



Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: <https://www.sciopen.com/journal/2097-0765>

Subtractive immunogenicity of super-deamidated wheat gluten: targeting the attenuation of immune-recognition of tissue transglutaminase–peptide complexes

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ABSTRACT: Super-deamidation can eliminate acyl donor sites in wheat gluten to subtract immunogenicity of celiac disease (CD) by blocking endogenous tTG-mediated deamidation of gluten. However, its efficacy and related mechanisms in reducing immunogenicity through modulation of tTG-peptide complexes remains unclear. In this study, the digestive stability and tTG-peptide complexes immunogenicity of super-deamidated wheat gluten (SDWG) were evaluated to clarify the above mechanisms in suppressed tTG-peptide complexes immune responses. SDWG showed a 23.41% increase in the degree of hydrolysis and a 1.44 mg/mL increase in peptide concentration, potentially reducing to ~66% in CD related immunogenic epitopes compared with those of nature wheat gluten (NWG). Digested SDWG peptides (150–2,400 µg/mL) decreased intracellular ROS, lowered cell permeability, increased TEER value, and enhanced Occludin expression compare to NWG. Moreover, SDWG reduced the binding capacity between digested peptides and tTG, decreased interfacial adsorbed mass by 25.64 ng/cm² and the enthalpy change (ΔH) by 1.6 kcal/mol as well as the immunogenicity potential of the complexes. These results indicate that SDWG could improve digestibility and attenuate tTG-peptide immune-recognition, thereby reducing immunogenicity. This research highlights the SDWG potential for applications in low-immunogenic gluten-based food ingredients.

Keywords: super-deamidated wheat gluten, celiac disease, tissue transglutaminase

1. Introduction

Celiac disease (CD) an autoimmune immune mediated systemic disorder that develops upon an inadequate immune response to dietary gluten on genetically susceptible individuals (HLA-DQ2/DQ8) [1]. Repetitive amino acid sequences within gluten peptides are implicated as critical determinants in the pathogenesis of CD. These peptides resist further proteolytic degradation mediated by gastric, pancreatic and intestinal brush-border membrane peptidases owing to their high proline content [2]. They cross the mucosal epithelium of the small intestine, where the glutamine residues are posttranslational modification modified by tissue transglutaminase (tTG). It forms an enzyme-substrate intermediate that can be nucleophilically attacked

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Received 29 September 2025

Received in revised from 26 January 2026

Accepted 8 May 2026

either by an amine or alternatively by water^[3]. When water involved in the reaction, generates CD gluten T cell epitopes with negatively anchor residues and with increased affinity the positively charged pockets of HLA-DQ allotypes binding grooves^[4–6]. When an amine involved in the reaction, a tTG-gluten complex is formed. This complex can be recognized by tTG-specific B cell receptors, allowing for the presentation of deamidated gluten epitopes to gluten-specific T cells^[2,7]. Thus, the elimination of glutamine residues presents a promising target for therapeutic intervention in CD by disrupting the interplay between endogenous tTG-mediated deamidation and tTG-peptide complex formation.

Deamidation, a chemical modification that converts glutamine residues into glutamic acid, reduces gluten immunogenicity by eliminating acyl donors required for tTG-mediated modification and by facilitating gastrointestinal degradation of immunogenic epitopes^[8,9]. The deamidation of wheat gluten caused a greater loss of epitopes, and thereby reduction of the immunodominant immunoglobulin E binding capacity^[10]. Previous studies have shown that deamidated wheat alcohol-soluble protein effectively reduced the reactivity of patient serum and intestinal permeability^[11]. However, the impact of varying degrees of deamidation (DD) on desensitization remains controversial. As reported by Tranquet et al.^[12], wheat gluten with a DD of 50–60% exhibited a stronger IgE affinity than those with a DD of 15%. This may be attributed to the stochastic of partial gluten deamidation, a higher DD can still preserve highly immunogenic sequences. These sequences may act as preferred substrates for tTG, promoting the formation of neo-epitope complexes and thereby enhancing immunoreactivity^[13,14].

The super-deamidation wheat gluten (SDWG) refers to DD > 99%. Compared with moderate deamidation (DD 50–60%), super-deamidation converts the majority of glutamine residues, thereby may reduce residual glutamine immunogenic motifs and attenuating the potential toxicity of gluten peptides in CD. Furthermore, it also induce complete unfolding of protein structures that promote the hydrolysis of protein in Gastrointestinal tract^[15]. These characteristics confers a significant advantage to SDWG in the diminishing of toxicity in CD. However, whether SDWG effectively attenuates epithelial immunotoxicity and the underlying mechanisms remain unclear. Therefore, the objective of this study was to systematically investigate how deamidation-induced epitope eliminate and structural alterations in wheat proteins affect the stability of immunogenic epitopes during gastrointestinal digestion and the associated immunotoxicity. Furthermore, we further elucidated the mechanisms underlying celiac toxicity by examining how these changes influence tTG–peptide complex formation.

In this study, we investigated the stability of SDWG epitopes in the gastrointestinal tract, the immunotoxicity, the role of tTG–peptide complexes in CD pathogenesis. The dynamic changes in immunogenic epitope were analysed using R5-ELISA and LC-ESI-MS, while the effects of digested peptides on cell viability, oxidative balance, and monolayer integrity were assessed using a Caco-2 cell model. Additionally, the interactions between tTG and peptides were characterized by molecular docking, physicochemical analysis, indirect ELISA, and western blotting. This study provides evidence of the effect of

super-deamidation on wheat gluten immunogenicity and theoretical support for the development of low-allergen cereal food ingredients.

2. Materials and methods

2.1 Materials

Commercial wheat gluten with protein contents 80% on a dry weight were purchased from Henan Midaner Trading Co., Ltd (Zhengzhou, China). Ithiothreitol (DTT) was purchased from Sigma-Aldrich Co., Ltd (Livonia, USA). RIDASCREEN® Gliadin R5 ELISA kit was purchased from R-Biopharm AG (Darmstadt, Germany). Pierce quantitative colorimetric peptide assay kit and Bradford protein concentration assay kit were purchased from Shanghai Beyotime Biotechnology Co., Ltd (Shanghai, China). Cell Counting Kit-8 was purchased from GLPbio (Montclair, USA). Porcine pepsin, pancreatin, reactive Oxygen Species (ROS) Assay Kit, WST-8 assay kit, 3,3',5,5'-tetramethylbenzidine (TMB), ELISA coating buffer, PBST buffer, and tris-buffered saline (TBS) were purchased from Solarbio (Beijing, China). Non-fat milk powder was purchased from Yili Group (Inner Mongolia, China). Biotinylated goat anti-human IgE was purchased from Vector (Burlingame, USA). Alkaline phosphatase color development buffer was purchased from Bio-Rad Laboratories (Hercules, USA). The other reagents were obtained from Sinopharm Chemical Reagent Co., Ltd (Beijing, China). Chemicals and reagents used in this paper were analytical or HPLC grade.

2.2 NWG and SDWG preparations

Crude SDWG was prepared using a hydrated natural deep eutectic solvent composed of betaine, citric acid, glucose, and water. Previous studies of our group have reported an optimal physicochemical stability and deamidation-promoting capacity that molar ratio of substances was 3:1:1:6. Thus, we have used this composition throughout the remaining studies. Wheat gluten was hydrated based on the mass ratio of citric acid in NADES to wheat gluten was 0.6:10 (w/w) and hydrated at 25 °C for 10 h. Subsequently, the mixture was subjected to steam treatment at 121 °C for 10 min to induce deamidated, followed by freeze-drying to obtain SDWG powder ^[15]. The DD of wheat gluten was quantified by titration using our previously published method ^[16]. The amide nitrogen was released by acid hydrolysis of native wheat protein (2 M HCl, 121 °C, 3 h), absorbed in boric acid (40 g/L), and quantified by titration with 0.02 mol/L HCl. The ammonia released from SDWG under alkaline conditions was collected and similarly titrated. The DD was calculated according to equation (1).

Subsequently, crude SDWG and NWG were purified according to the protocol described previously with some modifications ^[17]. Briefly, the powders of wheat gluten were suspended in 10 volumes of 50% (v/v) isopropanol and ultrasonicated for 10 min. After centrifugation at $2,000 \times g$ for 2 min, the supernatant was collected, and the pellet was washed twice with 10 volumes of 50% (v/v) isopropanol containing 1% DTT. The combined supernatants were then centrifuged again under the same conditions, and the pellet was discarded. Finally, the resulting supernatants were mixed with 4 volumes of acetone and dried overnight at 4 °C to obtain NWG and SDWG.

$$DD(\%) = \frac{\text{ammonia produced by deamidation}}{\text{ammonia produced by deamidation completely}} \times 100 \quad (1)$$

2.3 *In vitro simulated digestion*

The digestion simulations were performed in accordance with the INFOGEST static digestion protocol with some modifications [18]. The oral phase was performed without salivary amylase, as the starch fraction had been removed from the wheat gluten samples. Specifically, NWG and SDWG solution (dissolved in distilled water, 80 mg/mL, 10 mL) were mixed with simulated gastric juice (4 mL), 2000 U/mL porcine pepsin solution (pepsin, 250 U/mg protein, 0.5 mL), $\text{CaCl}_2(\text{H}_2\text{O})_2$ (0.15 mol/L, 0.0025 mL), HCl (6.0 mol/L, 0.2 mL) and incubated at 37°C for 2 h. Then, gastric digested sample was mixed with simulated intestinal juice (4 mL), 100 U/mL pancreatin solution (porcine pancreas, 100 U/mg protein, 2.5 mL), $\text{CaCl}_2(\text{H}_2\text{O})_2$ (0.15 mol/L, 0.02 mL), NaOH (1.0 mol/L, 0.8 mL) and incubated at 37 °C for 2 h. Digested products were collected every 30 min, and enzymes were inactivated by boiling for 5 min. The digested samples were subsequently centrifuged at 10,000 g for 10 min at 4°C to remove insoluble aggregates, and the supernatants were collected and stored at −80°C for further analysis.

2.4 *Analysis of physicochemical properties of SDWG digestion product*

2.4.1 *Degree of hydrolysis (DH) and peptide concentration*

The DH was determined using the o-phthalaldehyde (OPA) method [19] and calculated according to equation (2).

$$DH(\%) = \frac{\alpha - n_i}{n_t} \times 100 \quad (2)$$

where α , n_i , n_t represent free amino acid concentration in the digestion product samples, in the undigested NWG, and the total amino acid concentration in the corresponding sample, respectively.

The peptide concentration was determined using a Pierce Quantitative Colorimetric Peptide Assay Kit, and the absorbance was measured at 480 nm.

2.4.2 *Reverse phase high-performance liquid chromatography (RP-HPLC) assay*

The molecular weight of the digestion products was analyzed using an LC-20A HPLC system (Shimadzu, Japan), according to the previous study with some modifications [20]. Samples were filtered through a 0.22 μm membrane prior to injection. Separation was performed on a C18 reversed-phase silica gel column (4.6 mm $\times$ 250 mm), and detection was conducted at 210 nm. The mobile phase consisted of (A) 0.1% (v/v) aqueous trifluoroacetic acid (TFA) and (B) 0.1% (v/v) TFA in acetonitrile. Gradient elution was applied as follows: the proportion of mobile phase B was increased from 20% to 60% over 0–60 min at a flow rate of 0.8 mL/min.

2.4.3 *Microstructures*

The microstructures of digestion products were observed using a confocal laser scanning microscope (CLSM, LSM 900, Carl Zeiss AG, Oberkochen, Germany) equipped with a 10 $\times$ objective. Samples were stained with rhodamine B (1 mg/mL) at a 10:1 (v/v) ratio and imaged at 530 nm excitation [21].

2.5 Immunogenic characteristics of SDWG digestion products

2.5.1 R5 competitive ELISA

The CD related immunogenic epitopes of samples were studied using R5 competitive ELISA assay kit. All procedures were performed strictly in accordance with the manufacturer's instructions.

2.5.2 Liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS)

The CD related immunogenic epitopes in the digested products were further analyzed using an HPLC system coupled to a timsTOF HT mass spectrometer (Bruker, Massachusetts, USA) [22]. LC-ESI-MS/MS analysis was carried out on a Phenomenex polar C18 column. Samples were eluted using a 22 min linear gradient from 3.5% to 1% of mobile phase B (80% acetonitrile containing 0.1% formic acid, v/v) in mobile phase A (0.1% aqueous formic acid, v/v). The eluate from chromatography system was subsequently introduced into the ESI ion source. Data-dependent MS/MS experiments were then performed with a resolution of 60,000 and a scan range of 100–800 m/z.

2.6 Cell viability and antioxidant activities of SDWG digestion products

2.6.1 Cell culture and cell viability

Caco-2 cells were obtained from American Type Culture Collection (ATCC HTB-37) and were cultured with Dulbecco's modified eagle medium (DMEM) containing 10% (v/v) FBS and 1% (w/v) penicillin-streptomycin double antibodies. Cells were maintained cultured in a 37 °C and 5% CO₂ incubator.

Cells viability was assessed by a CCK-8 method. Caco-2 cells (2.5×10^5 cell/well) were seeded into 96-well microplates. Then cells were treated with different concentrations of digestion products (0, 150, 300, 600, 1,200, and 2,400 µg/mL) and further cultured in a 37°C and 5% CO₂ incubator for 48 h. Cells maintained in DMEM alone served as the blank group, while those cultured without digestion products served as the control group. Subsequently, 10% CCK-8 solution was added to all the tested wells and continuously cultured for 2h. The absorbance of the CCK-8 was measured by the Synergy HT microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) at 450 nm, and calculated using the following equation (3):

$$\text{Cell viability (\%)} = \frac{A_1 - A_0}{A_2 - A_0} \times 100 \quad (3)$$

where A_1 , A_2 , A_0 were represented the absorbance of test, control, and blank cells at 450 nm, respectively.

2.6.2 Cellular antioxidant activities

Cellular antioxidant activity was characterized by quantifying ROS using a ROS Assay Kit, while superoxide dismutase (SOD) was assessed using a WST-8 assay kit.

2.7 Cell permeability of SDWG digestion products

2.7.1 Establishment of the Caco-2 cell transport model

200 µL of Caco-2 cells (2.5×10^5 cells/mL) were seeded into the apical (AP) chamber of 24-well Transwell insert with polycarbonate membranes pre-equilibrated with DMEM at 37 °C for 12 h. After that,

cell-free DMEM (500 μL) was added to the basolateral (BL) chamber. To reach the desired value of trans-epithelial electrical resistance (TEER, $> 500 \Omega/\text{cm}^2$), the culture medium was changed every 2 days and TEER was monitored every 2 days. Caco-2 monolayers lasted for 21 days. Only monolayers reaching a TEER value of $\geq 500 \Omega \cdot \text{cm}^2$ was used for subsequent treatments.

2.7.2 Trans-endothelial electrical resistance change (ΔTEER)

The integrity of Caco-2 monolayer was further monitored by measuring ΔTEER using an EVOM2 voltohmmeter (World Precision Instruments, Aston, UK) according to the methods described by Gu et al. [23]. The ΔTEER value was calculated using the following equation (4).

$$\Delta\text{TEER} (\Omega) = T_8 - T_0 \quad (4)$$

where T_8 denotes the resistance (Ω) of Caco-2 monolayer subjected to digestion products for 8 h, and T_0 represents the Ω wheat gluten of initial monolayer.

2.7.3 Effect of SDWG Treatment on Occludin Protein expression in Caco-2 Cells

The expression of Occludin in the Caco-2 monolayer was analyzed by a western blot experiment as pervious described with slight modifications [24]. Specifically, the DMEM in Transwell insert was replaced with 200 μL of wheat gluten digestion products at different concentrations, and the plates were incubated at 37°C for 24 h. Subsequently, the monolayers were gently rinsed with Hank's solution and maintained in the same solution for 30 min. After that, cellular proteins were extracted with RIPA buffer, separated by 10% SDS-PAGE, and transferred to polyvinylidene fluoride membranes. After blocking with 5% non-fat milk in TBST (Tris-buffered saline with 0.1% Tween-20), membranes were incubated with anti-Occludin primary antibody (Sevier Biotechnology Co., Ltd., Wuhan, China) at a dilution of 1:1000 overnight at 4°C . Subsequently, the membranes were probed with Goat Anti-Mouse IgG (H+L)-HRP Conjugated secondary antibody (Wanlei Life Sciences, Shenyang, China) at a dilution of 1:5000 for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence substrate and imaged with a transilluminator system (SGGW2000, Sevier Biotechnology Co., Ltd., Wuhan, China).

2.8 The tTG-mediated immunogenicity of SDWG

2.8.1 Preparation of tTG-peptide samples

Peptides were extracted from the intestinal digestion products of NWG and SDWG (**prepared in Section 2.3**) using a high-speed centrifuged method. Briefly, digestion products were centrifuged at 7,000 g for 20 min (4°C), and the supernatants were then centrifuged twice at 140,000 g for 30 min (4°C). The resulting pellets were freeze-dried to obtain gluten digested peptides. Then, cross-linked samples were prepared according to the method described by Lexhaller et al. [25] with slight modifications. The digested peptides (3.56 mg/mL) were incubated with tTG (final concentration 0.02 mg/mL) at 37°C for 120 min, while the blank control was prepared under the identical preparations without tTG. All reactions were terminated by heat inactivation (95°C , 10 min).

2.8.2 Molecular docking on tTG-peptide complex

The molecular docking on tTG-peptide complex was performed according to the previous study [26]. To examine the binding of digested peptides with tTG enzyme, a molecular docking simulation was conducted using AutoDock (4.2.6, Scripps, USA), and a docking visualization was performed using Pymol (3.12, DeLano Scientific LLC, USA). The peptides detected from LC-ESI-MS/MS were used to construct 3D structures using Chemdraw 3D (20.0, PerkinElmer, MA, USA) and minimized using the MM2 potential field to eliminate geometric conflicts in molecular bond lengths, bond angles, and dihedral angles, thereby obtaining stable conformations. The crystal structures of the tTG enzyme (PDB code: 9BC2) was obtained from the PDB database (<https://www.rcsb.org/>). After removing water residues and original ligands from the receptor protein and hydrogenation, a grid box was constructed to cover all possible binding sites, followed by docking. The results were evaluated based on binding energy and interaction bonds.

2.8.3 Binding kinetic

Based on the method of Yang et al. [27], quartz crystal microbalance with dissipation (QCMD) analyzer (Q-Sence E1, Biolin Scientific Q-sense AB, Sweden) was used to investigate the binding kinetic behavior between digested peptides and tTG. Solutions of tTG (0.0114 mg/mL) and digested peptides (0.2 mg/mL) were dispersed in Tris-HCl buffer (0.1 M, pH 7.4). Regarding QCM-D measurement, the system was first equilibrated with buffer to establish a stable dissipation baseline. Then, tTG was immobilized on the sensor surface until frequency stabilization, after which peptide solutions were injected until binding equilibrium was reached. All experiments were conducted at 37 °C with a flow rate of 100 μ L/min. Data were analyzed using QSense DFind software to track frequency (Δf) and dissipation (ΔD) shifts.

2.8.4 Binding thermodynamics

Peptides and tTG was dissolved in Tris-HCl buffer (0.1 M, pH 7.4) to achieve final concentrations of 3 mg/mL and 1.14 mg/mL, respectively. Then, thermodynamics of the combined process were measured using isothermal titration calorimetry (ITC, MicroCal PEAQ-ITC; Malvern Panalytical, UK). Briefly, 280 μ L of tTG solution was carefully loaded into the sample cell, and 40 μ L of peptides solution was aspirated into the injection syringe. The data were processed using MicroCal PEAQ-ITC software (v1.4.1; Malvern Panalytical Ltd, UK).

2.9 Antigenicity of tTG-peptide

2.9.1 Indirect ELISA

Indirect ELISA was performed as described by Benedé et al [28].

2.9.2 Western blot analysis

The Western blot was performed as previously described [29,30], and the results are presented as images to visualize the number and molecular-weight distribution of immunoreactive bands. The tTG-peptide complex was separated by SDS-PAGE and transferred onto PVDF membrane. Human serum samples used for serum immunoblotting were commercially purchased from PlasmaLab International (Everett, WA, USA). The pooled serum was prepared by mixing equal volumes from five independent donors (Bleed No. PL 24172, PL

24714, PL 26732, PL 27387, and PL 27419). According to the vendor-provided ImmunoCAP/FEIA results, all donors were positive for wheat-specific IgE (F4). The membrane was blocked with 5% non-fat milk and incubated with human patient serum samples diluted 1:15 in Tris-buffered saline containing 0.1% Tween-20 (TBST) at 4°C for 24 h. After rinsing by TBS buffer for 3 times, the membrane was incubated for 2 h with biotinylated goat anti-human IgE (diluted 1:1000 in 5% non-fat milk-TBST), followed by another 3 washes. The membranes were then incubated with BCIP/NBT substrate for 10 min. Finally, Milli-Q water was added to rise the membrane and the western blot bands were visualized using a Bio-Rad GS-710 Calibrated Imaging Densitometer (Bio-Rad Laboratories, Hercules, USA).

2.10 Statistical analysis

All the experiments were conducted at least three times. Data were analyzed using SPSS 16.0 (IBM, USA) with one-way or two-way analysis of variance, and $P < 0.05$, as determined by Tukey's test, was considered to indicate significant differences.

3. Result and discussion

3.1 *In vitro* digestive characteristic of SDWG

The extent of wheat gluten digestion is considered to be closely associated with their immunogenic potential in genetically predisposed individuals, as incomplete proteolysis leads to the persistence of immunoreactive peptide fragments, and interact with tTG to induce CD [21]. In this study, we aimed to verify the effect of super-deamidation approach on the degradation of wheat proteins sensitizing peptides. With increasing digestion time, the DH (**Figure 1A-B**) and peptide concentration (**Figure 1C-D**) of wheat gluten exhibited an initial slowly increased during the gastric digestion stage followed by a rapidly increased during the intestinal digestion stage. This was attributed to the fact that wheat gluten tends to aggregate in an acidic environment, reducing the number of sites exposed to pepsin action sites [31,32]. Moreover, SDWG showed improved hydrolysis properties, as it exhibited higher DH and peptide concentrations than NWG throughout digestion time, with increases of 23.41% and 1.44 mg/mL, respectively, at the end of intestinal digestion. These results may be explained by super-deamidation induced structural changes that the breakage of a large number of unstable disulfide bonds and a reduction in the strength of the internal hydrogen bonding interactions. These changes increasing solubility of SDWG, promoting the hydrolysis of protein peptide bonds by digestive enzymes [33].

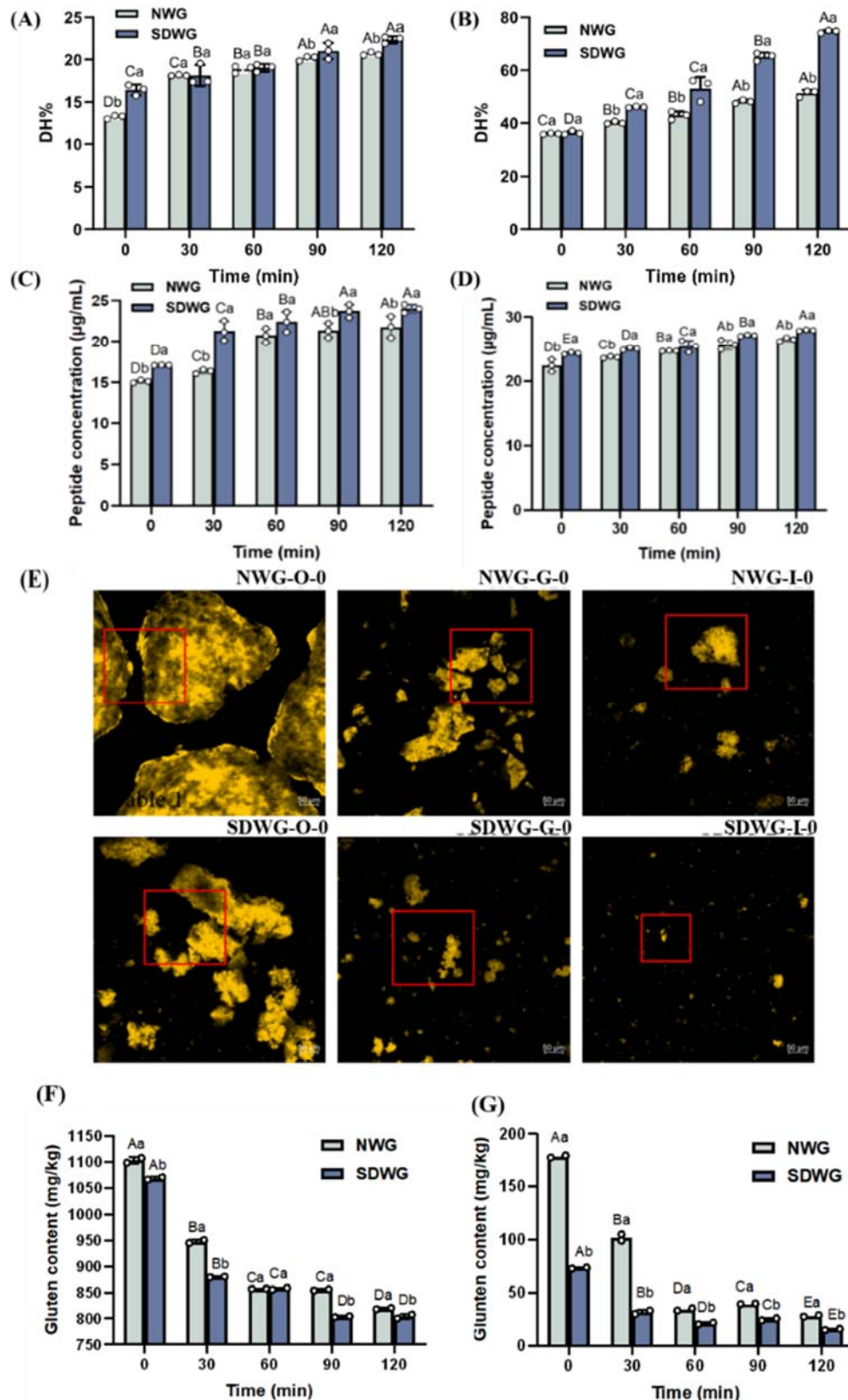


Figure 1 In vitro digestive characteristic and allergenic properties of SDWG. (A) The change of degree of hydrolysis of gluten proteins in the gastric digestion. (B) The change of degree of hydrolysis of gluten proteins in the intestinal digestion. (C) The change of peptide concentration of gluten proteins in the gastric digestion. (D) The change of peptide concentration of gluten proteins in the intestinal digestion. (E) CLSM plots of gluten proteins in gastrointestinal tract (NWG-O-0: The end of oral digestion of NWG; NWG-G-120: The end of gastric digestion of NWG; NWG-I-120: The end of intestinal digestion of NWG; SDWG-O-0: The end of oral digestion of SDWG; SDWG-G-120: The end of gastric digestion of SDWG; SDWG-I-120: The end of intestinal digestion of SDWG). (F) Gluten content of wheat gluten in gastric digestion stage. (G) Gluten content of wheat gluten in intestinal digestion stage. Different letters (a,b) are significantly different among groups at the same time point ($P < 0.05$). Different letters (A-E) are significant different over time within the same group ($P < 0.05$).

HPLC chromatogram suggested that the retention peak advanced and the formation of more small peptides was detected in SDWG as compared NWG after digestion (**Figure S1**). CLSM was used to examine the structural features of protein in the gastrointestinal tract. **Figure 1E** presents micrographs obtained with samples of NWG and SDWG digest products. Both of the digestion products were large agglomerates of protein visible at the oral digestion stage. After gastric stage of digestion, the proteins were broken down into smaller particles but still remained visible particles. However, the size of the SDWG clusters was smaller than NWG. After intestinal phase, only the protein clusters of NWG remained and the sample of the SDWG were almost disappeared. These results suggested that the higher degradation compared with NWG in the gastrointestinal digestion. We have previously reported the super-deamidated modification significantly altered the secondary structure of the gluten, resulting in decreased α -helix content and increased β -turn content. This attribute to large amounts of Glu and Asp produced by deamidation were exposed to the protein surface. It also reduced aggregation and increased random coil formation. These conformational changes caused the aggregated protein to diffuse outward from the inside, forming a slender network^[15]. The significant changes in structure can be attributed to the more enzymatic sites in the hydrophobic core region of the protein, thereby promoting digestion of gluten in the gastrointestinal tract.

3.2 The allergenic properties of SDWG digestion products

The content of immunogenic gluten epitopes within gluten dictates the magnitude of the pathogenic T-cell response in celiac disease. R5 sandwich competition ELISA recognizes the presence of multiple epitopes of α/β -, γ -, and ω -alcohol-soluble proteins, providing an effective method to assess changes in gluten content of samples during digestion^[34]. The gluten protein content of SDWG digestion products was assessed using the R5 sandwich competition ELISA kit. The gluten content decreased significantly with increasing digestion time (**Figure 1G–F**), indicating epitopes are hydrolyzed by digestive enzyme. The gluten content of SDWG have a lower content compare with NWG at all digestion stage. SDWG resulted in a decrease from 1107.47 mg/kg to 1070.08 mg/kg in the gluten content (**Figure 1G**). This reduction is contributed to the glutamine residue loss by super-deamidation treatment. After gastrointestinal tract digestion, the gluten content of SDWG significantly decrease to 98% in relation to the pre-digestion, as well as the decrease observed on 66% in relation to the NWG. These findings may attribute to the structure alterations induced by super-deamidated treatment. The super-deamidation disrupts CD related sequences by converting the glutamine amide groups into carboxyl groups, leading to a reduced abundance of detectable CD related immunogenic epitopes in the digestive products. Moreover, super-deamidation increases the thiol-to-disulfide bond ratio, enhances structural disorder, and promotes extensive protein unfolding, thereby improving enzymatic accessibility during gastrointestinal digestion. This facilitates proteolytic cleavage and the conversion of allergenic peptide segments into smaller, non-toxic peptides^[35]. This result is consistent with the previously observed increases in hydrolysis degree and peptide concentration.

To further substantiate our findings, LC-ESI-MS was used to systematically identify the peptide information. The digested peptides of wheat gluten were identified by primary mass spectrometry by

comparing the database of celiac disease-associated T-cell epitopes established by the study of Sollid et al. [5]. As shown in Table 1, nine T-cell epitope peptides with different abundances were identified in the digestion products of NWG and SDWG. The identified peptides could be classified into three major epitope groups, including γ -gliadin-derived epitopes (DQ8-glia- γ 1a, DQ2.5-glia- γ 4c, and DQ2.5-glia- γ 5), glutenin-derived epitopes (DQ8.5-glut-H1), and hordein-derived epitopes (DQ2.5-hor-2). These epitopes are characterized by conserved proline-rich and glutamine-containing sequence motifs, which confer resistance to gastrointestinal proteolysis. In particular, DQ8-glia- γ 1a and DQ2.5-glia- γ 4c represent immunodominant CD-associated epitopes due to their high binding affinity and structural compatibility with HLA-DQ molecules. The HLA-DQ2.5 form stable peptide–HLA complexes with gluten-derived T-cell epitopes. The HLA-DQ8-restricted epitopes exhibit lower stringency in proline positioning, allowing a broader range of peptide conformations to be recognized, thereby contributing to their pathogenic potential in CD [36]. The quantitative analysis showed that the total peak area of the epitopes in the SDWG was significantly lower than NWG. Notably, the epitope abundance in SDWG (670,200) was significantly lower than that in the NWG group (918,342), corresponding to approximately 72% of the NWG. Specifically, the peak area of the HLA-DQ8–restricted core epitope DQ8-glia- γ 1a (QPQQPFPQ) decreased from 778,000 to 369,000. In DQ8-positive patients, immune responses are most commonly directed against the DQ8-glia- α 1 epitope [37]. In addition, SDWG resulted in the deletion of the DQ8-glia- γ 1a and DQ2.5-hor-2 epitopes. No significant differences were observed in the total peak areas of DQ8-glia- γ 1a, DQ8.5-glut-H1, and DQ2.5-glia- γ 4c epitopes were increased after super-deamidated treatment. This finding attributed to the super-deamidation treatment induced protein conformational changes, which exposed certain peptides or generated fragments partially overlapping known epitopes [38]. These results show that SDWG significantly reduces the potential immunogenicity of wheat gluten epitopes.

Table 1 Peptide sequence information generated from gluten protein digestion products

Peptide	m/z	RT	Epitope	Area		p-value
				NWG	SDWG	
EQPQQPFPQ	549.76038	12.2133	DQ8-glia- γ 1a	47,400±65	73,000±77	<0.0001
GYYPPTSPQ	456.70581	10.6683	DQ8.5-glut-H1	1,270±101	2,000±142	ns
QGYYPPTSPQ	520.7345	10.7535	DQ8.5-glut-H1	632±4	1,000±15	ns
QPEQPFP	421.70163	13.5307	DQ2.5-hor-2	2,510±104	nd	/
QPEQPFPQ	485.73209	11.909	DQ2.5-glia- γ 4c	59,200±70	117,000±752	<0.0001
QPFPEQPQ	485.73175	11.2005	DQ2.5-glia- γ 5	nd	35,200±115	/
QPQQPFPQ	969.48456	11.6342	DQ8-glia- γ 1a	778,000±70	369,000±144	<0.0001
QQPEQPFPQ	549.7605	12.4472	DQ2.5-glia- γ 4c	23,900±90	73,000±62	<0.0001
QQPFPQ	372.68549	11.0918	DQ8-glia- γ 1a	5,430±48	nd	/
SUM				918342	670200	

m/z: the peptide mass-to-charge ratio; nd: not detected;

3.3 Cytotoxicity and Cellular Antioxidant of SDWG on Caco-2 cells

As displayed in **Figure 2A**, the cell viability of digestion product of wheat gluten digestion product decreased gradually with the increase of concentration in the range of 150–2400 μ g/mL. Cell viability of

digestion product of gluten was >80% at all test concentrations, which could be considered safe. Thus, concentrations of 0–2400 $\mu\text{g/mL}$ were selected as safe doses for further experiments. Cell viability was significantly higher in SDWG than in the NWG at the highest concentration (2400 $\mu\text{g/mL}$), indicates that SDWG increases cell viability.

The oxidative stress is one of the mechanisms that can play a role in gliadin toxicity. The accumulation of toxic peptides in cells leads to activation of some signal transduction pathways and to increased levels of ROS and decreased activity of SOD [39]. As shown in **Figure 2B**, the ROS levels gradually increased with rising concentrations of wheat gluten in Caco-2 cells. While the SDWG significantly ($P < 0.05$) reduced the ROS levels in Caco-2 cells at highest concentrations compare to the NWG.

Meanwhile wheat gluten derived peptides led to a concentration-dependent decline in cellular SOD activity, indicating impaired antioxidant defense with increasing peptide exposure (**Figure 2C**). At 300 $\mu\text{g/mL}$, SDWG treatment resulted in a higher SOD activity than NWG treatment, increasing from 0.218 U/mg prot to 0.2795 U/mg prot. At the low concentrations (300–1,200 $\mu\text{g/mL}$), the limited amounts of peptides are likely insufficient to effectively activate cellular antioxidant defenses. Conversely, at the highest concentration (2,400 $\mu\text{g/mL}$), excessive peptide exposure may induce substantial ROS accumulation and overwhelm the SOD mediated antioxidant defense system, resulting in a complete disorder of the oxidative system and eliminating significant differences between SDWG and NWG. Wang et al [40] reported similar changes of SOD in Caco-2 cells treated with silver carp muscle hydrolysates. These results suggested that SDWG digestion products might alleviate oxidative stress by limiting ROS accumulation and enhancing SOD activity.

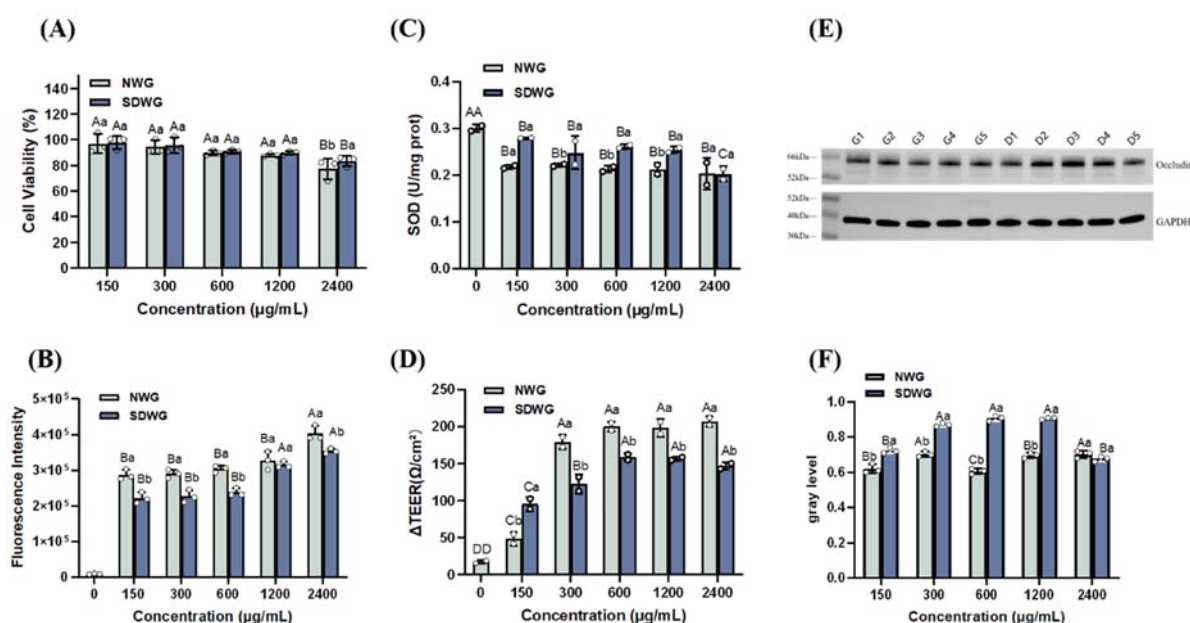


Figure 2 Evaluation of cytotoxicity, oxidative stress, and permeability of SDWG on Caco-2 cells. (A) Cellular activity of wheat gluten digestion products. (B) The change of ROS levels in wheat gluten digestion products. (C) The change of SOD activity in wheat gluten digestion products. (D) Effect of different concentrations of wheat gluten digestion products on cell monolayer TEER values. (E) Expression of Occludin tight junction proteins in monolayer cells at different concentrations of wheat gluten. G1–G5: concentration of NWG in the range of 150–2400 $\mu\text{g/mL}$; D1–D5: concentration of SDWG in the range of 150–2400 $\mu\text{g/mL}$. (F) The grayscale analysis of Occludin tight junction proteins. Different letters (a,b) are significantly different among groups at the same time point ($P < 0.05$). Different letters (A–E) are significant different over time within the same group ($P < 0.05$).

3.4 Effect of SDWG on Caco-2 mechanical barrier

The TEER and the expression of TJ proteins Occludin in Caco-2 monolayers were measured to elucidate the impact of SDWG on intestinal barrier function. Caco-2 intestinal epithelial cells were treated in different concentrations of wheat gluten and the changes in TEER over time were recorded. Of note SDWG showed a greater reduction in TEER at low concentrations (150 $\mu\text{g/mL}$), compared with NWG. However, it resulted in improved TEER value reduction as concentrations increased (**Figure 2D**), indicating that SDWG showed improved protective on the compactness of monolayer across a broader concentration range. This may be attributed to the lower molecular weight and increased solubility of peptides generated from SDWG during gastrointestinal digestion, which facilitate epithelial interaction at low concentrations. This finding is consistent with the observations of Picariello et al ^[41], which reported that smaller peptides exhibit greater epithelial accessibility and interaction potential due to their higher solubility and diffusibility. In contrast, NWG retains a higher abundance of intact immunogenic peptides capable of interacting with epithelial cells at higher concentrations, thereby inducing a greater reduction in TEER.

To further investigate the SDWG in regulating barrier permeability and dynamic tight junction architecture, we measured the expression of tight junctions Occludin protein. The impact of wheat gluten digestion products on Occludin expression in Caco-2 cells was assessed via immunoblotting (**Figure 2F**). As shown in **Figure 2G**, Occludin expression decreased in a concentration-dependent manner with increasing wheat gluten digestion products, indicating disruption of epithelial tight junctions. The cells treated with SDWG digestion products showed significantly higher Occludin expression compared to those treated with NWG at equivalent concentrations ($P < 0.05$). These results suggest that SDWG mitigates the inhibitory effects of wheat gluten digested on Occludin expression. The upregulation of Occludin on SDWG suggests that surper-deamidation treatment may protect tight junctions and attenuate gluten-induced barrier dysfunction.

3.5 Binding kinetic of tTG-peptides

The adsorption kinetics between tTG enzyme interface and SDWG digestive peptides was evaluated using QCM-D. QCM-D can monitor the real-time kinetics of the combine process between tTG enzyme and SDWG peptide, which allows us to calculate the changes in mass adsorption at the interaction interface ^[42,43]. **Figure 3A-B** illustrates a four-phase interaction profile: i) Buffer establishing the baseline, ii) tTG phase showing enzyme adsorption to saturation, iii) Buffer rinsing removing loosely bound tTG, and iv) Sample phase capturing interactions between digestive products and surface-immobilized tTG. When tTG was fed at (ii) phase, a rapid decreased in the frequency shift (Δf) while dissipation shift (ΔD) increased, indicating adsorption of tTG to the bare surface of the quartz sensor. During the sample phase (iv), with the introduction of the NWG and SDWG digestive products the Δf decreased by 21.68 Hz and 20.22 Hz to reach a plateau, respectively. This result suggested that more adsorption of NWG onto the tTG enzyme interface compare to SDWG ^[44]. As show in **Figure 3C-D**, the mass and thickness properties show that during the sample phase, the mass and thickness of SDWG reduced by 25.64 ng/cm² and 0.14 nm reduction than NWG. These results

indicate that SDWG exhibits a lower binding affinity for the tTG enzyme compared to NWG. Consistent with another reports, deamidated peptide inhibited the activity of Caco-2 and guinea pig liver tTG, which indicating the deamidation effectively reducing the binding capacity between peptides and tTG [45].

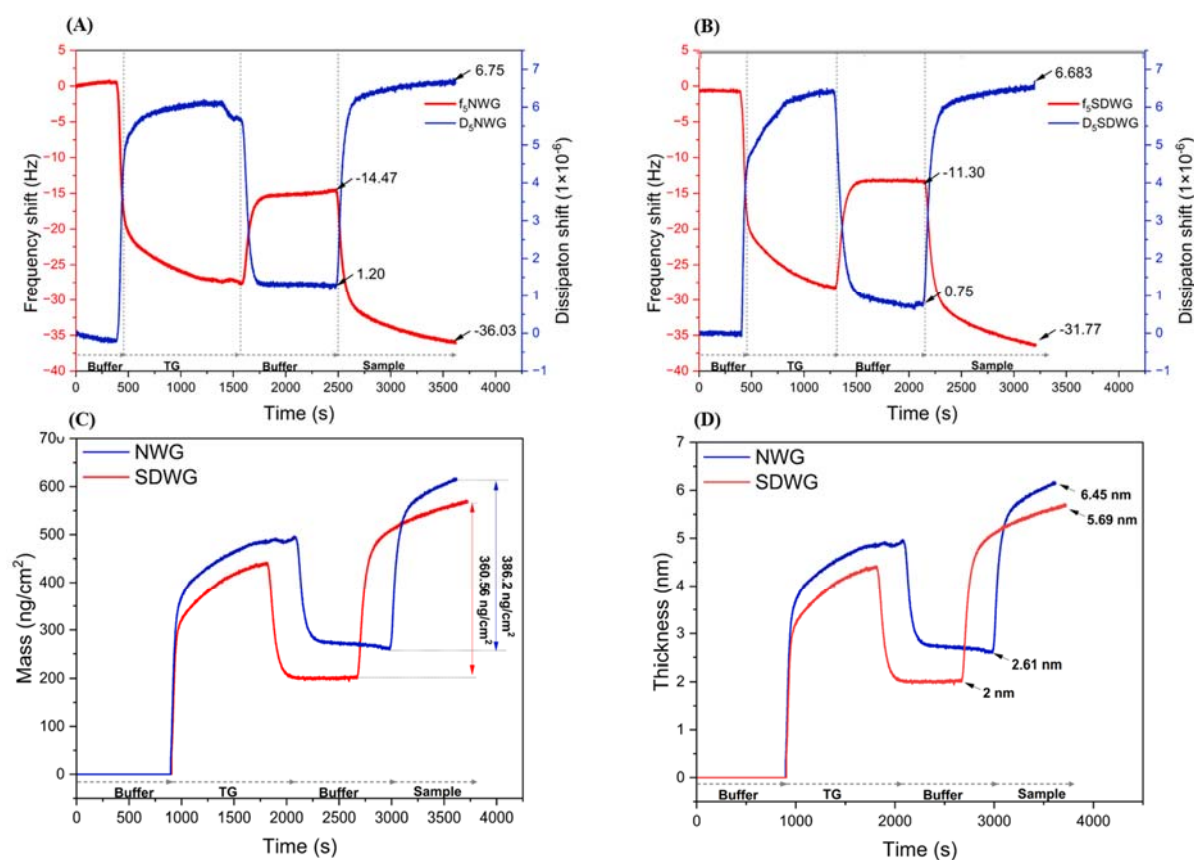


Figure 3 QCM-D determination of the interaction of wheat gluten digestion products with tTG enzyme. (A-B) Changes in vibronic frequency (Δf) and dissipation factor (ΔD) induced by the adsorption of NWG and SDWG digestion products in the tTG enzyme layer, where the pan frequency $n=5$; Voigt model fitting to assess the process of adsorption of gluten protein digestion products to the vibronic surface of the gold plating layer and the adsorption of gluten protein digestion products to the tTG enzyme induced changes in (C) the mass, (D) the thickness change.

3.6 Binding thermodynamics of tTG-peptides

ITC allows a full description of thermodynamic parameters arising from the binding interaction by monitors dynamic thermal characteristics of molecular interactions [46]. As shown in **Figure 4A**, both NWG and SDWG titration curves exhibited downward exothermic peaks, indicating an exothermic binding process. The exothermic peak intensity decayed exponentially during titration until reaching equilibrium, consistent with a single-site saturation binding model. This result suggested that tTG progressively occupied binding sites on digestive peptides, forming stable complexes [47]. Moreover, ΔH curves provided a visual distinction of the driving mechanisms of binding actions. The ΔH of NWG and SDWG exhibited similar trends with increasing molar concentration (**Figure 4B-C**). **Table 2** showed the further analyze of binding curves ITC analyze by the model NanoAnalyze software. The negative changes in Gibbs free energy indicate a spontaneous interaction. the ΔKD of SDWG ($(2.11 \pm 0.24) \times 10^{-6}$) was higher than of NWG ($(1.94 \pm 0.61) \times 10^{-6}$), indicating that NWG exhibited a higher binding affinity to tTG than SDWG. Negative ΔH values indicated the interaction can be associated to the presence of hydrogen bonding and van der Waals forces.

Both wheat gluten groups displayed entropy-driven characteristics ($-T\Delta S > \Delta H$), signifying dominant hydrophobic interaction-driven solvation entropy [48]. Notably, SDWG exhibited a lower negative ΔH and a less $-T\Delta S$ contribution, indicating that a typical enthalpy–entropy compensation effect. Consistent with other report, the stronger specific interactions enhance enthalpic stabilization while restricting conformational flexibility, thereby reducing entropy [49]. Together, these data indicated the super-deamidation weakens binding affinity of tTG-peptides.

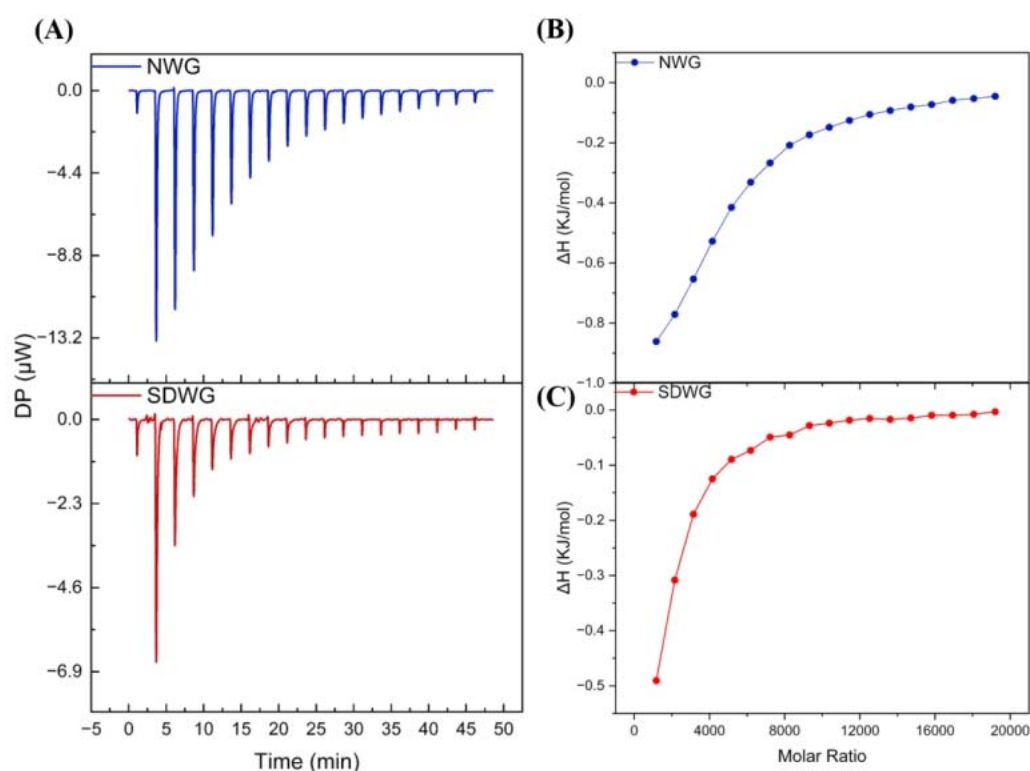


Figure 4 ITC determination of the interaction of gluten protein digestion products with tTG enzyme. (A) Binding isotherm plots of digestion products titrated into tTG enzyme solution NWG and SDWG; (B-C) enthalpy changes (ΔH) of digestion products titrated into tTG enzyme solution NWG and SDWG.

Table 2 Binding constants, Gibbs free energy, molar enthalpy and molar entropy of wheat gluten digestion products titrated with tTG enzyme

Group/Indicator	$\Delta K_D(M)$	$\Delta G(kJ/mol)$	$\Delta H(kJ/mol)$	$\Delta TS(kJ/mol)$
NWG	$2.11 \pm 0.24 \times 10^{-6}$	-32.63 ± 0.32	-8.04 ± 0.64	-24.59 ± 0.16
SDWG	$1.94 \pm 0.61 \times 10^{-6}$	-32.42 ± 0.13	-8.79 ± 0.34	-23.62 ± 0.29

3.7 Molecular docking

To predict the potential binding sites and estimate the relative interaction strengths between wheat gluten peptides and tTG enzyme, molecular docking analyses were performed on the nine peptide fragments (P1-P9) detected in Section 2.3. **Figure 5 A-I** suggested that all peptides exhibited interactions with tTG, predominantly mediated by hydrogen bonding and electrostatic forces. This finding was consistent with the ITC results, which showed that polar interactions contribute substantially to binding stabilization. The peptides were bound predominantly to an acidic residue cluster within tTG, involving critical sites such as

Glu-535, Glu-502, Glu-501, Asp-409, Glu-618, and Glu-646. Among these, Glu-535 and Glu-502 appeared to form stable interactions with the peptides, characterized by an average interaction distance of about 2.8 Å. Both residues carried strong negative charges, enabling electrostatic complementarity with positively charged lysine and arginine residues present in the peptides. This result was consistent with the findings of Vader et al. ^[50], who reported that celiac disease-associated HLA-DQ alleles preferentially recognize negatively charged anchor residues at defined positions within the nine-residue binding core. The P2–P3, P5–P6, P7, and P9 peptides showed the most stable binding patterns, suggesting that these peptides are major contributors to tTG–peptide interactions. Of note, compared with NWG, these key peptides revealed markedly lower abundance in SDWG. This depletion of high contributing peptides, which may account for the weakened tTG–peptide interactions observed for SDWG.

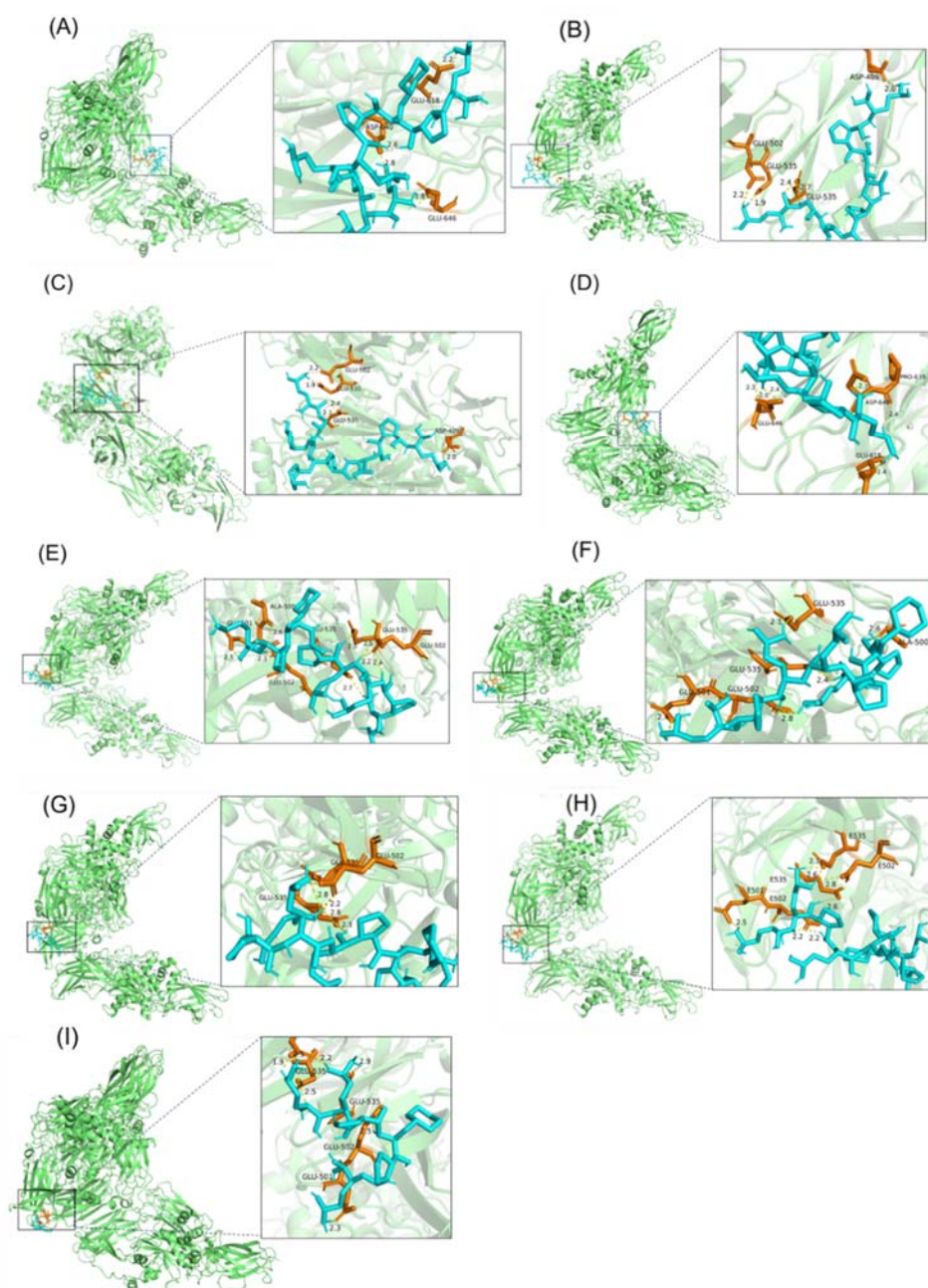


Figure 5 Molecular docking simulation between wheat gluten peptides and tTG enzyme. (A) EQPQQPFPPQ; (B) GYYPTSPQ; (C) QGYPTSPQ; (D) QPEQPFP; (E) QPEQPFPQ; (F) QPFPEQPQ; (G) QPQQPFPPQ; (H) QQPEQPFPQ; (I) QQPFPQ.

In addition, the binding free energy calculations were presented in **Table 3**. With the exception of peptide P2, all peptides exhibited binding energies lower than -5 kJ/mol, indicating strong binding affinity with the tTG enzyme [50]. Some high-affinity peptides, including P5 (QPEQPFQ), P6 (QPFPEQPQ), and P7 (QPQQPFQ), exhibited binding energies of -7.05, -7.52, and -7.36 kJ/mol, respectively. These peptides have a common structural feature, namely the presence of glutamine–proline repeats at both termini. This is attributed to the tTG enzyme specifically recognizes proline residues at the C-terminal position of sensitizing peptides and catalyzes the deamidation of adjacent glutamine residues, thereby stabilizing peptide-enzyme complexes and enhancing binding affinity [51]. Importantly, the relative abundance of P7 in SDWG was reduced to 52% of that observed in the NWG. The decreased of this key high-affinity peptide may account for the attenuated cross-linking activity observed between SDWG peptides and the tTG enzyme.

Table 3 Binding energy of different wheat gluten sensitizing epitopes with tTG enzymes

	Peptide sequence	Binding site	Binding energy(kJ/mol)
P1	EQPQQPFQ	GLU-618 ASP-640 GLU-646	-5.77
P2	GYYPSTPQ	GLU-502 ASP-409 GLU-535	-4.06
P3	QGYYPSTPQ	GLU-502 GLU-535 ASP-409	-6.02
P4	QPEQPFQ	GLU-646 ASP-640 PRO-639 GLU-618	-5.95
P5	QPEQPFQ	ALA-500 GLU-501 GLU-535 GLU-502	-7.05
P6	QPFPEQPQ	GLU-535 ALA-500 GLU-502 GLU-501	-7.52
P7	QPQQPFQ	GLU-535 GLU-502	-7.36
P8	QQPEQPFQ	E535 E502 E501	-5.03
P9	QQPFQ	GLU-535 GLU-502 GLU 501	-6.97

3.8 IgE-binding capacity of tTG-peptides

To investigate the effect of tTG-SDWG digested complexes on CD, we further performed the indirect ELISA and WB to analyze IgE-binding capacity of tTG-SDWG digested complexes. In ELISA IgE from pooled patient sera ($n = 5$) showed clear binding to the wheat gluten digested products, as well as lower IgE values to the SDWG (**Figure 6A**). In immunoblotting showed clear IgE binding to the peptides of wheat gluten and their complex with tTG (**Figure 6B**). Subsequent tTG treatment of the whole peptic digest formed large peptide aggregates. These cross-linked aggregates of wheat gluten digestion with tTG showed more intense IgE binding in immunoblotting than did the NWG digestion. The SDWG digestion into smaller peptides that were cross-linked by tTG to a lesser extent than the peptic digested and showed weaker IgE binding in immunoblotting. These results indicate that the super-deamidation treatment reduced the IgE binding to wheat gluten before and after tTG enzyme treatment.

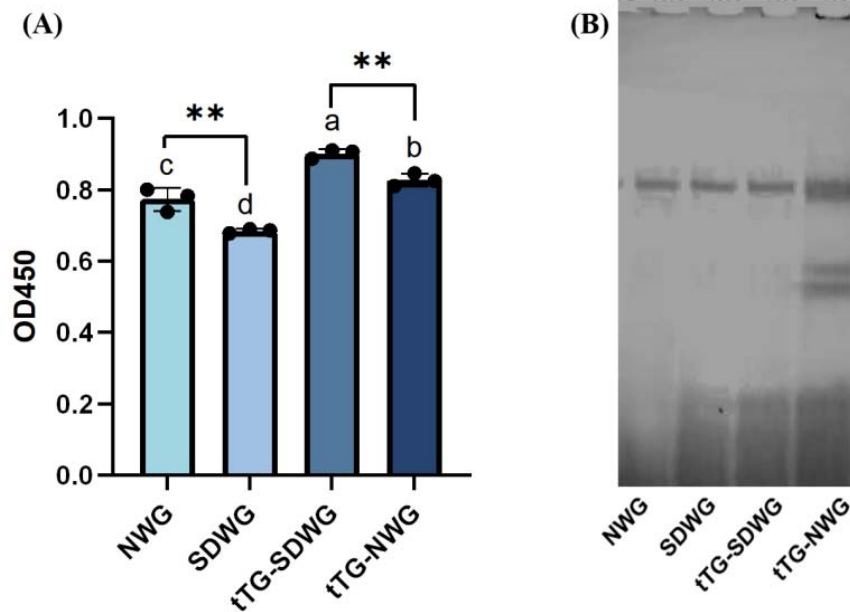


Figure 6 Immunological analysis of cross-linked complexes between tTG enzyme and digestive products. (A) IgE binding capacity of tTG enzyme cross-linked complexes with gluten digestion products; (B) Immunoblotting of wheat gluten digested peptides and crosslinking products with tTG. Different letters (a–d) are significantly different ($P < 0.05$). Compared with the NWG, $**P < 0.01$; compared with the tTG-NWG, $**P < 0.01$.

3.9 Potential underlying mechanism of SDWG in reducing the triggering and development of CD responses

As shown in **Figure 7**, super-deamidated removes Q residues in wheat gluten, converting them to E. This increases the negative charge density of proteins, disrupts intramolecular hydrogen bonding networks, and exposes more enzymatic cleavage sites. Consequently, peptide sequences resistant to digestion are further degraded by enzymatic hydrolysis. Simultaneously, the original repetitive peptide sequences are disrupted, leading to loss of glutamine residues that serve as tTG modification sites. This prevents tTG-mediated cross-linking and reduces the initiation of celiac disease immune responses. Furthermore, the increased negative surface charge from super-deamidation enhances electrostatic repulsion between particles and increases steric hindrance. This reduces the interaction energy barrier between tTG catalytic centers and potential modification sites, thereby diminishing damage to intestinal epithelial cells and cellular oxidative stress. It also mitigates disruption of junctional proteins, protects cellular barriers, and obstructs the development of wheat protein-mediated celiac disease-toxic responses. Given that the super-deamidated induced irreversible hydrolysis of glutamine residues into glutamic acid, resulting in permanent immunogenic epitope modifications in wheat gluten. The SDWG are expected to remain low-immunogenic in actual food processing.

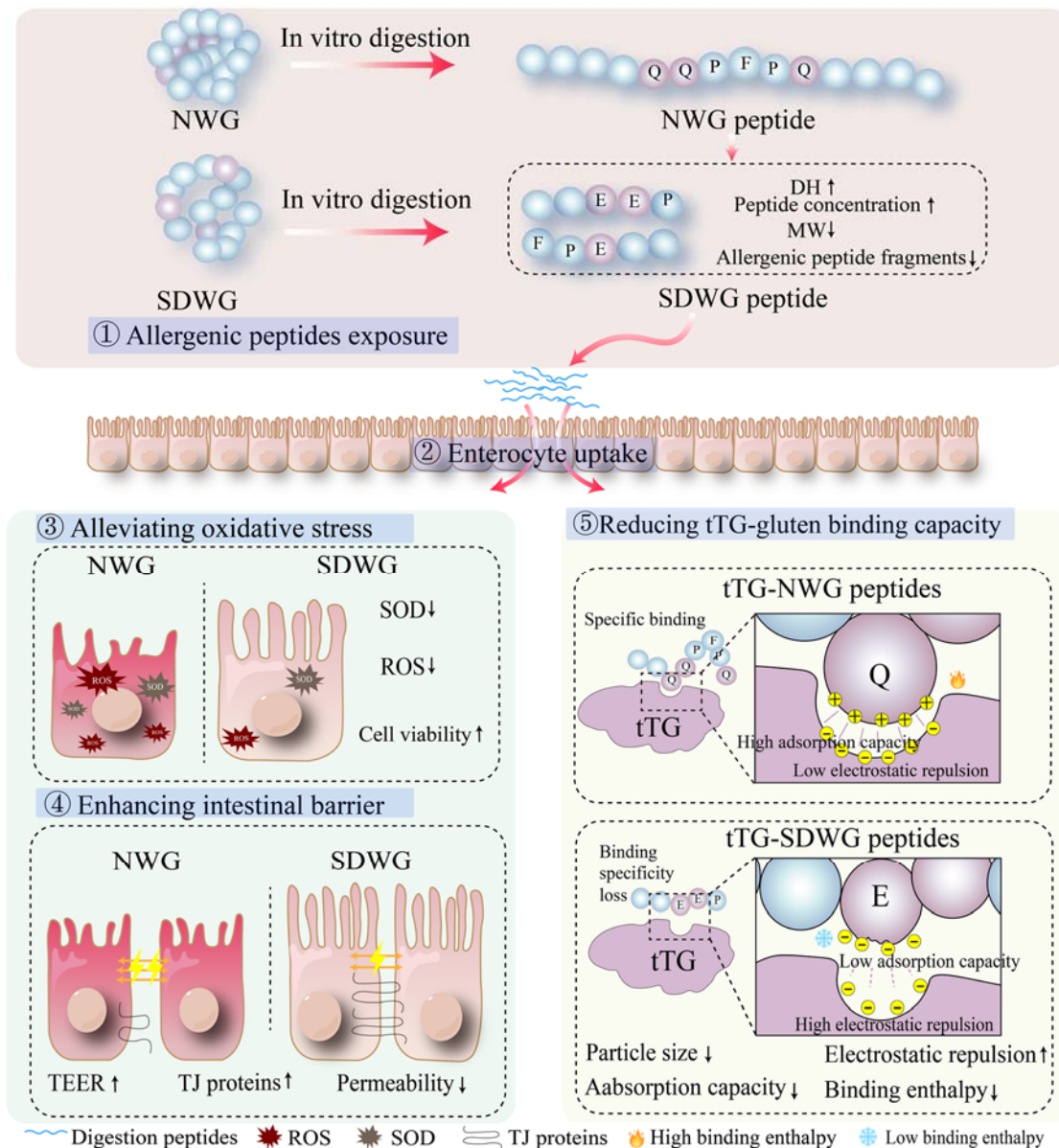


Figure 7 Proposed mechanism diagram of reduced wheat protein immunogenicity by super-deamidated

4. Conclusions

This study shows that, the super-deamidation significantly reduces the CD-related immunogenic potential of wheat gluten through a dual mechanism, which involving disruption of immunogenic epitope structures and attenuation of tTG-peptide interactions. The extensive conversion of glutamine residues destabilizes immunogenic sequences and enhances proteolytic susceptibility, thereby reducing the availability of intact pathogenic epitopes during gastrointestinal digestion. The binding analyses of tTG-peptides revealed that super-deamidation reduces the strong binding between gluten peptides and tTG. These findings highlight the potential of super-deamidated for developing low-immunogenic gluten-based food ingredients. Additionally, the irreversible epitope modifications induced by super-deamidation allow SDWG to preserve low-immunogenic epitope structures under practical food-processing conditions, including heating, mixing, drying, and shearing. This processing stability supports its potential application in cereal-based food systems. However, the immunological effects of super-deamidated gluten require further

validation in animal models and clinical systems. Future studies should focus on evaluating in vivo immune responses, investigating the stability and functionality of super-deamidated gluten during food processing.

Declaration of competing interest

The authors declare that no conflicts of interest are involved in this paper.

Acknowledgments

The authors are grateful to National Key Research and Development Program of China (2023YFD2101005-2), the Talent Development Strategy Special Fund of Guangdong Province (YF2025NYRC01), the National Key Research and Development Program of China (2024YFF1106000), the Guangdong Basic and Applied Basic Research Foundation (2023B1515040014).

Appendix A. Supplementary data

Supplementary data to this article can be found online.

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