we observed a unique banding pattern around the 70-kDa marker in CS and the N1AT1B, N1AT1D, N1DT1A, and N1DT1B, but not N1BT1A and N1BT1D (Fig. 1). Immunoblot analysis with mono-O5B-1C10 confirmed the presence and absence of these unique bands in the same CS lines (Fig. 1). N1BT1A and N1BT1D CS lines lack chromosome 1B, on which omega-5 gliadins are encoded. Therefore, our data demonstrate the specificity of the mono-O5B-1C10 antibody for omega-5 gliadin proteins.

SDS-PAGE and immunoblot analyses of omega-5 gliadins in standard wheat varieties

Since 2-DE separates proteins by pI and molecular weight but requires considerable time and effort to perform, we conducted one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analyses to further confirm the specificity of mono-O5B-1C10 for omega-5 gliadins. We subjected gliadins extracted from CS lines specified above to SDS-PAGE using NuPAGE gels. Proteins separated by molecular weight (Fig. 2a). Coomassie staining of the gels revealed specific bands between 56 and 70 kDa that did not appear in the N1BT1A and N1BT1D lines. Immunoblot analysis with mono-O5B-1C10 antibody displayed this same pattern (Fig. 2b). Thus, immunoblot analysis was sufficient to confirm the deficiency of omega-5 gliadins.

Identification of omega-5-deficient germplasms by RP-UPLC

To further demonstrate the specificity of mono-O5B-1C10 for omega-5 gliadins, we tested our antibody on additional wheat germplasms lacking omega-5 gliadins. To select germplasms which have lack of omega-5 gliadins to test the antibody, we analyzed gliadins of 665 wheat germplasms by RP-UPLC. A previous study using reverse-phase column in liquid chromatography, RP-HPLC with the same conditions identified peaks corresponding to omega-5 gliadins as those with retention times (RT) of 2–4 min [27, 28]. Based on this, we identified RP-UPLC chromatograms from

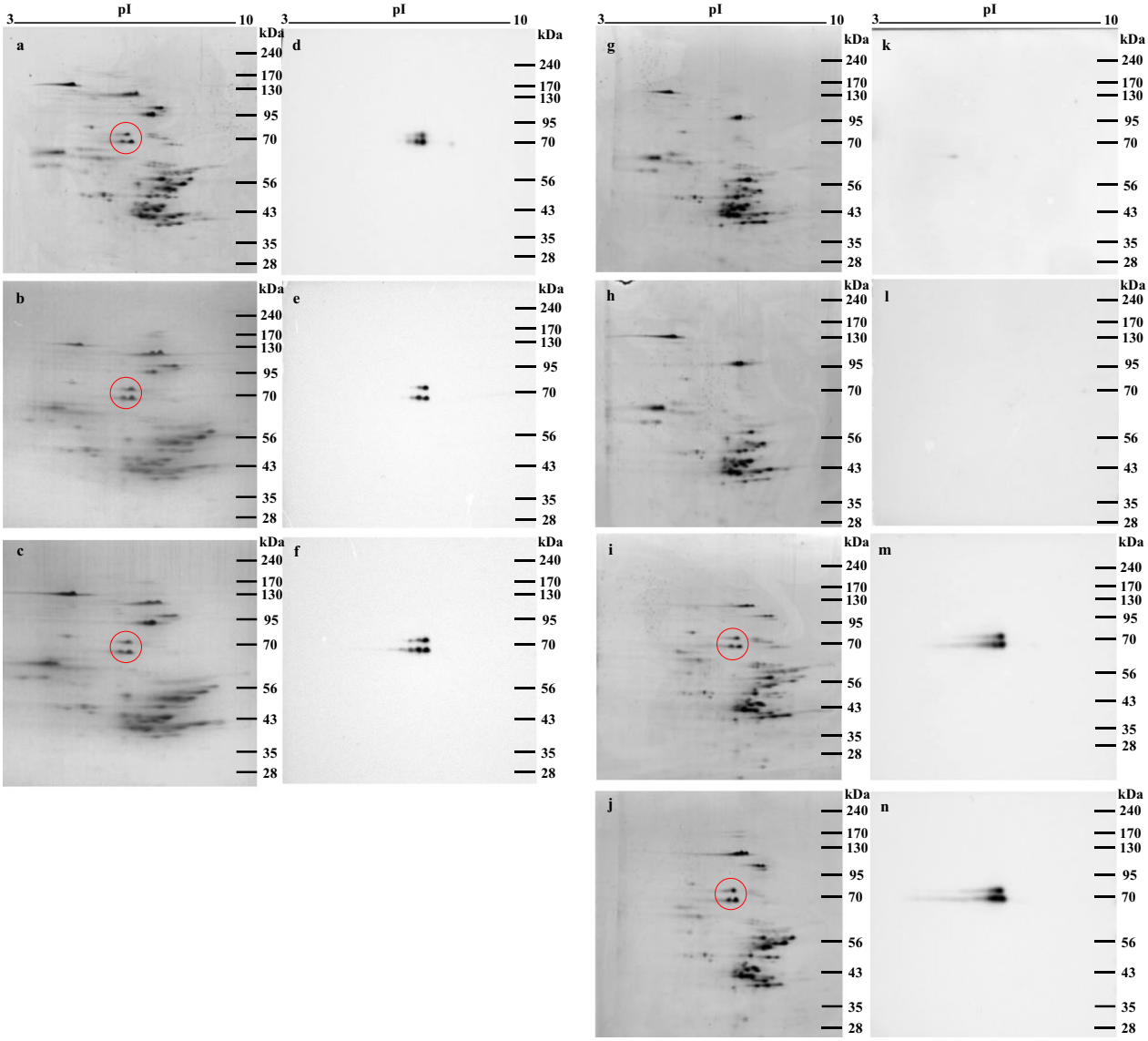

Fig. 1 Coomassie staining (**a**–**c**, **g**–**j**) and immunoblotting (**d**–**f**, **k**–**n**) analyses of total flour proteins from CS (**a**, **d**) and N1AT1B (**b**, **e**), N1AT1D (**c**, **f**), N1BT1A (**g**, **k**), N1BT1D (**h**, **l**), N1DT1A (**i**, **m**), and N1DT1B (**j**, **n**) CS NT lines subjected to 2-DE. Protein bands representing omega-5 gliadins are indicated by red circles

five of our germplasms that contained peaks representing omega-5 gliadins in the 2- to 4-min retention time span (Fig. 3). We included Keumkang (KK, commercial wheat of South Korea) as well as CS and CS NT lines in our analysis as controls. Within the 2- to 4 min retention time span, N1BT1A, germplasms 29, 62, 158, 278 and 280 displayed minor peaks, which are lack of omega-5 gliadins (Fig. 3). However, those of the KK and CS, N1AT1B and N1DT1A consisted of distinct, tall peaks in 2- to 4- min retention time (Fig. 3). Hence, we selected germplasms 29, 62, 158, 278, and 280 as candidates of omega-5 gliadin–deficient lines to test immunoblot analysis using mono-O5B-1C10 antibody.

Validation of mono-O5B-1C10 specificity using omega-5 gliadin–deficient germplasms

To further test the specificity of mono-O5B-1C10 for omega-5 gliadins, we investigated the reactivity of mono-O5B-1C10 with proteins from the five germplasms identified as lacking omega-5 gliadins. We subjected proteins extracted from KK and germplasms 29, 62, 158, 278, and 280, as well as from the CS (positive control) and CS NT aneuploid N1BT1A and N1BT1D

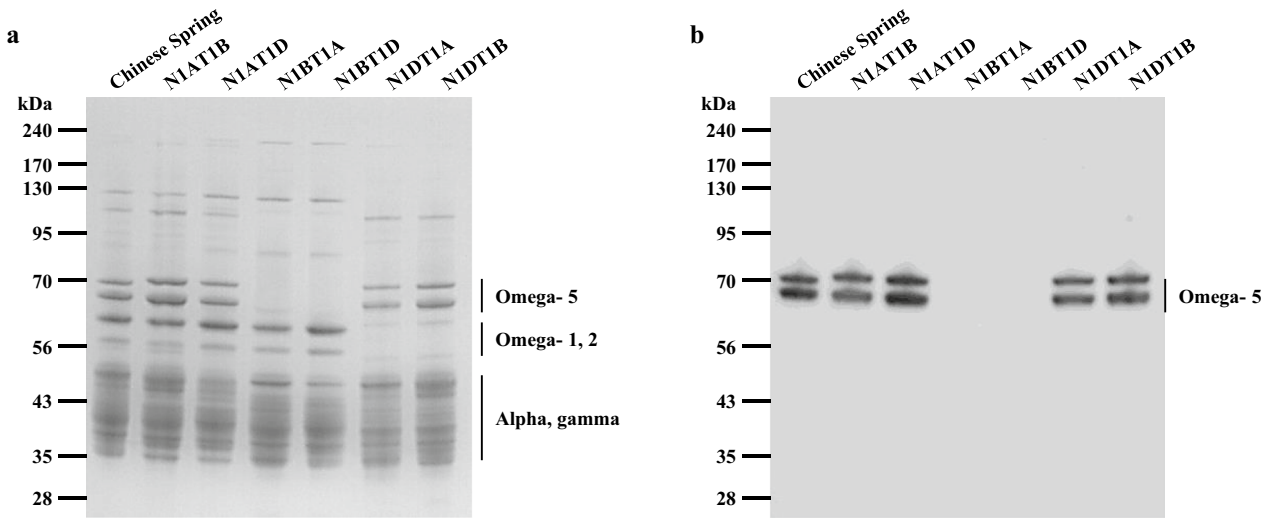

Fig. 2 Analysis of CS and CS NT gliadins separated by SDS-PAGE and subsequently stained with Coomassie (**a**) or immunoblotted with mono-O5B-1C10 (**b**)

(negative controls) CS lines, to SDS-PAGE and subsequent Coomassie staining or immunoblot analysis (Fig. 4a, b). As we had observed previously in the CS but not N1BT1A and N1BT1D CS sample lanes of the Coomassie-stained gel, several bands appeared between 56 and 70 kDa, which correspond to omega-5 gliadins (Fig. 4a). Unexpectedly, these bands similarly appeared in the sample lane of germplasm 62 (Fig. 4a). [However, observation of bands corresponding to omega-5 gliadins in the other samples was difficult (Fig. 4a).] We also performed an immunoblot analysis to better assess the presence of omega-5 gliadins in these samples (Fig. 4b). As expected, bands representing omega-5 gliadins appeared in sample lanes of the CS but not the N1BT1A and N1BT1D CS lines (Fig. 4b). However, contrary to our expectations based on the RP-ULPC analysis, we also detected bands corresponding to omega-5 gliadins in samples from germplasms 29, 62, and 158, although not in samples from germplasms 278 and 280 (Fig. 4b). Thus, we sought an additional method to verify whether germplasms 29, 62, and 158 actually express omega-5 gliadins. We probed the gene of omega-5 gliadins in all nine wheat lines at the genetic level via PCR analysis with primers specific for *omega-B6*, a gene encoding an omega-5 gliadin (Fig. 4c, d). Our analysis revealed *omega-B6* DNA (1212 bp) in the CS, KK, germplasms 29, 62, and 158 samples, but not in the N1BT1A, N1BT1D, germplasm 278 and 280 samples (Fig. 4d). Thus, in line with the PCR analysis and contrary to the RP-ULPC analysis, mono-O5B-1C10 immunoblotting demonstrated that germplasms 29, 62, and 158 contain omega-5 gliadins. These data confirm the specificity of mono-O5B-1C10 for omega-5 gliadins and demonstrate the superiority of this antibody as a credible tool to detect omega-5 gliadins compared to alternative methods such as RP-UPLC.

Discussion

Omega-5 gliadins are encoded at the *Gli-1* locus on the short arms of chromosomes 1B and 1D in hexaploid wheat. These proteins comprise the major class of allergen that confers hypersensitivity in WDEIA, a severe and life-threatening form of food allergy that occurs in patients who exercise after eating wheat [21]. Omega-5 gliadins specifically encoded on chromosome 1B are thought to be the major antigen that causes WDEIA [23, 29]. The *Gli-Bi* locus consist of two full-length omega-5 gliadin genes (*omega-B3* and *omega-B6*) and six omega-5 gliadin pseudogenes (*omega-B1*, *omega-B2*, *omega-B4*, *omega-B5*, *omega-B7*, and *omega-B8*) [30]. At 20 days post-flowering in CS endosperm development, transcript levels of omega-5 gliadin genes were much higher for *omega-B3* and *omega-B6* genes located on chromosome 1B than for *omega-D4* on chromosome 1D, encoding a truncated form of omega-5 gliadin [30]. Furthermore, a quantitative 2-DE analysis coupled with tandem mass spectrometry (MS/MS) revealed that protein expression of *omega-B6* was much greater than that of *omega-D4* in mature CS seeds [26]. These reports indicate that omega-5 gliadins encoded on chromosome 1B represent the majority of omega-5 gliadins expressed in wheat flour and therefore are likely the relevant omega-5 gliadin antigens that stimulate WDEIA. Consistent with this likelihood, we determined that protein extracts from N1BT1A and N1BT1D CS lines did not react with mono-O5B-1C10 (Figs. 1k, l; 2b and

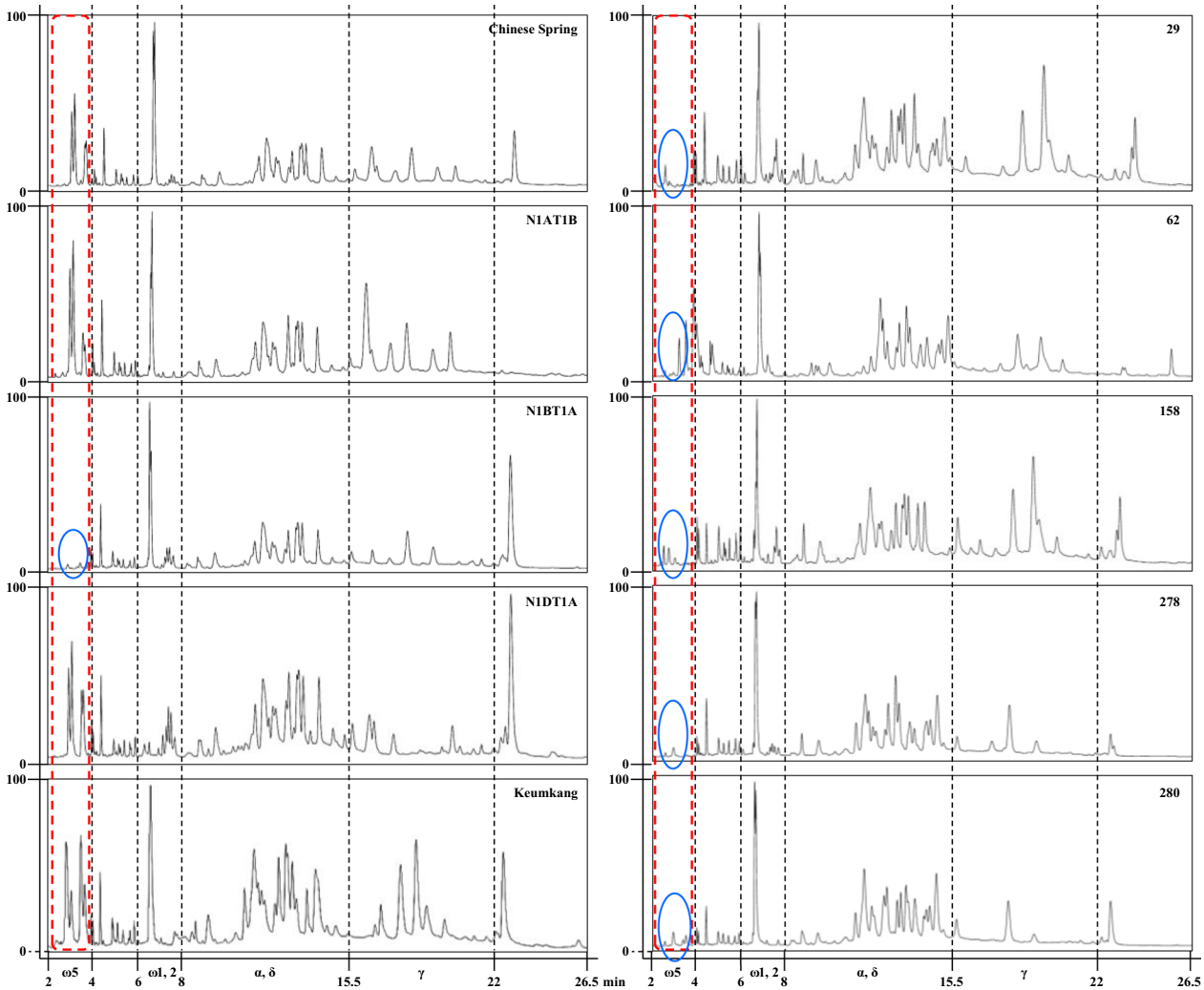

Fig. 3 RP-UPLC chromatograms of gliadin samples from KK and CS and CS NT lines (left column) as well as selected wheat germplasms (right column). Red, dotted boxes represent peaks corresponding to omega-5 gliadins. Blue, ovals indicate the minor peaks of omega-5 gliadins in N1BT1A and selected candidates of wheat germplasms. Retention times (RT) 2- to 4-min, 6- to 8-min, 8- to 15.5-min and 15.5- to 22-min indicate omega-5 gliadins, omega-1,2 gliadins, alpha/delta gliadins and gamma gliadins respectively

4b). These CS NT lines have 1 A and 1D tetrasomes in place of a 1B chromosome. Our results are consistent with the notion that omega-5 gliadins are primarily encoded on chromosome 1B. We hypothesize that the two bands observed in the CS lines correspond to *omega-B3* and *omega-B6* gliadins encoded by the full-length genes on chromosome 1B [30].

To detect omega-5 gliadin–deficient varieties in wheat, various analytical methods can be used, such as 2-DE, SDS-PAGE, RP-UPLC, and PCR. However, each method has weak points. For example, in 2-DE and SDS-PAGE, omega-5 gliadins may not be detected if the samples have only trace amounts. In the case of RP-UPLC analysis, low levels of omega-5 gliadins or sample contamination will prevent correct determination of the proportion of omega-5 gliadins present. When PCR analysis is performed using a primer specific for omega-5 gliadin, omega-5 gliadin genes or adjacent loci must be deleted for confirmation. Utilizing an antibody with an immunological reactivity specific to the omega-5 gliadin protein is thus required to address these issues.

Numerous investigations have been carried out using patient serum to characterize the immunological response to WDEIA [12, 13, 29]. However, the available antibodies were detecting both omega-5 gliadins encoded on the 1B and 1D chromosome and were

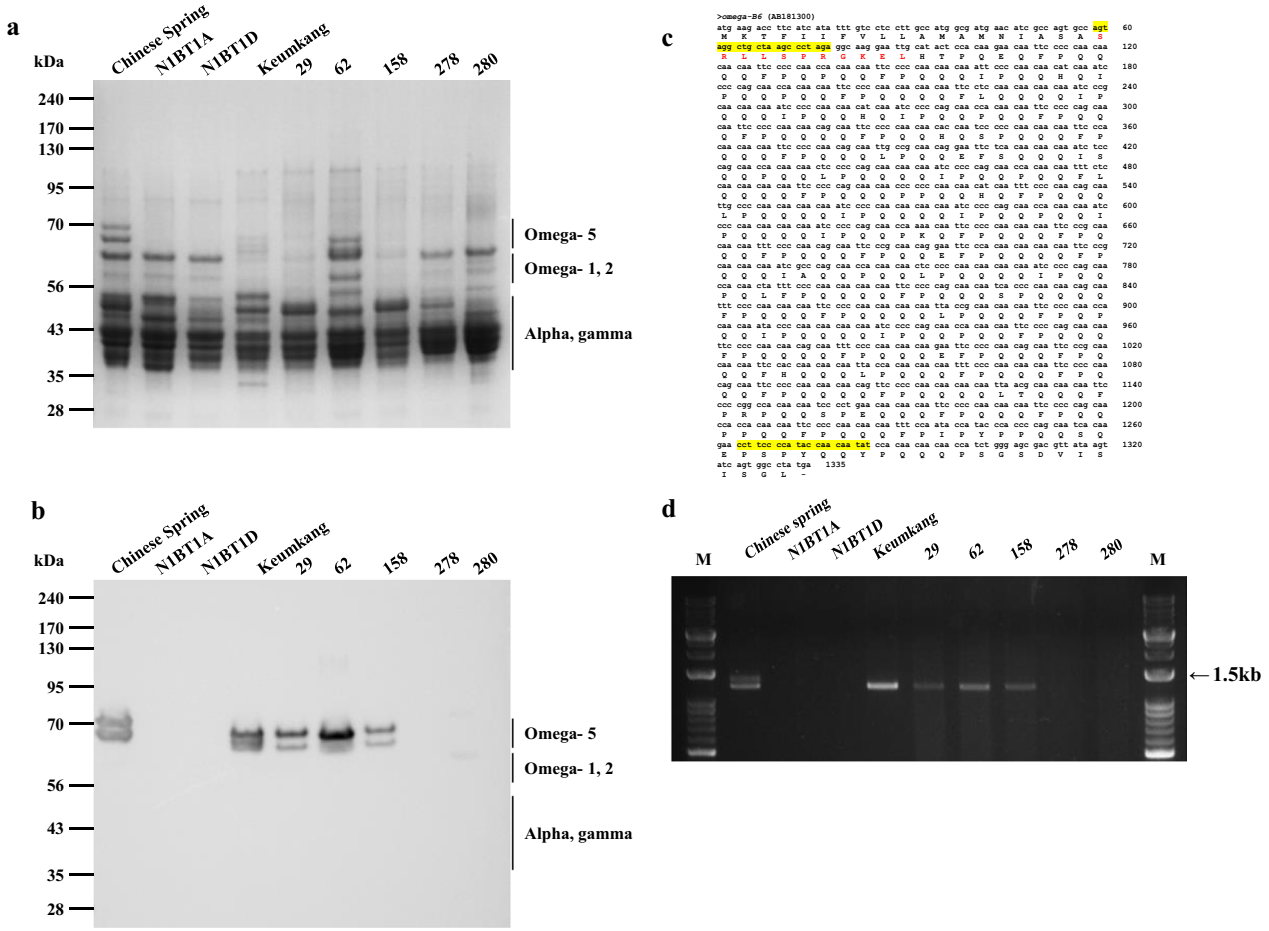

Fig. 4 Analysis of gliadins separated by SDS-PAGE and subsequently stained with Coomassie (**a**) and immunoblotted with mono-O5B-1C10 (**b**). The nucleotide sequence of omega-B6 (GenBank accession number AB181300) and its codon sequences, with forward and reverse primers highlighted in yellow and the codon sequences marked in red, representing the target site of the mono-O5B-1C10 antibody (**c**). Confirmation of *omega-B6* gene deletion by PCR analysis (**d**)

insufficient to identify omega-5 gliadin–deficient germplasms which gliadins encoded in 1B chromosome [12]. In this study, we generated a monoclonal antibody specific in omega-5 gliadins encoded on the 1B chromosome (Table 1) and confirmed its specificity using CS and CS NT lines (Figs. 1 and 2).

In order to produce antibody to detect specific omega-5 gliadins encoded in 1B chromosome, Cys residues were conjugated during production. Cys residues serve essential roles in protein structure and function, as their highly reactive thiol side chains that form inter- or intra- molecular disulfide bonds to enable correct protein folding [31]. We also purified large amounts of mono-O5B-1C10 by IgG purification that were extremely sensitive to omega-5 gliadins, thereby enabling the use of small amounts of gliadin protein samples. We achieved high-resolution detection of omega-5 gliadins with our antibody. For example, we detected omega-5 gliadins in our immunoblots even at a dilution of 1:5000, in contrast to other studies that used monoclonal antibodies at a dilution of 1:80 [12].

Prior to Coomassie or immunoblot analyses, we employed both one-dimensional SDS-PAGE and 2-DE to separate gliadin proteins (Figs. 1 and 2). We specifically used Bis-Tris gels for electrophoretic protein separation because compared to Tris-glycine gels, Bis-Tris gels have a wider range of molecular weight separation, a more neutral pH that reduces unintended protein modification, and a shorter run time requirement to achieve separation.

We further tested the utility of our monoclonal antibody for determination of omega-5 gliadin deletion in germplasms. In selecting candidates of omega-5 deficient wheat germplasms, there are numerous way such as PCR analysis in gene level and SDS-PAGE, liquid chromatography in protein level. We selected several

wheat germplasms by RP-UPLC chromatograms compare to the peak AU levels of N1BT1A and confirmed through SDS-PAGE (Figs. 3 and 4a). However, at the protein level, the selection of candidates for omega-5 gliadin-deficient germplasms were not clear on SDS-PAGE or chromatograms. Using mono-O5B-1C10 antibody, reactivity was observed clearly in rest of selected wheat germplasms except #278 and #280 (Fig. 4b) and they were confirmed in PCR analysis (Fig. 4d). These results indicate that in protein level, immunoblot analysis using mono-O5B-1C10 is much clear to verify whether omega-5 gliadins are deficient than SDS-PAGE followed by Coomassie staining. Also, mono-O5B-1C10 reacted in not only in standard wheat varieties such as CS and NT lines of CS, but also reacted in various wheat germplasms. Therefore, the monoclonal antibody could be useful as a confirmation method for determining elimination of omega-5 gliadins in various wheat germplasms.

In summary, we generated a purified monoclonal antibody specific for omega-5 gliadin by conjugating Cys to the SRLLSPRGKELG (mono-O5B-1C10). Our monoclonal antibody (mono-O5B-1C10) showed specific immunoreactivity to omega-5 gliadin protein in 2-D and 1-D immunoblots for the reference variety Chinese Spring (CS) and nullisomic-tetrasomic lines of CS (N1AT1B, N1AT1D, N1BT1A, N1BT1D, N1DT1A and N1DT1B). In addition, we identified that mono-O5B-1C10 showed immunoreactivity in various wheat germplasms. We conclude that mono-O5B-1C10 produced in this study can be used to select wheat germplasms in which the omega-5 gliadin protein is missing or lowered, which will contribute to the production of safe wheat-related products.

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Author contributions
The initial manuscript draft and experiments were performed by JRS, while JYL contributed an idea and carried out data analysis. SK was responsible for the overall manuscript conceptualization and supervision. All authors have reviewed and endorsed the final version of the manuscript.

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Availability of data and materials
All data generated or analysed during this study are included in this published article.

Declarations

Competing interests
The authors declare that they have no competing interests.

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