Dissecting the Disulfide Linkage of the N-Terminal Domain of HMW 1Dx5 and Its Contributions to Dough Functionality

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Supporting Information

ABSTRACT: The N-terminal domain of HMW-GS 1Dx5 (1Dx5-N) contains three cysteine residues (Cys10, Cys25, Cys40), which are the basis of gluten network formation through disulfide bonds. Disulfide linkage in 1Dx5-N was dissected by site-directed mutagenesis and LC-MS/MS, and its contributions to structural and conformational stability of 1Dx5-N and dough functionality were investigated by circular dichroism, intrinsic fluorescence, surface hydrophobicity determination, size exclusion chromatography, nonreducing/reducing SDS–PAGE, atomic force microscopy, and farinographic analysis. Results showed that Cys10 and Cys40 of 1Dx5-N were the active sites for intermolecular linkage. Meanwhile, Cys40 also exhibited the ability to form intrachain disulfide linkage with Cys25. Moreover, Cys10 and Cys40 played a functionally important role in maintaining the structural and conformational stability and high surface hydrophobicity of the N-terminal domain of HMW-GS, which in turn facilitated the formation of HMW polymers and massive disulfide linkage of HMW-GS through hydrophobic interaction. Additionally, the 1Dx5-N mutants in which Cys were replaced by serine (Ser) presented different effects on dough functionality, while only the C25S mutant produced positive effects compared with wild type 1Dx5-N. Na2CO3-induced β-elimination of cystine might occur in glutenin without heating, which would make it much easier to reduce the nutritional quality of flour products by the cost of lysine. Therefore, these results give a deep understanding of the disulfide linkage of the N-terminal domain of HMW-GS and its functional importance, which will provide a practical guide to effectively generate a superior HMW-GS allele by artificial mutagenesis.

KEYWORDS: N-terminal domain of high molecular weight glutenin subunit 1Dx5, site-directed mutagenesis, disulfide bond, dough functionality

INTRODUCTION

High molecular weight glutenin subunit (HMW-GS), with the molecular weight ranging from 68 to 89 kDa, is a kind of protein in the grain solely as components of gluten polymers, and HMW-GSs account for about 45−70% of the variation in breadmaking performance within European wheat, despite corresponding to around 1−1.7% of the flour dry weight.† HMW-GS is composed of a nonrepetitive N-terminal domain and a nonrepetitive C-terminal domain flanking a central repetitive domain, which form the molecular basis of HMW-GS in determining the gluten functionality.‡−4 Therefore, the importance of domains of HMW-GS in gluten viscoelasticity and flour quality has been appreciated for many decades.⁄

The repetitive domain of HMW-GS consists of 60−80% of the entire amino acid sequence, which is rich in glutamine, proline, and glycine and poor in sulfur (0 or 1 cysteine). The structural investigation indicates that the repetitive domain may form an unusual spiral supersecondary structure based on β-reverse turns.⁵ Meanwhile, the functional analysis shows that the individual repetitive domain of HMW-GS does not present the capability to improve the strength of wheat dough.⁶ However, the hydrophilic nature of the repetitive domain can contribute to the formation of intra- and intermolecular hydrogen bonds between HMW-GSs, due to a high level of glutamine residues.⁷−⁹ In dough, these hydrogen bonds give rise to unbonded mobile regions (loops) and bonded regions (trains), which account for the restoring elastic force of gluten.⁵,¹⁰ Consequently, much attention has been paid to the repetitive domain of HMW-GS to investigate its amino acid composition and structural and functional properties.

Despite the above, cereal scientists have gradually realized the importance of terminal domains in dough quality.⁸ The structure model shows that nonrepetitive N-terminal domain and C-terminal domain appear to be more similar to globular proteins, containing α-helix and aperiodic structure.² The
function of the N-terminal domain of HMW-GS 1Dx5 (1Dx5-N) in our group indicates that 1Dx5-N can improve the quality of dough by enhancing the formation of macromolecular weight aggregates and massive protein networks. Such functional differences between repetitive domain and terminal domains can be greatly attributed to the important roles of cysteine residues (Cys), because Cys residues in terminal domains have been demonstrated to be involved in “elastic backbone” formation by interchain disulfide bonds (SS) between HMW-GS. Meanwhile, it has been demonstrated that the sole Cys residue in C-terminal domain of HMW-GS participated in the formation of “head-to-tail” disulfide bonds between HMW subunits. Therefore, disulfide bonds, especially intermolecular SS, are widely considered to be essential for the processing quality of dough. The genomic DNA of HMW-GS indicates that N-terminal domains contain most of Cys (generally 3 to 5), which provides the main reaction sites for disulfide cross-linking, because of its contributions to protein structure and one of the important physicochemical properties of protein surface hydrophobicity and disulfide cross-linking sites. However, the results of this study suggested that the functional differences among various HMW-GSs are thought to be greatly affected by the numbers and positions of Cys, which determine whether HMW-GSs act as chain extenders or chain terminators during polymerization. Therefore, dissecting the SS cross-links in the N-terminal domain of HMW-GS is essential for effectively understanding and improving crop quality attributes. However, such a task is often hard to achieve, because it is difficult to isolate the target domains or whole HMW-GS from other wheat storage proteins. Therefore, detailed knowledge about the role of Cys in SS cross-linking and dough functionality is still limited.

Additionally, the amino acid composition of the N-terminal domain indicates its hydrophobic nature. Hydrophobicity is one of the important physicochemical properties of protein systems because of its contributions to protein structure and functionality. Furthermore, protein surface hydrophobicity greatly determines the aggregation tendency of protein molecules, and the exposure of buried hydrophobic region also promotes the cross-linking of proteins by covalent bonds. Previously, few studies have paid attention to the hydrophobic nature of the N-terminal domain and its potential functionality, and even Buonocore et al. concluded that the terminal domains had little functional importance except for providing cross-linking sites. However, the results of this study suggested that the high surface hydrophobicity was important for the formation of aggregates and massive SS of the N-terminal domain, which was greatly maintained by the cross-linking of Cys residues. Therefore, clarifying the relationship between surface hydrophobicity and disulfide linkages not only facilitates our understanding of the structural and functional properties of the N-terminal domain but also paves the way for the potential application of HMW-GS.

After great efforts, an N-terminal domain of typical representative of HMW-GS 1Dx5 (1Dx5-N) has been successfully cloned and purified in our group, and it is composed of 89 amino acids containing 3 cysteine residues which are located at 10 (Cys10), 25 (Cys25), and 40 (Cys40) sites, respectively. Moreover, the structural and functional properties of 1Dx5-N have been investigated. Therefore, the objective of this paper was to dissect Cys cross-links of N-terminal domain and their functional importance in dough functionality by employing liquid chromatograph—mass spectrometer/mass spectrometry (LC-MS/MS) analysis and developing missense mutants (C2S5, C40S, C10S, C25S/C40S, C10S/C40S). These results will be beneficial to provide the information for generating superior HMW-GS alleles through artificial mutagenesis.

**Materials and Methods**

**Site-Directed Mutagenesis for 1Dx5-N.** Five mutants of 1Dx5-N (C25S, C40S, C10S, C25S/C40S, C10S/C40S) by replacing Cys with serine (Ser) were obtained using the overlap extension PCR, since Ser is similar to Cys in that the hydroxyl group mimics the sulfhydryl group except for SS linkage. PCR was performed using the pMD19 T-1dx5 template containing a 267 bp target DNA fragment in a previous study and the primers in Table S1, and then initiated by the addition of Pfu polymerase, and the cycling parameters were as follows: one cycle at 98 °C for 2 min; 11 cycles of 98 °C for 30 s, 68 °C for 3.5 min; and a final step at 72 °C for 5 min. The samples (25 μL) were then incubated with 10 units of DpnI for 3 h at 37 °C to remove the methylated parental template. The PCR products were digested by NdeI and Xhol sites, purified by agarose gel electrophoresis, and subcloned into the pET-30b vector to produce the recombinant plasmids. The constructed products were transfected into Escherichia coli DH5α (DE3) cells. The plasmids were then isolated as individual colonies and were sequenced to confirm the altered sites.

**Expression and Purification of Recombinant 1Dx5-N and Its Mutants.** Based on the previous study, the recombinant plasmids were transformed into E. coli BL21 (DE3) for expression. The harvested E. coli cells were resuspended with Tris–HCl containing 0.5 M NaCl (pH 7.0), sonicated on ice for 10 min, and centrifuged at 8000g, 4 °C for 30 min. The target proteins were purified by affinity chromatography on a Ni²⁺-charged HiTrap chelating HP column (GE, Healthcare) and identified by SDS–PAGE. Subsequently, the pooled proteins were desalted with a HiPrep TM 26/10 column using 20 mM phosphate buffer solution (PBS, pH 7.4), and concentrated with a Centricon 3 kDa cutoff device. The concentration of target proteins was determined spectrophotometrically at 280 nm based on a previous method.

The stock of 1Dx5-N and its mutants was diluted to 5 mg/mL in 20 mM PBS containing 0.5 M NaCl (pH 7.0) and then stored at 25 °C for 72 h to characterize the changes in structure and aggregation. For the effect of hydrophobic interaction on SS formation, all target proteins were diluted to 5 mg/mL in 20 mM PBS containing 0.5 M NaCl (pH 7.0) or 0.5 M NaCl and 15 mM sodium dodecyl sulfate (SDS), and then stored at 25 °C for 72 h. For non-SS linkage, target proteins (2 mg/mL) were treated by 0.5 M Na₂CO₃ for 48 h at 25 °C.

**LC-MS/MS Analysis of In-Gel Digested Protein.** Monomer, dimer, and trimer of 1Dx5-N were obtained from non-reducing SDS–PAGE and in-gel chymotryptic digested at a chymotrypsin-to-protein sample ratio 1:10 (w:w), 37 °C overnight in ammonium acetate buffer pH 6.0. The digested peptides were aliquoted to two equal fractions: the first fraction was reduced with 50 mM DTT to reduce SS and created free cysteine groups, and the other one was served as a disulfide-intact control. All samples were reconstituted in 10% TFA and analyzed by nano-LC-MS/MS using a rapid separation LC system interfaced with a Thermo LTQ-Orbitrap Velos Pro ETD mass spectrometer (Thermo Fisher, San Jose, CA). Samples were loaded onto a self-packed 100 μm × 2 cm trap packed with Magic C18AQ, 5 μm 200 A (Michrom Bioresources Inc., Auburn, CA) and washed with buffer A (0.2% formic acid) for 5 min with flow rate of 10 μL/min. The trap was brought in-line with the homemade analytical column (Magic C18AQ, 3 μm 200 A, 75 μm × 50 cm), and peptides were fractionated at 300 nL/min with a multistepped gradient [4 to 15% buffer B (0.16% formic acid 80% acetonitrile) in 5 min and 15–50% B in 25 min]. Mass spectrometry data was acquired using a data-dependent acquisition procedure with a cyclic series of full scan acquired with a resolution of 60,000 followed by MS/MS scans (35% of collision energy in the ion trap) of 20 most intense ions with dynamic exclusion duration of 30 s. The MS/MS data were searched against a custom FASTA database including target protein sequences...
Reducing Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (Reducing SDS–PAGE) and Nonreducing SDS–PAGE. In order to reveal the intermolecular SS cross-linking, nonreducing and reducing SDS–PAGE were employed. Protein solutions (20 μL, 2 mg/mL) were diluted 1:1 with loading buffer containing 2% 2-mercaptoethanol (2-ME, v/v) and heated at 100 °C for 10 min. The protein samples for nonreducing SDS–PAGE were prepared by the same process but without 2-ME. SDS–PAGE was performed on a discontinuous buffer system using 12% separating gel and 3% stacking gel. The gel was stained using Coomassie brilliant blue (R-250) stain solution (45% methanol, 10% acetic acid, and 0.25% R-250) and destained in methanol–water solution containing 10% acetic acid (methanol/acetic acid/water = 1:1.8, v/v/v). The molecular weight of standard markers ranges from 18.4 kDa to 116 kDa.

Circular Dichroism (CD) Spectra and Intrinsic Fluorescence Spectra. Protein samples were diluted at 0.1 mg/mL using 20 mM PBS (pH 7.4) in a quartz cell with a 1 mm path length. CD spectra measurement was recorded on a circular dichroism polarimeter (Chirascan, U.K.) by scanning the sample from 190 to 260 nm at 25 °C with a bandwidth of 1 nm, a step size of 1 nm, an averaging time of 0.5 s, and a slit width of 0.02 nm. The CD spectra data of background was subtracted from sample to obtain the representative data. The percentages of secondary structure (α-helix, β-sheet, β-turn, and random coil) were predicted using CDNN spectral fitting software.

Fluorescence spectra of target proteins (0.3 mg/mL) were measured by a Hitachi spectrophotofluorometer (F-7000, Hitachi Ltd., Japan) using a 5 nm slit width at 25.0 °C. The intrinsic fluorescence spectra were recorded in the 300–500 nm range using a rectangular quartz cuvette with 1 mm path length by excitation at 295 nm, which was solely contributed by tryptophan residue.

Surface Hydrophobicity Determination (Hs). Surface hydrophobicity (Hs) of samples was determined using 8-anilino-1-naphthalenesulfonic acid (ANS). Briefly, ANS solution (20 μL, 8.0 mM in 20 mM PBS, pH 7.0) was added to serial dilutions of samples containing 31.3–1000 μg/mL target proteins. Fluorescence intensity was determined using a fluorescence spectrophotometer by setting 390 and 470 nm as excitation and emission wavelengths with a 5 nm slit width. The initial slope of the fluorescence intensity versus protein concentrations was calculated by linear regression analysis and used as Hs.

Size Exclusion Fast Protein Liquid Chromatography (SE-FPLC). SE-FPLC was used to monitor the effects of Cys substitution on the aggregation of target proteins. The experiments were performed on a fast protein liquid chromatography system (FPLC, GE Health, USA) equipped with a Superdex 200 Increase 10/300 GL column (100–600 kDa). The proteins (5 mg/mL) treated with 0.5 M NaCl with/without 15 mM SDS were filtered (0.45 μm) to obtain the injected samples. Protein samples were eluted isocratically at a flow rate of 0.7 mL/min with mobile phase containing 20 mM PBS (pH 7.0) with 0.5 M NaCl or 0.5 M NaCl and 15 mM SDS. Absorbance was monitored at 280 nm. The column was manually calibrated with standard protein: ferritin (440 kDa), BSA (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), and ribonuclease (13.7 kDa).

Atomic Force Microscopy (AFM). The morphological changes of the target protein aggregates were characterized by AFM. AFM images were recorded in tapping mode, and the scan rate was 1.0 Hz using a MultiMode SPM microscope equipped with a Nanoscope IIIa Controller (Digital Instruments, Veeco, Santa Barbara, CA). PointProbe NCHR silicon tips of 125 μm in length with a spring constant of 42 N/m were used. Aliquots (5 μL) of target protein dispersions (0.1 mg/mL) were placed on a freshly cleaved mica disk and air-dried for 20 min at room temperature. Images were analyzed using Digital Nanoscope software (version 5.30r3).

Dough Mixing. Dough mixing assay could reveal the functionality of 1DXs-N (WT) and the mutants on the flour processing quality. Microfarinograph measurements and the evaluation of farinogram were performed by AACC-approved methods. Low-gluten flour (11.0% protein, 12.1% moisture, 0.4% ash) was used, and microscale mixing tests were carried out using a 4 g Mixograph. Effects of WT, C2SS, C40S, and C10S (0.25%, w/w, flour base) on dough functionality were investigated. Water absorption (WA, %), dough development time (DDT, min), dough stability time (DST, min), and degree of softening (DS, FU) were recorded. Flour samples without added target proteins were prepared as control.

Statistical Analysis. The experimental data were expressed as the mean ± standard deviation. One way analysis of variance was used to compare the value differences (P < 0.05) using SPSS 17.0 (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Disulfide Bonding Pattern of 1DXs-N by Site-Directed Mutagenesis and LC-MS/MS. Figure S1 shows the reducing SDS–PAGE results of wild type 1DXs-N (WT) and its five mutants (C2SS, C40S, C10S, C2SS/C40S, and C10S/C40S). The five missense mutants were soluble and exhibited the same electrophoretic mobility compared with WT, indicating that the full length coding sequences of mutants were translated.

In fact we also attempted, but failed, to prepare C10S/C2SS and C10S/C2SS/C40S mutants in heterologous overexpression. Based on the previous study, nonreducing and reducing SDS–PAGE were used to reveal the formation of intermolecular SS of target proteins. In Figure 1A, WT exhibited main bands of monomer, dimer, and polymers, and C2SS presented similar SDS–PAGE profiles, indicating that Cys25 substitution did not influence the formation of interchain covalent bonds. However, C40S presented an obvious decrease in polymers. Furthermore, C10S showed a decrease not only in the formation of polymers but also in the formation of dimer. C2SS/C40S and C10S/C40S lost the ability to form polymers and only showed the bands of monomer and dimer. Moreover, all dimers and polymers of target proteins could be reduced by 2-ME in Figure 1B, indicating that the covalent bonds were disulfide linkages. Therefore, the above facts suggested that Cys10 and Cys40 were collectively involved in the formation of polymers through intermolecular SS, and Cys10 might present a much stronger ability in intermolecular linkage.

Previous studies indicated that salt stress (NaCl) could greatly induce the formation of glutenin polymers through SS, thus NaCl-induced changes in disulfide linkages were characterized to further explore the cross-linking ability of Cys in 1DXs-N. In Figure 1C, WT and C2SS presented an increased band intensity of polymers after 72 h storage, while C40S and C10S still lost their potential to form more aggregates by SS. However, it is noteworthy that C10S exhibited a more significant decrease in dimer than C40S, implying that Cys10 was the predominant site for dimer formation by intermolecular SS. For C2SS/C40S and C10S/C40S, massive dimer formed at the cost of monomer, suggesting that the remaining Cys could still form the SS. Previously, the addition of NaCl (0–750 mM) did not produce an obvious effect on the amount of 1DXs-N dimer for 72 h, implying that few free sulphydryl groups of Cys were available in dimer, and most of them should highly involve in the formation of disulfide bonds. Therefore, it could be inferred that Cys10 was prone to form intermolecular SS, and the adjacent cysteine residues (Cys25, Cys40) formed...
intramolecular SS in 1Dx5-N, which was also supported by the results of LC-MS/MS.

In order to determine the disulfide bonding pattern in 1Dx5-N, enzymatic digestion followed by LC-MS/MS analysis was used. After the digestion of monomer, dimer, or trimer of 1Dx5-N (Figure S2), native target peptide fragments containing Cys10, Cys25, and Cys40 were detected by high-resolution mass spectrometry: QC10ERELQELQEREL, KAC25QQVMDQQL and DQQLRDISPEC40HPVVVPVAGQY. In Figure 2, the results from digested fragments treated without/with DTT indicated that Cys10, Cys25, and Cys40 residues participated in the SS formation but presented different cross-linking degrees, supported by the increased peak area of target peptides with DTT treatment.19,25 Moreover, native SS linking peptide fragments, including the intermolecular linkages between QC10ERELQELQEREL and QC10ERELQELQEREL, DQQLRDISPEC40HPVVVPVAGQY and DQQLRDISPEC40HPVVVPVAGQY, QC10ERELQELQEREL and
KAC_{25}QQVMDQQL, QC_{10}ERELQELQEREL and DQQLRDISPEC_{40}HPVVVSPVAGQY, KAC_{25}QQVMDQQL and DQQLRDISPEC_{40}HPVVVSPVAGQY, and intramolecular linkage KAC_{25}QQVMDQQLRDISPEC_{40}HPVVVSPVAGQY were detected by LC-MS/MS (Figure 3A−F), suggesting that the disulfide linkages between Cys10, Cys25, and Cys40 were not conserved strictly. The detection of a large amount of intramolecular linkage between Cys25 and Cys40 supported the deduction from the site-directed mutagenesis results. Meanwhile, the results also suggested that Cys10 and Cys40 were the active sites for intermolecular linkage, because they exhibited a great ability to participate in the intermolecular cross-linking. However, Cys10 presented a much stronger potential than Cys40 in intramolecular linkage, because, to some extent, the intramolecular cross-linking between Cys40 and Cys25 limited the availability of Cys40 for intermolecular disulfide linkage. Therefore, the SS mapping results from LC-MS/MS supported the disulfide linkage results from the site-directed mutagenesis.

Intermolecular SS has been widely considered to be essential for gluten viscoelasticity, and, as a result, dissecting the disulfide linkage in gluten has been appreciated for many years. A previous disulfide linkage model of 1Dx5-N from Shewry et al. showed that the adjacent cysteine residues (Cys10, Cys25) formed intramolecular SS, and the remaining one (Cys40) participated in intermolecular SS. However, this study directly demonstrated that Cys10 residue should be the predominant site for intermolecular SS, and the adjacent cysteine residues (Cys40, Cys25) were the main sites for intramolecular SS. Therefore, based on a widely held view of gluten structure, it can be proposed that the whole 1Dx5 subunit forms an “elastic backbone” consisting largely of head-to-tail polymer through intermolecular SS from Cys10 in N-domain and Cys806 in C-terminal domain (Figure 4), and the “head-to-tail” HMW-GS polymers construct the basis for low molecular weight (LMW) subunit “branches”.

**Effects of Cysteine Residues of 1Dx5-N on Structural and Conformational Stability and Surface Hydrophobicity.** Disulfide bonding is crucial for protein structure and function,
and changes in the disulfide arrangement have been associated with altered activity of proteins. To detect the roles of Cys in the structural and conformational stability of I Dx5-N, CD and intrinsic fluorescence spectra measurements were performed (Table 1 and Table 2). In Table 1, the β-sheet conformation (40.0%) was found to dominate in WT, followed by α-helix (24.3%), β-turn (18.9%), and random coil (16.8%), which was similar to our previous results. Such structural composition, followed by β-sheet (27.7%), β-turn (20.1%), and random coil (5.7%). Although an increased content of α-helix, β-sheet, and β-turn at the cost of random coil indicated more formation of the ordered structure, the reduction of β-sheet resulted in a decrease of protein molecular interactions by hydrogen bonds, which to some degree suggested the dissociation of protein aggregates. Meanwhile, the α-helix conformation had a more compact structure than β-sheet, indicating the enhanced protein folding and the decreased structure flexibility. Therefore, Cys10 and Cys40 were beneficial for the aggregation of N-terminal domain of HMW-GS by maintaining the secondary structure stability. Moreover, the results from fluorescence spectra further supported the aggregation and structural changes which were revealed by CD. In Table 2, the λmax of WT had a blue shift ranging from 336.9 to 335.8 nm, with the maximum difference value being 1.1 nm after 72 h storage. Meanwhile, the λmax of C2SS, C40S, C10S, and C2SS/C40S also presented a slight blue shift with the maximum value of 0.8 nm, 1.0 nm, 1.2 nm, and 0.8 nm, respectively. However, the λmax of C10S/C40S presented a marked red shift with the maximum value of 7.1 nm after 72 h storage. A red shift indicated that the tryptophan residues exposed to solvent and the peptide chains became extended, which was an indicator of protein dissociation. Therefore, the results also showed that Cys10 and Cys40 were responsible for the structural and conformational stability of N-terminal domain of HMW-GS.

Because protein surface hydrophobicity (Hₒ) greatly determines the tendency of protein molecules to form aggregates, exploring the changes in Hₒ of WT and its mutants will unravel the contributions of cysteine residues to target protein aggregation. ANS binds to the exposed hydrophobic patches in partially unfolded protein, and the increase in Hₒ is accompanied by an increase in the relative fluorescence intensity, which is indicative of the exposure of aromatic amino acids to the reaction solvent. Compared with WT in Figure SA, Hₒ of C2SS, C40S, and C10S did not present significant changes. However, C2SS/C40S had an obvious (P < 0.05) decrease in Hₒ compared to WT, and C10S/C40S showed a much lower Hₒ than C2SS/C40S. The decreased Hₒ was mainly due to the reduced exposure of nonpolar groups, which was greatly caused by the transition of β-sheet to α-helix induced by Cys substitution (Table 1), because the geometrical difference between the α-helix and β-sheet showed that more side chains of the hydrophobic amino acids on the β-sheet were

| Table 1. Secondary Structural Analysis of 1Dx5-N (WT) and Its Five Mutants from CD Data |
|-----------------|-------|-------|-------|-------|-------|-------|-------|
| secondary structure | WT    | C2SS  | C40S  | C10S  | C2SS/C40S | C10S/C40S |
| α-helix (%)     | 24.3 (±1.4) | 25.5 (±0.4) | 24.9 (±6.4) | 22.4 (±1.1) | 27.1 (±0.0) | 46.5 (±2.8) |
| β-sheet (%)     | 40.0 (±0.8) | 39.4 (±0.5) | 38.6 (±2.2) | 40.6 (±1.5) | 35.8 (±3.9) | 27.7 (±2.2) |
| β-turn (%)      | 18.9 (±0.3) | 19.1 (±0.0) | 18.8 (±1.3) | 18.6 (±0.1) | 19.4 (±0.1) | 20.1 (±0.1) |
| random coil (%) | 16.8 (±0.9) | 16.0 (±1.1) | 17.7 (±8.1) | 18.4 (±0.3) | 17.7 (±4.0) | 5.7 (±0.5) |

| Table 2. Fluorescence Maximum Emission Spectra (λmax) of 1Dx5-N (WT) and Its Five Mutants Stored at 25°C for 72 h |
|-----------------|-------|-------|-------|-------|-------|-------|
| time (h)       | WT    | C2SS  | C40S  | C10S  | C2SS/C40S | C10S/C40S |
| λmax (nm)      |       |       |       |       |         |         |
| 0               | 336.9 (±0.0) | 338.2 (±1.1) | 338.7 (±0.5) | 338.2 (±0.5) | 339.5 (±0.2) | 339.1 (±0.3) |
| 36              | 336.5 (±0.2) | 337.4 (±1.0) | 337.6 (±1.1) | 337.3 (±0.1) | 338.6 (±0.3) | 343.9 (±1.1) |
| 72              | 335.8 (±0.3) | 337.4 (±0.2) | 337.7 (±1.1) | 337.0 (±0.0) | 338.7 (±0.5) | 346.2 (±0.8) |

Figure 5. Changes in the surface hydrophobicity (A) and aggregate formation (B) of 1Dx5-N (WT) and its five mutants.
exposed to the surrounding conditions, while the α-helix was a more compact structure and had a reduction in side chain exposure, leading to a decreased contributions of nonpolar groups to protein hydrophobicity.\textsuperscript{31,32}

In order to verify the changes in surface hydrophobicity, SE-FPLC was employed to further study the aggregation changes of WT and its mutants. Figure 5B shows the elution profiles of WT and its mutants incubated at 25 °C for 72 h. For WT sample, a tall and sharp peak could be observed at the retention volume of \( \sim 9.5 \text{ mL} \), which indicated the elution of larger aggregates (66.2−440 kDa). Meanwhile, two tiny bumps were barely noticeable at \( \sim 14.7 \text{ and } \sim 16.4 \text{ mL} \), which corresponded to the molecular range of dimer and monomer (13.7−35.0 kDa). The same results were observed in C25S, C40S, and C10S, suggesting that individual Cys mutation did not produce a negative effect on the formation of larger aggregates. However, the elution peaks at \( \sim 9.5 \text{ mL} \) became weaker for C25S/C40S and C10S/C40S, and the peaks of dimer and monomer at \( \sim 14.7 \text{ and } \sim 16.4 \text{ mL} \) significantly increased especially for C10S/C40S, indicating that the larger aggregates dissociated and the monomer/dimer became the predominant fractions. Identical with the above results, such a dissociation phenomenon for C25S/C40S and C10S/C40S was mainly caused by the decreased surface hydrophobicity (Figure 5A). Therefore, the cross-linking of Cys residues provided a special conformation for the N-terminal domain of HMW-GS to maintain its high surface hydrophobicity, which greatly determined the aggregation extent of HMW-GS.

The above results have clearly demonstrated that the disulfide linkages of the N-terminal domain contributed to its high surface hydrophobicity, and in turn, the knowledge of surface hydrophobicity could help to predict, control, and manipulate the influence of hydrophobic interaction on protein aggregation and precipitation during food processing and storage.\textsuperscript{33} Therefore, this study employed denaturant SDS to decipher the role of hydrophobic interaction in the aggregation of 1Dx5-N and formation of intermolecular SS\textsuperscript{34} with the purpose of better understanding and then manipulating N-terminal domain of HMW-GS. Figure 6 shows the nonreducing SDS−PAGE profiles and SE-FPLC results of WT and its mutants with SDS treatment. In Figure 6A, WT, C25S, C40S, and C10S exhibited main bands of monomer and dimer, and slight amount of polymers. C25S/C40S and C10S/C40S only presented the bands of monomer and dimer. After 72 h storage (Figure 6B), all WT, C25S, C40S, and C10S lost the ability to form polymers, and C25S/C40S and C10S/C40S did not form massive dimer by the cost of monomer compared to those treated by NaCl in Figure 1C.

In addition, Figure 6C shows the elution profiles of target proteins treated by SDS at 25 °C for 72 h. For all samples, the elution peaks at \( \sim 9.5 \text{ mL} \) disappeared, and the peak intensity of monomer or dimer significantly increased. However, without SDS treatment, WT and its mutants possessed the ability to form polymers through hydrophobic interaction and SS (Figure 1 and Figure 5B). Meanwhile, the morphological details of WT and its five mutants with/without SDS treatment were also investigated by AFM. As shown in Figure S3A, massive aggregates could be observed in WT, C25S, C40S, and C10S without SDS after 72 h storage, which was identical with the results from SE-FPLC. However, C25S/C40S and C10S/C40S presented the dissociation of aggregates, which was mainly attributed to the decreased protein hydrophobicity caused by Cys substitution (Figure 5B). After destroying the hydrophobic interaction (Figure S3B), no larger aggregates were observed in all samples, and smaller size proteins became the predominant component. Therefore, the hydrophobic interaction was the predominant driving force on improving the formation of disulfide bonds and HMW aggregate formation of 1Dx5-N (WT) and its five mutants indicated by nonreducing SDS−PAGE (A, B) and SE-FPLC (C).
of polymers and the massive SS of N-terminal domain of HMW-GS.

Besides the above, the hydrophobic interaction was indispensable for establishing the unique viscoelastic properties of dough, because it contributed to the stabilization of gluten structure during baking. Moreover, the hydrophobic interaction exerted a synergistic effect on the formation of gluten aggregates and network structure in hydrated dough with SS. In addition, due to its hydrophobic nature, N-terminal domains were the surface active components of glutenin, and consequently their interfacial properties could be of importance for the gas-retention capacity of dough, while this study found that the disulfide linkages were of functional importance in maintaining the high surface hydrophobicity of N-terminal domain, and the high surface hydrophobicity greatly facilitated the aggregation of HMW-GS through SS and then would enhance the functional properties of wheat dough. Therefore, clarifying the relationship between SS and surface hydrophobicity of 1Dx5-N provided a practical strategy to optimize or manipulate the potential application of HMW-GS by genetic engineering.

Effects of Cysteine Residues of 1Dx5-N on the Dough Functionality. Because "head-to-tail" glutenin polymers linked by interchain SS were the structural basis for gluten network, WT, C25S, C40S, and C10S were chosen to investigate the functionality of Cys residues of 1Dx5-N on dough processing quality. The flour mixing response with WT, C25S, C40S, C10S, and control sample are shown in Figure S4 and Table 3. Compared with control, WA of flour treated by 0.25% WT presented a significant (P < 0.05) increase by 1.0%. Moreover, the DDT and DST related to dough strength were also significantly (P < 0.05) increased from 0.87 and 1.40 min to 1.10 and 5.20 min, respectively. Meanwhile, the DS was much lower (P < 0.05) than that of control, also indicating that WT strengthened the wheat dough. However, the dough functionality could be further enhanced by the addition of C25S. In Table 3, the DST and DS with C25S treatment were significantly (P < 0.05) changed, reaching 6.70 min and 67.5 FU, which was much better (P < 0.05) than those of WT. However, C10S and C40S treatment, especially for C40S, lost the ability to improve the dough quality, which was indicated by a stable and even a significantly (P < 0.05) decreased DST value. Moreover, the DS of dough treated by C10S and C40S also increased compared to C25S treatment, suggesting the decreased dough strength.

A previous study demonstrated that WT possessed positive effects on improving the dough quality by enhancing the formation of gluten polymers and network structure. According to SS pattern, the improved dough quality treated by C25S could be attributed to the altered structure of intrachain disulfide bonds induced by the substitution of Cys25, because Cys25 substitution allowed portions of Cys40 usually involved in the intrachain SS with Cys25 being available for interchain cross-linking, which contributed to the formation of gluten network and HMW polymers. Additionally, the cysteine residues (Cys25 and Cys40) in C10S mainly formed intrachain cross-linking, and few free Cys could participate in the interchain SS between protein and gluten matrix. For C40S, it contained Cys10 and Cys25, which had preferential ability of intrachain and intrachain cross-linking, respectively. However, during the formation of gluten network structure, proteins carrying an odd number of cysteine residues with preferential ability of interchain linkage, like C40S, might act as chain terminators, and hence impeded the formation of gluten network structure by SS. Thus, these comparisons indicated that there were significant differences in the contributions of Cys10, Cys25, and Cys40 to dough functionality, and the Cys25 substitution was responsible for the observed improvement, implying that Cys25-based artificial mutagenesis might provide an effective way to generate a superior HMW-GS allele.

Although it has been generally accepted that disulfide linkages between glutenin had a major effect on gluten network and dough functionality, non-SS covalent linkage might well contribute to the gluten functionality under specified reaction conditions. A previous study showed that Na2CO3 at concentration >200 mM greatly induced non-SS covalent linkage between amino acids in 1Dx5-N at 25 °C. In this study, it was clarified that cysteine residues participated in the irreversible non-SS cross-linkage (Figure S5), supported by Cys mutation's production of negative effects on non-SS polymers. However, the other amino acids involved in the covalent bonds have not been determined. Similar cross-linking has been found in gliadin during heating (130 °C) at alkaline pH, reporting the occurrence of β-elimination of cystine. In theory, cystine, cysteine, serine, and threonine can undergo the initiating β-elimination reaction, and then cysteine, lysine, and histidine can react with the resulting (methyl)dehydrolalanine. Therefore, this study suggested that β-elimination of cysteine might occur in the N-terminal domain of HMW-GS under alkaline conditions (Na2CO3) but without heating. From a structural point of view, non-SS linkages in HMW-GS induced by Na2CO3 to some extent strengthened the covalent cross-linking and the network structure formation, which facilitated the improvement of dough functionality. However, it might be much easier for Na2CO3-induced non-SS linkages in 1Dx5 to reduce the nutritional quality of wheat dough due to lysine losses (7 lysine residues in 1Dx5), because the reaction could be initiated at room temperature. Therefore, the results revealed the role of Cys residues of the N-terminal domain of HMW-GS in dough functionality and related nutritional quality of flour products.

**ASSOCIATED CONTENT**

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b02449.
Primer sequences, SDS–PAGE profiles, AFM morphology, and mixing curves (PDF)

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Notes
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■ ABBREVIATIONS USED
HMW-GS, high molecular weight glutenin subunit; 1DX5-N, N-terminal domain of 1DX5; C/Cys, cysteine; S/Ser, serine; LC-MS/MS, liquid chromatograph–mass spectrometer/mass spectrometry; SS, disulfide bonds; H₀, surface hydrophobicity; PCR, polymerase chain reaction; PBS, phosphate buffer solution; TFA, trifluoroacetic acid; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; 2-ME, 2-mercaptoethanol; CD, circular dichroism; SE-FPLC, size exclusion fast protein liquid chromatography; AFM, atomic force microscopy; WA, water absorption; DDT, dough development time; DS, dough strength; ANS, 8-anilino-1-naphthalenesulfonic acid; WT, wild type; LMW, low molecular weight

■ REFERENCES