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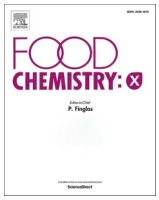

Enhancing zein functionality through sequential limited Alcalase hydrolysis and transglutaminase treatment: Structural changes and functional properties

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ABSTRACT

This study investigated the effects of sequential enzymatic hydrolysis using Alcalase, followed by transglutaminase conjugation on the secondary and tertiary structures, hydrophobicity, free amine content, protein-protein interactions, and functional properties of zein. Fourier-transform infrared spectroscopy showed that the most significant secondary structural changes, characterized by a decrease in α-helix content and an increase in β-turns, occurred at a higher degree of hydrolysis. At a 2 % degree of hydrolysis, it revealed notable emulsifying activity (65.96 m^2/g), while at 5 % hydrolysis, it achieved the highest solubility (75.06 %). Additionally, the zein hydrolysate with a 7 % hydrolysis degree, treated with transglutaminase, demonstrated improved H_0 values (2992.33), enhanced foam capacity (65.95 %), and increased solubilized protein content in a dithiothreitol extractant (31.35 %). Meanwhile, native zein treated with transglutaminase showed the highest water holding capacity (4.47 g/g). Overall, the combined enzymatic approach modified zein structure and properties, suggesting potential for improving functionality in plant-based food applications.

1. Introduction

The expanding global population and evolving dietary trends are placing greater demand on conventional sources of protein, particularly animal-based proteins. This increasing global demand for protein has prompted a critical need for sustainable and innovative alternatives (Glusac et al., 2018). Plant proteins offer a promising solution but often require modification to enhance their functional properties (Mattice & Marangoni, 2021).

Zein is a heterogeneous polypeptide byproduct of corn starch production, having a molecular weight that ranges from 21 to 25 kD and is soluble in alcohol. This protein shows promise in structuring plant-based food products due to its ability to self-assemble into aqueous viscoelastic dispersions, similar to gluten (Luo & Wang, 2016; Mattice & Marangoni, 2021). However, unlike wheat gluten, zein-based networks in water are notably brittle and exhibit low yield strength. Furthermore, the low amount of polar-charged amino acids in zein causes its insolubility in water, which limits its functional properties due to its tendency to aggregate (Cabra et al., 2007). This finding implies that to overcome this problem and create plant-based products, some form of functionalization will be necessary (Mattice & Marangoni, 2020). Therefore, various techniques are used to improve proteins' physical and functional characteristics, including physical processes, chemical modifications, or enzyme treatments (Glusac & Fishman, 2021).

In recent years, protein-modifying enzymes have been introduced to facilitate the functionalization of proteins, which has led to their application in various fields. The mechanisms of action of these enzymes include crosslinking, deamidation, glycosylation, and hydrolysis (Glusac & Fishman, 2021).

Due to the ability to improve texture, stability, and other functional properties without negatively affecting nutritional or sensory quality, food-grade transglutaminase (TGase) has become widely used (Sulaiman et al., 2022). It is effectiveness in forming crosslinks or inducing deamidation in protein structure relies on the accessibility of glutamine and lysine residues (Glusac & Fishman, 2021). Zein contains substantial amounts of glutamine and hydrophobic residues, which facilitate the creation of compact structures that can be conjugated using TGase (Glusac & Fishman, 2021). Alcalase, has been widely used to produce protein hydrolysates with better nutritional or functional properties than intact protein (Yust Del et al., 2010). It can hydrolyze

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zein into numerous amphipathic polypeptide fragments, generating of micelles of zein hydrolysate (ZH) in a water medium (Glusac & Fishman, 2021). However, excessive hydrolysis may result in the formation of bitter-tasting peptides and diminished protein functionality (Zhang et al., 2021). Thus, limited hydrolysis of zein by Alcalase can increase the exposure of glutamine sites, facilitating conjugation by TGase (He et al., 2021; Zhang et al., 2021). Sequential enzymatic hydrolysis and TGase treatments were found to be effective in improving the functional characteristics, especially foaming capacity and emulsifying properties of protein hydrolysates, including wheat gluten (Agyare et al., 2009), peanut (Meng et al., 2020) soybean protein (Zhang et al., 2021) and zein (He et al., 2021). Another study by Noman et al. (2018) revealed that using the papain enzyme to produce protein hydrolysate led to strong emulsification properties, high solubility, and good water holding capacity and oil holding capacity.

A few studies have focused on enzymatic modification of zein to improve its functionality and structure. Hence, this study utilized Alcalase for zein limit hydrolysis and then conjugated the resulting hydrolysate using TGase. The secondary and tertiary structures of ZH were investigated using multiple Fourier-transform infrared spectroscopy (FTIR) and spectroscopic techniques. Furthermore, the impact of these modifications on zein's functional properties, including solubility, emulsification, foam properties, and water and oil holding capacities, were evaluated.

2. Materials and methods

2.1. Materials

Corn gluten meal (CGM) with ~55 % protein was supplied by Zar Ind. Co. (Alborz, Iran). Alcalase 2.4 L, a protease sourced from *Bacillus licheniformis* (E.C. 3.4.21.62, ≥2.4 U/g), was acquired from Novozyme® (Bagsvaerd, Denmark). ACTIVA® EB Transglutaminase (100 U/g) was purchased from Ajinomoto (Tokyo, Japan). Bovine serum albumin (BSA) (96 %), 2,4,6-trinitrobenzene sulfonic acid solution (TNBS) (95 %), sodium dodecyl sulfate (SDS) (≥99.0 %), Sodium carbonate (≥99.5 %), Copper (II) sulfate (≥99 %), potassium sodium tartrate tetrahydrate (99 %) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Ammonium salt of 1-anilino-8-naphtalene-sulphonic acid (ANS) (≥90 %) was obtained from Sigma-Aldrich (Vallensbaek Strand, Denmark). Analytical grade Urea, Thiourea, and Dithiothreitol (DTT) were obtained from VWR International (Radnor, PA, USA). Other chemicals were obtained from Merck (Darmstadt, Germany).

2.2. Preparation of zein from CGM

Zein was purified from CGM according to the method of Tang and Zhuang (2014). CGM was soaked for 2 h at 50 °C in a mixture of 0.1 mol/L NaOH and 95 % ethanol (45:55, *v*/v). The mixture was centrifuged (10,000 ×*g*, 10 min at 4 °C). Then, the supernatant was mixed with distilled water, and the pH was set to 6.3 with 1 mol/L HCl. A 2 % NaCl solution was added (1:5, v/v), and the mixture was incubated for 1 h, then centrifuged and freeze-dried using an Alpha 2–4 LD plus freeze dryer (Christ, Germany). The Kjeldahl method determined the crude protein content (92 %) using Automated Kjeldahl (Foss, Kjeltec™ 8200) (AACC International, 2000).

2.3. Modification of zein

2.3.1. Preparation of zein hydrolysates

Protein solutions of zein (~ 92 % protein) were prepared at 3 % *w*/w. Enzymatic hydrolysis was carried out using Alcalase at a temperature of 50 °C, pH of 9.0, and 2 % w/w of the enzyme to substrate ratio. Hydrolysis time was considered based on the degree of hydrolysis (DH) reaching 2, 5, and 7 %. Following the reaction, the mixtures were heated (95 °C, 10 min) to end the enzyme's action. Subsequently, the solutions were centrifuged (10,000 ×*g*, 10 min), and supernatants were separated. The ZH 2, 5, and 7 were then set to a pH of 7.0 with 1 mol/L HCl and freeze-dried (Wang et al., 2015).

2.3.2. Degree of hydrolysis

The pH-stat method developed by Adler-Nissen (1986) was employed to calculate the DH.

$$DH = \left(B \times N_b/\alpha \times M_p \times h_{tot}\right) \times 100 \quad (1)$$

In this equation, N_b refers to the normality of the base used, and B represents the volume (mL) of NaOH required to maintain a constant pH. Mp indicates the mass of zein protein. The quantity of α-NH_2 released during the proteolysis reaction is represented by α (1.01), h_{tot} denotes the total number of peptide bonds in the protein substrates, which is 9.2 mmol/g for CGM (Wang et al., 2015).

2.3.3. Post-hydrolysis polymerization

Native zein and ZH 2, 5, and 7 % (1 %, *w/v*) were dissolved in ultrapure water, and the mixtures were adjusted to pH 6 using 0.5 mol/L NaOH and 0.5 mol/L HCl. TGase was dispersed in solutions under the conditions of 4 h, an enzyme to substrate ratio of 100 U/g, and a temperature of 44 °C. After the reaction, the mixtures (ZH-Tg2, ZH-Tg5, and ZH-Tg7) were heated (95 °C, 5 min) to inactivate the enzyme, followed by freeze-drying (He et al., 2021).

2.4. Determination of modified zein structure

2.4.1. Fourier-transform infrared spectroscopy (FTIR)

The FTIR spectra for both native and modified zein were recorded with an AVATAR 370 (Thermo Nicolet Corporation, USA), using KBr pellets as detailed by Zhang et al. (2022). The spectroscopic data, obtained in the 4000–400 cm^{-1} range with a resolution of 4 cm^{-1} and 32 scans, were analyzed using OriginPro 2022 software (OriginLab Corporation, Northampton, MA, USA).

2.4.2. Intrinsic fluorescence spectra

Fluorescence spectra of samples were obtained using a fluorescence spectrophotometer (LS45, PerkinElmer, Waltham, MA, USA) following the method outlined by Zhang et al. (2022). The samples were dispersed in ethanol 70 % (0.2 mg/mL). The emission wavelength was determined between 300 and 400 nm, with an excitation wavelength set at 290 nm and an emission slit width of 5 nm.

2.4.3. Ultraviolet-visible (UV–visible) absorbance

UV–visible spectra of native and modified zein were obtained using a UV–visible spectrophotometer (Agilent Cary 60, USA), as described by Fathi et al. (2021). Solutions at 2 mg/mL were scanned from 200 to 400 nm at 2 nm intervals with a scanning speed of 60 nm/min.

2.5. Free amine group content

The content of free amine groups in the native and modified zein was measured as described by He et al. (2021). A 250 μL solution (5 mg/mL) was combined with 2 mL of phosphate buffer (0.2 mol/L, pH 8.2) and 1 mL of TNBS solution (0.01 % *w/v*), and then heated for 30 min at 50 °C. The reaction was stopped by adding 2 mL of sodium sulfite (0.1 mol/L), and the absorbance was measured at 420 nm. The content of free amine groups was calculated using eq. (2) and an L-leucine standard curve ranging from 0 to 20 mmol/L.

$$Y = 0.2392 \times + 0.4902 \quad (2)$$

where Y is the absorbance at 420 nm and X is the free amine group content (mmol/L).

2.6. Surface hydrophobicity (H_0)

According to Yu et al. (2019), the fluorescent probe ANS was employed to measure the H_0. The samples (0–4.0 mg/mL) and ANS solution were prepared in phosphate buffer (pH 7.0) and mixed in a 96-well microplate. Then, the fluorescence intensity was measured with a Cytation 3 multi-mode plate reader (BioTek Instruments, Inc., Winooski, Vermont, USA). H_0 was calculated using the slope of the curve plotting fluorescence intensity against protein concentration.

2.7. Protein-protein interactions

The method described by Dent et al. (2023) and Chen et al. (2021) was used to evaluate interactions between proteins, including both noncovalent and covalent interactions. A phosphate buffer solution (PBS) (100 mmol/L, pH 7.5) containing urea (8 mol/L) or thiourea (2 mol/L) was used to extract proteins involved in hydrogen bonding (HBs) and hydrophobic interactions. Additionally, PBS with 50 mmol/L DTT was used for extracting proteins with disulfide bonds, while a combination of urea and DTT in PBS was used to extract proteins that exhibit both disulfide bonds and hydrogen bonding. The samples (0.5 g) were added into the above extractant (20 mL), stirred at room temperature (1 h), and centrifuged (13,750 ×*g*, 20 min, 4 °C). The supernatants were collected, passed through 0.2 μm cellulose acetate filters, and subsequently diluted 2 to 7 times with the same extractant. The Bradford method ((Bradford, 1976) was applied to evaluate the solubility of supernatants.

2.8. Functional properties

2.8.1. Protein solubility

Protein samples were prepared (5 mg/mL) and stirred for 30 min. The pH was then adjusted to 4.0, 7.0, and 10.0 with 1 mol/L HCl or NaOH. Subsequently, the sample solutions were centrifuged (6000 ×*g*, 15 min) at 20 °C (Zhang et al., 2022). The protein content was determined using Lowry's method (Lowry, Rosebrough, Farr, & Randall, 1951; Zheng et al., 2015), with BSA as the standard. Briefly, the analytical reagent was prepared by mixing 50 mL of reagent A (2 % Na_2CO_3 in 0.10 mol/L NaOH) with 1 mL of reagent B (1.56 % $CuSO_4$ in 2.37 % potassium sodium tartrate tetrahydrate). Then, 200 μL of supernatant was added to 2 mL of the analytical reagent. Subsequently, the Folin-Ciocâlteu phenol reagent was added to the solution, and the absorbance was then measured at 750 nm after 30 min. The protein solubility was computed using the following formula.

$$\text{Solubility (\%)} = \text{protein content in the supernatant/total protein content} \times 100 \quad (3)$$

2.8.2. Emulsifying properties

A turbidimetric method, as explained by Zhang et al. (2022), was employed to measure the emulsifying properties of the samples. A high-speed homogenizer (IKA-RCR-B) was used to mix sample solutions (2 mg/mL) with corn oil for 1 min at 10,000 rpm after the pH was adjusted to 7.0. A mixture of 50 μL of emulsion (from the bottom of the container) and 5 mL of SDS solution (1 mg/mL) was made, and the absorbance at 500 nm was measured immediately and after 10 min. Eqs. (4) and (5) were used to measure the emulsifying activity index (EAI) and emulsion stability index (ESI).

$$\text{EAI}\left(\text{m}^2/\text{g}\right) = 2 \times 2.303/\text{C} \times (1-\phi) \times 10^4 \times \text{A}_0 \times \text{D} \quad (4)$$

$$\text{ESI (\%)} = \text{A}_{10}/\text{A}_0 \times 100 \quad (5)$$

where A_{10} and A_0 denote the absorbance values at 500 nm after 10 min and 0 min, respectively; D represents the dilution factor applied to the sample; C is the initial concentration of the protein; ϕ is the volume fraction of the dispersed oil phase.

2.8.3. Foaming properties

The method outlined by He et al. (2021) was used to evaluate foaming capacity (FC) and foam stability (FS). A protein solution (1 %, *w/v*) in a 20 mL sample was homogenized at 16,000 rpm for 2 min. The volume of the whipped sample was measured at 0 and 30 min. To calculate FC and FS, the following formulas were used:

$$\text{FC (\%)} = (V_0 \blacksquare \text{V})/\text{V} \times 100 \quad (6)$$

$$\text{FS (\%)} = \text{V}_{30}/\text{V}_0 \times 100 \quad (7)$$

where V, V_0, and V_{30} represent the volume before whipping (mL), volume after whipping (mL), and volume following standing (mL), respectively.

2.8.4. Water holding capacity and oil holding capacity

The method described by Noman et al. (2018) was used to calculate the water holding capacity (WHC) and oil holding capacity (OHC) of native and modified zein. A 0.5 g of the sample was dissolved in 10 mL of deionized water to determine the WAC. The mixtures were centrifuged (2000 ×*g*, 10 min) at 25 °C after being vortexed for 2 min. Then, the excess water was poured off and the residue was weighed. The OAC was determined by suspending a 0.5 g sample in 10 mL of corn oil. The dispersions were then vortexed for 2 min and centrifuged. Then, the excess oil was poured off, and the precipitate was weighed. Eq. (8) was used to compute the WHC and OHC.

$$\text{WHC/OHC (g/g)} = (\text{weight of residue} - \text{weight of dry sample})/\text{weight of dry sample} \quad (8)$$

2.9. Statistical analysis

All experiments were carried out in triplicate, and the mean ± SD results are shown. A one-way ANOVA was employed to analyze the data using JMP 10 statistical software. Significant differences ($p < 0.05$) between the samples were observed using Tukey's test.

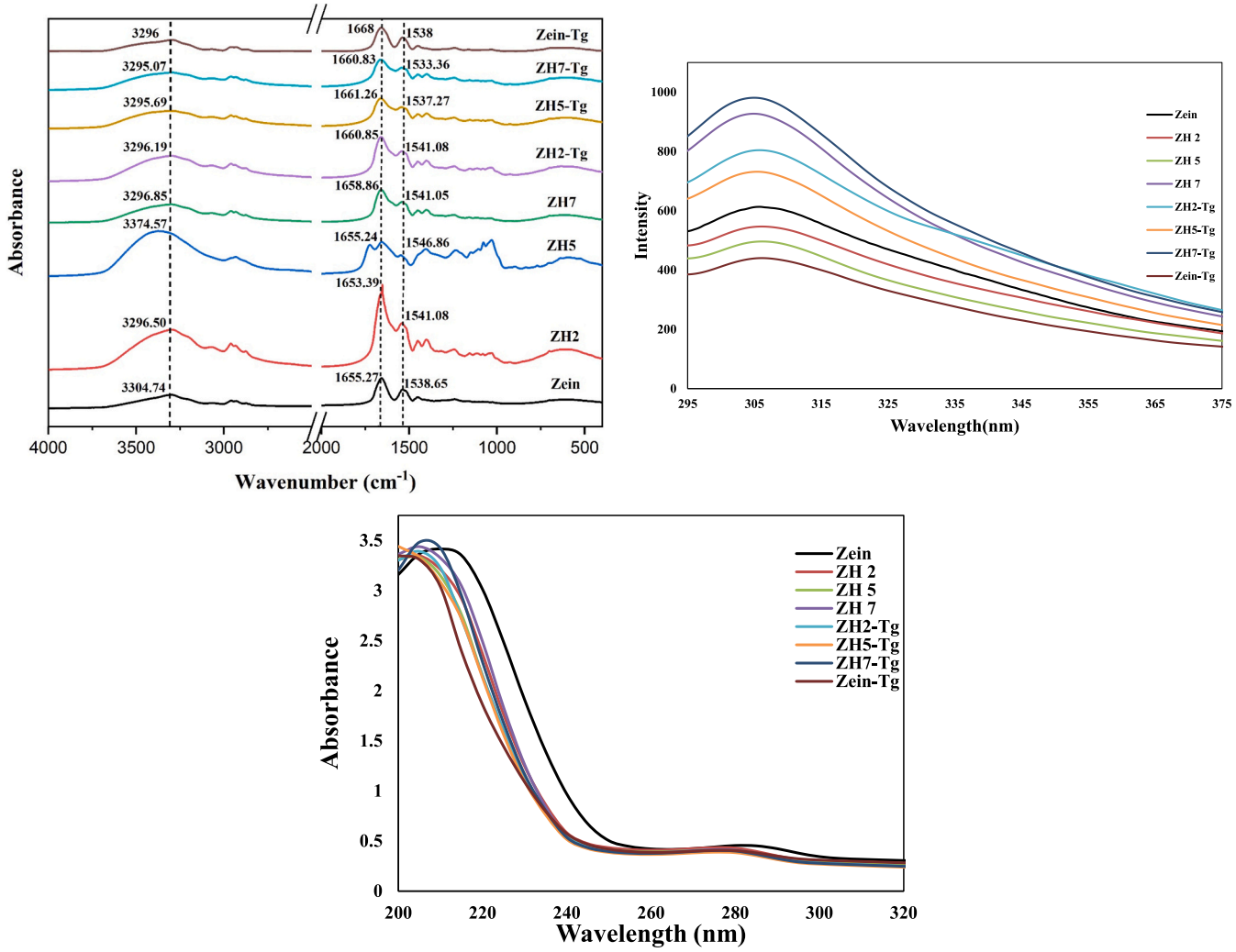

Fig. 1. FTIR spectra of native and modified zein (a), Fluorescence spectra of native and modified zein (b), UV–visible absorbance spectra of native and modified zein (c).

Table 1
Secondary structure in amide I region (1600–1700 cm^{-1}) by FTIR analysis.

Sample	Area (%)						
	β-Sheet			α-helix	Random Coils	β-Turns	α-helix/β- Sheet
	Intermolecular	Intramolecular					
	1620–1625 (cm^{-1})	1630–1640 (cm^{-1})	1670–1684 (cm^{-1})	1648–1658 (cm^{-1})	1640–1648 (cm^{-1})	1668–1670 (cm^{-1})	
Zein	3.94 ± 0.06^{c}	10.75 ± 0.25^{bc}	17.13 ± 0.12^{b}	19.32 ± 0.12^{b}	14.06 ± 0.06^{b}	2.90 ± 0.10^{abc}	0.6 ± 0.02^{bc}
ZH2	3.80 ± 0.2^{c}	9.71 ± 0.44^{c}	16.80 ± 0.20^{b}	20.89 ± 0.10^{a}	13.57 ± 0.07^{bc}	3.06 ± 0.06^{ab}	0.68 ± 0.01^{a}
ZH5	4.97 ± 0.17^{a}	12.41 ± 0.40^{a}	13.12 ± 0.12^{c}	20.75 ± 0.25^{a}	15.34 ± 0.05^{a}	2.68 ± 0.07^{c}	0.67 ± 0.00^{a}
ZH7	4.31 ± 0.1^{b}	10.53 ± 0.50^{bc}	16.74 ± 0.25^{b}	18.25 ± 0.05^{e}	13.41 ± 0.37^{c}	2.9 ± 0.10^{abc}	0.65 ± 0.00^{ab}
ZH2-Tg	4.04 ± 0.05^{bc}	10.33 ± 0.35^{bc}	17.50 ± 0.50^{b}	18.66 ± 0.07^{d}	13.38 ± 0.35^{c}	3.04 ± 0.05^{ab}	0.59 ± 0.01^{cd}
ZH5-Tg	3.75 ± 0.05^{c}	10.26 ± 0.25^{bc}	18.48 ± 0.40^{a}	18.89 ± 0.10^{cd}	13.50 ± 0.25^{bc}	3.14 ± 0.05^{a}	0.57 ± 0.02^{cd}
ZH7-Tg	3.77 ± 0.06^{c}	11.02 ± 0.56^{bc}	17.45 ± 0.79^{a}	18.10 ± 0.82^{bc}	13.76 ± 0.23^{bc}	3.16 ± 0.05^{a}	0.54 ± 0.03^{d}
Zein-Tg	4.68 ± 0.07^{a}	10.46 ± 0.50^{b}	18.57 ± 0.50^{b}	18.89 ± 0.32^{f}	13.40 ± 0.15^{c}	2.79 ± 0.20^{bc}	0.59 ± 0.01^{cd}

* Different letters in each row indicate significantly different mean values ($p < 0.05$).

3. Result and discussion

3.1. FTIR secondary structure of protein

FTIR analysis was performed to evaluate modified zein's conformational and structural changes (Fig. 1a). The FTIR spectrum of zein revealed peaks associated with hydroxyl groups from different origins amide A (3600–3100 cm^{-1}), amide I (1700–1600 cm^{-1}) and amide II (1500–1400 cm^{-1}) bands (He et al., 2021). Specifically, the amide I band reveals the presence of α-helices, β-sheets (both intramolecular and intermolecular), β-turns, and random coils among the secondary structures. The presence of hydroxyl groups is shown by the broad peak around 3650–3200 cm^{-1} (Mattice & Marangoni, 2021; Cao et al., 2023). As shown in Fig. 1a, the vibration of the -OH in ZH2 (3296.50 cm^{-1}),

ZH7 (3296.85 cm^{-1}), Zein-Tg (3296 cm^{-1}), ZH2-Tg (3296.19 cm^{-1}), ZH5-Tg (3295.69 cm^{-1}) and ZH7-Tg (3295.07 cm^{-1}) moved to lower wavenumbers compared to zein (3304.74 cm^{-1}), which may be due to weakening HBs. ZH5 (3374.57 cm^{-1}) also exhibited the red-shifted phenomena, indicating the formation of additional HBs (Cao et al., 2023).

Peak fitting was also performed on the FTIR spectra (Table 1) to determine the relative quantity of each secondary structure (Mattice & Marangoni, 2021). ZH7-Tg displayed the lowest content of α-helices (18.10 %) and the highest β-turns (3.16 %) among all the samples. In terms of the β-sheet content, Zein-Tg exhibited the strongest intramolecular interactions (29.03 %), while ZH5 displayed the highest level of intermolecular interactions (4.97 %). Notably, all treatments except ZH5 resulted in a decrease in random coil content. These changes in the FTIR spectra confirmed that the sequential processes of enzymatic hydrolysis and TGase reaction reorganized the ZH by inducing conformational structural modifications. The α-helix to β-sheet ratio reveals molecular flexibility and lower ratios are associated with greater flexibility (Cao et al., 2023). The limited hydrolysis increased molecular flexibility, while the TGase reaction decreased it, with ZH2 (0.68) and ZH7-Tg (0.54) showed the highest and lowest molecular flexibility, respectively. The polypeptide chains unfold, exposing HBs and hydrophobic regions. The subsequent interaction between these exposed areas leads to the formation of irregular protein structures, increasing overall protein disorder. Disrupting HBs within α-helix regions alters FTIR absorption intensity, indicating reduced α-helix content and unfolded secondary structures (Zhang et al., 2022). Xu et al. (2020) found that the hydrolysis process alters the conformation of the protein, rising β-turn structures, a decrease in ordered structures (β-sheet and α-helix), and the formation of new random coils. Jiang et al. (2019) found that cross-linking whey protein isolates using TGase reduced α-helix content while increasing β-sheet content. In contrast, Mattice and Marangoni's (2021) study revealed that TGase significantly enhanced β-sheet and random coil content in zein, while decreasing α-helical structures due to non-covalent bonding. Zhang et al. (2021) study revealed that TGase treatment increased bounded N—H groups in treated samples, confirming conformational structural changes in soybean protein hydrolysates polypeptides through covalent bonding.

3.2. Fluorescence absorption spectra

Fluorescence spectroscopy analyzes the fluorescence emitted by tryptophan, tyrosine, and phenylalanine, with tryptophan and tyrosine residues being more sensitive to surrounding changes (Zhang et al., 2022). Fluorescence emission spectra in Fig. 1b illustrated the conformational changes in zein and zein treated by sequential enzymatic hydrolysis and TGase reaction. Tyrosine residues in zein, with an emission maximum wavelength (λ_{max}) of 304 nm, provide information about the protein's folding state in spectra between 295 and 375 nm (Dai et al., 2016). Fluorescence intensity results showed different intensity and λmax for modified zein compared to native zein (305.5 nm, 613.103). The wavelength of fluorescence spectra for the samples ranged from 305 to 308.5 nm, indicating a change in zein conformation due to enzyme modification. The maximum emission peak for ZH7-Tg was observed at 306.5 nm, while Zein-Tg exhibited the lowest at 306 nm. Hydrolysis of zein resulted in a decreased intrinsic fluorescence intensity, possibly due to the unfolded protein structure. Hydrolysates with varying DH may comprise peptides of different sizes, side-chain groups, and amounts of hydrophobicity. These peptides can form polymers with various tryptophan and tyrosine polar environments, suggesting that aromatic amino acid residues are involved in the polymerization process and resulting in different spatial structures (Zhang et al., 2021). Conversely, the TGase-treated ZH revealed increased fluorescence intensity. Exposure of more fluorescent amino acid residues to hydrophilic environments increases protein fluorescence intensity, followed by a red shift in emission wavelength, suggesting protein structure changes (Jiang et al., 2019). Zhang et al. (2022) reported that increases in fluorescence intensity were correlated with an increased fluorescence quantum yield, influenced by the substance's structure following sequential enzymatic hydrolysis and TGase crosslinking of whey protein isolate. Zhang et al. (2021) found significant changes in the fluorescence intensities of soybean protein hydrolysates following enzyme treatments.

3.3. UV–visible absorption spectra

UV–visible absorption spectra in the 200–320 nm range confirmed the change in conformation and aromatic amino acid residues in native and treated zein (Fig. 1c) (Xing et al., 2024). The maximum UV–visible absorption peak of native zein was approximately 210 nm, which results from the electronic transition of n-π* of the carbonyl group on the protein's peptide bond (Meng et al., 2022). Among all treatments, ZH7-Tg exhibited the highest UV–visible absorbance (3.48), while ZH5 showed the lowest absorbance (3.33). Changes in absorbance and the shifting of absorption peaks can be used to compare protein structural alterations. Slight blue shifts in λ_{max} at approximately 210 nm were also observed for all treatments. TGase-treated ZH showed enhanced UV absorption intensity at this wavelength, possibly due to protein unfolding and extended covalent crosslinking, causing more chromophoric groups to migrate to the protein surface (Shi et al., 2023).

In a similar conclusion, Xing et al. (2024) found that TGase cross-linking significantly increases UV absorption intensity in soy protein isolate due to conformational changes, exposing more chromophoric groups. Zhang et al. (2021) found that Alcalase hydrolysis, followed by TGase cross-linking, changed the structure of soybean proteins, causing λmax to red-shift and peak absorption intensities to rise in the UV–visible absorbance range.

3.4. Free amine group content

The effects of enzyme treatments on the free amino groups of protein/peptides are illustrated in Table 2. Native zein has a low free amine content (0.05 mmol/L), due to its intact peptide bonds and compact structure, which limit reagent access. (He et al., 2021). Results revealed that enzymatic hydrolysis significantly increased free amine content, ranging from 1.11 mmol/L to 1.85 mmol/L. Additionally, as DH increased, the free amine content also increased. ZH7 exhibited the maximum amount at 1.85 mmol/L, indicating that enzyme hydrolysis enhanced this content due to the partial depolymerization of zein. This has been reported enzymatic hydrolysis of proteins, such as proteins from pigeon pea (Ratnayani et al., 2023) and zein (He et al., 2021) led to an increase in free α-amino content. The amount of free amine content is correlated with the number of broken peptide bonds (Ratnayani et al., 2023). Nevertheless, the study reveals that compared to the 1st-step hydrolysis and the 2nd-step polymerization, TGase treatment significantly decreased free amine content, ranging from 0.91 mmol/L to 1.68 mmol/L. This indicates the effectiveness of TGase in modifying protein structures. TGase may have catalyzed a transfer reaction between two amino acids within proteins, resulting in crosslinks between the protein

Table 2
Free amine, and surface hydrophobicity of native and modified zein.

Sample	Free amine content (mmol/L)	Surface hydrophobicity
Zein	0.05 ± 0.01^{g}	2468.00 ± 10.00^{c}
ZH2	1.11 ± 0.01^{e}	1121 ± 7.21^{f}
ZH5	1.59 ± 0.05^{c}	786.01 ± 3.44^{h}
ZH7	1.85 ± 0.01^{a}	892 ± 11.26^{g}
ZH2-Tg	0.91 ± 0.00^{f}	1574.66 ± 88.63^{e}
ZH5-Tg	1.23 ± 0.01^{d}	2855.667 ± 22.54^{b}
ZH7-Tg	1.68 ± 0.05^{b}	2992.33 ± 12.42^{a}
Zein-Tg	0.06 ± 0.00^{g}	2328.333 ± 35.90^{d}

* Different letters in each row indicate significantly different mean values ($p < 0.05$).

molecules and lowering the free amine group (Ali et al., 2010). Zein-TG also had low free amine content (0.06 mmol/L), indicating that TGase treatment without prior hydrolysis did not affect the free amine group. Jiang et al. (2019) and Ali et al. (2010) found that the TGase reaction decreases the number of free amino groups in whey protein isolate and legume protein isolate.

3.5. Surface hydrophobicity (H_0)

The H_0 of proteins is employed to evaluate the functional property and the alterations resulting from conformational variations (Shi et al., 2023). As shown in Table 2, zein exhibited a high level of H_0 (2468), suggesting that its hydrophobic parts were more exposed and its structure was relatively loose (Yu et al., 2019). ZH7-Tg and ZH5 had the highest and lowest (2992.33 and 786.01 respectively) H_0 among all the treatments. As the DH increased, the H_0 of the hydrolysates decreased. This is probably attributed to the hydrophobic amino acid side chains becoming increasingly buried within the peptide structure or being lost during hydrolysis (Yu et al., 2019). As illustrated in Fig. 1b, the TGase reaction showed an increase in the fluorescence intensity of the ZH with an increasing DH, indicating an increase in H_0. The structural changes in TGase-treated ZH, such as the unfolding of peptide chains and the exposure of previously hidden hydrophobic regions, likely contributed to the increase in H_0 (Shi et al., 2023). Similar findings were reported by Yu et al. (2019), revealing that the hydrolysis of whey protein isolate resulted in losses of surface hydrophobicity while cross-linking partially salvaged these properties. Meng et al. (2020) reported that peanut protein/hydrolysates treated with TGase displayed significantly increased H_0, suggesting the unfolding of the protein/peptides. In contrast, Agyare et al. (2009) found that wheat gluten hydrophobicity decreased after limited hydrolysis with chymotrypsin and TGase treatment, possibly due to partially occluded hydrophobic residues in cross-linked molecules.

3.6. Interactions between proteins

The contribution of various protein interactions based on the solubility of native proteins and hydrolysates in solutions containing PBS, urea, thiourea, or DTT was measured (Fig. 2). These solutions can disrupt covalent and noncovalent bonds (Dent et al., 2023). The primary extracting solution used in this study PBs, which are known to extract proteins in or near their intact state (Zheng et al., 2015). Zein was solubilized to approximately 6.23 % in PBS. The addition of the above solutions enhanced protein solubility. In general, all samples exhibited enhanced solubility when dissolved in the urea solution, suggesting that HBs is the primary interaction responsible for the decreased solubility. Zein showed the highest solubility (68.02 %), while ZH7-Tg showed the lowest (25.64 %). HBs is a key factor in the aggregation of hydrolysates, as the hydrolysis process releases peptides, which in turn increases the total amount of N and C termini. These termini provide more potential partners for HBs (Dent et al., 2023). A similar study reported that the hydrolysis of soy and chickpea (Dent et al., 2023) and peanut protein (Zheng et al., 2015) increased their solubility during urea extraction due to HBs.

Approximately 21.85 % of zein protein was extracted by thiourea. The varying solubility of ZH underscores the influence of DH on protein structure and interactions. A higher DH reduces hydrophobic interactions, leading to a decrease in solubility in the presence of thiourea, from 33.73 % for ZH2 reaching 19.57 % for ZH7. Additionally, TGase treatment further decreased the amount of protein solubilized by thiourea as DH increased. In the case of hydrolysates, thiourea may even decrease solubility, suggesting that altering hydrophobic interactions can cause the protein structure mediated by the hydrophobic effect to become unstable (Dent et al., 2023). DTT disrupted disulfide bonds that caused covalent crosslinking. Sequential enzymatic hydrolysis and TGase reactions enhanced the solubility of the hydrolysates in DTT. ZH7-Tg exhibited a substantial increase in solubility in DTT, reaching 31.35 %, whereas zein's solubility remained below 12 %. This result suggests that the TGase-treated ZH may have introduced additional disulfide bonds. It appears that the combined effect of urea and DTT on protein solubility by disrupting both covalent and noncovalent bonds is markedly greater than that of either solvent alone, suggesting a synergistic interaction between these two solutions (Chen et al., 2021). Zein and ZH2 reveal the highest (84.39 %) and lowest (32.44 %) amount of solubility in these solvents. However, hydrolysis followed by cross-linking of zein protein was more affected by HBs interactions than disulfide bonds. However, the combined addition of urea may change the structure of proteins by influencing the non-covalent bonds, which leads to the access of hidden disulfide bonds to DTT (Zheng et al., 2015). Similar effects of DTT + Urea solution on solubility have been reported on soy and chickpea hydrolysates (Dent et al., 2023), Pea Protein Gel containing green tea polyphenols (Chen et al., 2021), and on peanut flour and peanut protein isolate hydrolysates (Zheng et al., 2015).

3.7. Functional properties of protein

3.7.1. Protein solubility

Solubility was determined as an essential measure of protein

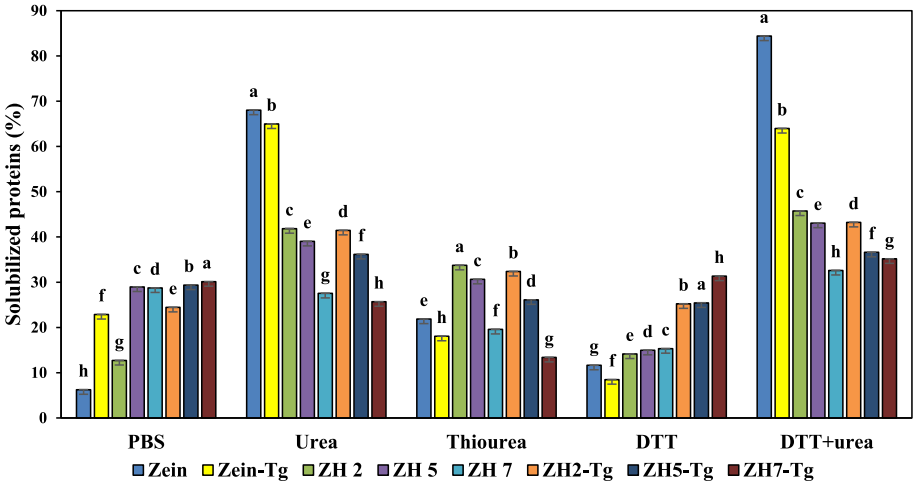

Fig. 2. Protein solubility of zein, Alcalase hydrolysate with different degrees of hydrolysis (ZH2, ZH5, ZH7), and transglutaminase treatments (ZH2-Tg, ZH5-Tg, ZH7-Tg) was measured in solvent systems of PBS, urea, thiourea, DTT, and DTT + urea to investigate protein intermolecular interactions leading to insoluble aggregate formation. Data are means ± SD of three replicates. Values with different letters are significantly different ($p < 0.05$).

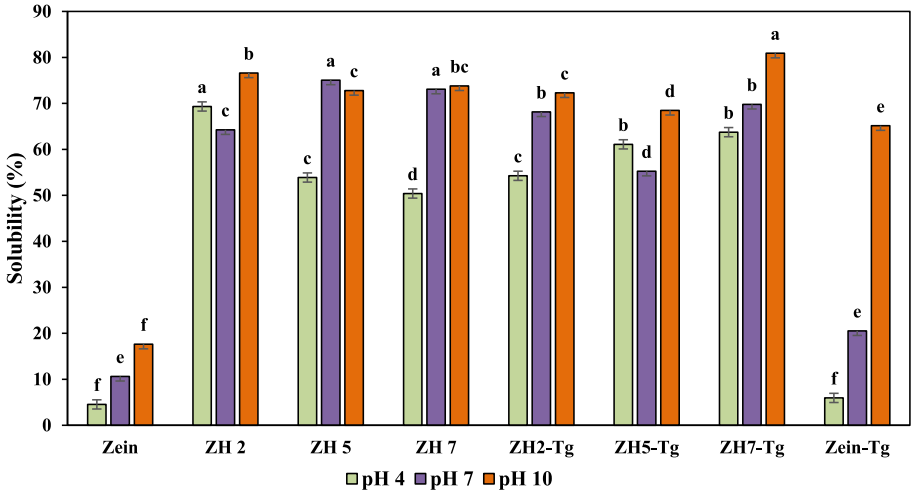

Fig. 3. Solubility of native and modified zein at pH 4.0, pH 7.0, and pH 10.0. Data are means ± SD of three replicates. Values with different letters are significantly different ($p < 0.05$).

functionality since many of the proteins' functional properties, including their ability to bind water, emulsification ability, and foaming capacity, are directly correlated with protein solubility (Dent et al., 2023). Protein Solubility profiles of native zein, ZH, and TGase-treated ZH at different pH values are shown in Fig. 3. Native zein's solubility is pH-dependent, with 10.67 % at pH 7.0, near the isoelectric point. The absence of a net charge at the isoelectric point diminishes repulsive forces, leading to insolubility, aggregation, and precipitation (Xu et al., 2020). ZH2, ZH5, and ZH7-Tg revealed the highest solubility at pH 4.0, 7.0, and 10.0 (69.32 %, 75.06 %, and 80.90 %, respectively). Comparing native zein, ZH, and ZH conjugated through Tgase, improved protein solubility was observed after enzyme treatment. Alcalase hydrolysis significantly improved protein solubility of ZH at all pH levels compared to unhydrolyzed zein, with increased DH further enhancing solubility at pH 7, decreasing at pH 4 and 10. Enzymatic hydrolysis can enhance plant protein solubility across pH ranges (Xu et al., 2020). This is especially evident for zein, where hydrolysis into smaller peptides using Alcalase significantly improves solubility (He et al., 2021). The same trend was observed by Xu et al. (2020), who found that enzymatic hydrolysis of chickpea protein enhances protein solubility by altering secondary protein structure. As a result, the number of disordered structures increases and the number of organized structures decreases. Conversely, subsequent TGase reactions, while improving solubility compared to native zein, decreased protein solubility, except for ZH-Tg7 at pH 10. This decline in solubility is attributed to the formation of conjugated peptides with fewer ionizable side chains, resulting in diminished protein-water interactions (Zhang et al., 2021; Zhang et al., 2022). Furthermore, protein-TGase binding can cause stretching and loosening of spatial protein structures (Li et al., 2023). It has been demonstrated that semantical enzyme hydrolysis TGase cross-linking enhances the solubility of zein (He et al., 2021) and soybean protein (Zhang et al., 2021).

3.7.2. *Emulsifying properties*

The emulsifying properties of the native zein, ZH, and TGase-treated ZH were determined and shown in Fig. 4a and Fig. 4b. Zein had an EAI of 5.57 m^2/g and ESI of 63.23 %, respectively. Enzymatic hydrolysis significantly improved the EAI and ESI of zein ($p < 0.05$). The EAI values ranged from 45.66 to 65.96 m^2/g, while the ESI values ranged from 123.73 to 154.46 %. Among the hydrolyzed samples, ZH2 had the highest ESI (154.46 %) and EAI (65.96 m^2/g). Limited hydrolysis (DH < 8 %) has been suggested to preserve or enhance protein functionalities (Zhang et al., 2021). The results show that higher DH leads to lower emulsifying properties, likely due to the smaller peptides produced having fewer interactions with both aqueous and non-aqueous phases. on the other hand, emulsifying properties decrease for hydrolysates, possibly because the partially unfolded protein molecules have greater molecular flexibility, which facilitates better rearrangement at the oil-water interface and inhibits coalescence (Xu et al., 2020). As expected, the zein protein's emulsifying qualities were improved by the TGase reaction following hydrolysis. The TGase reaction modified the distribution of hydrophobic and hydrophilic amino acid residues in the tertiary structures of cross-linked molecules, resulting in conjugated peptides with enhanced amphiphilicity to preserve at the interfaces (Zhang et al., 2021). Additionally, prior research has demonstrated that zein conjugated with chitosan oligosaccharide (He et al., 2021) and milk proteins (Yu et al., 2019) can both have their emulsifying qualities improved by TGase cross-linking through an enzymatic reaction. This results from the cross-linked proteins' arrangement of hydrophobic and hydrophilic amino acids and the creation of polypeptides with better interfacial characteristics. Conversely, Jiang et al. (2019) found that cross-linked caseins with extended protein structures and high molar mass molecules can impede protein adsorption and encourage aggregation, leading to decreased emulsifying characteristics.

3.7.3. *Foaming properties*

The foaming properties of zein, ZH and TGase-treated ZH at pH 7.0, were determined by measuring the FC and FS (Fig.4c and Fig. 4d). Zein exhibited the lowest FC (5.04 %) and FS (82.74 %) of all samples. This is attributable to zein's low solubility in water at pH 7.0 (He et al., 2021). ZH7-Tg exhibited the highest FC (65.95 %) and FS (92.42 %). The results suggested that hydrolysis of the protein using Alcalase could improve foaming properties. Lowering the molecular weight of hydrolysates increases their flexibility, allowing them to form a stable interfacial layer and diffuse more quickly to the surface, which enhances their foaming feature (Yust Del et al., 2010). In addition, the TGase-treated ZH at all DH have better FC than the protein hydrolysates. These results suggest that partial hydrolysis and subsequent polymerization could improve FC and FS. TGase treatment modifies the arrangement of hydrophobic and hydrophilic amino acids within cross-linked proteins, resulting in peptides with improved amphiphilic properties. These modified peptides are better suited for interacting at interfaces (Zhang et al., 2021). Several studies found that hydrolysis followed by TGase treatment can improve the foaming properties of zein protein (He et al., 2021), soy protein (Song et al., 2021; Zhang et al., 2021), whey protein isolate (Yu et al., 2019).

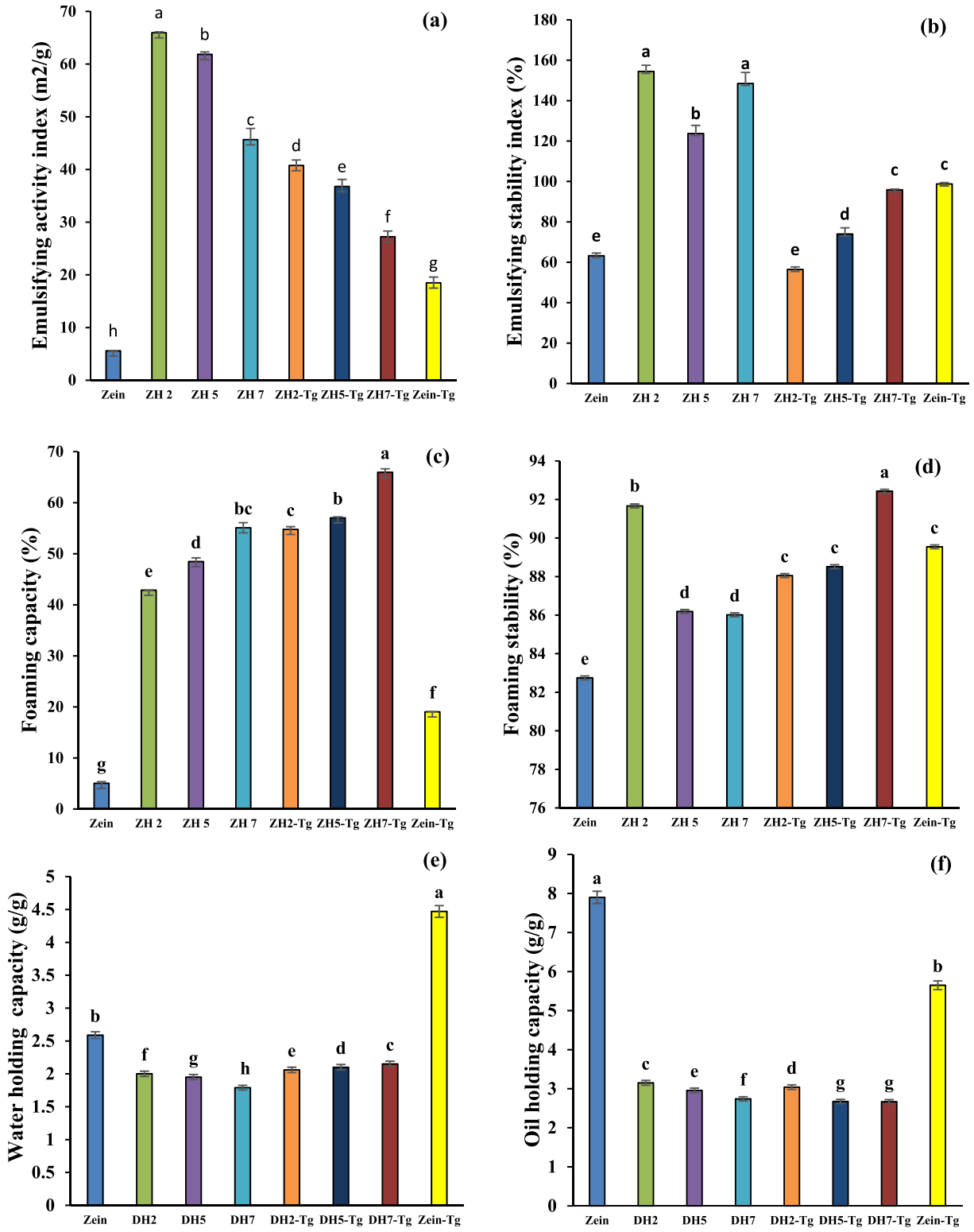

Fig. 4. Emulsifying activity (a) and stability (b), foaming capacity (c) and stability (d), water holding capacity (e), and oil holding capacity (f) of native and modified zein. Data are means ± SD of three replicates. Values with different letters are significantly different ($p < 0.05$).

3.7.4. Water holding capacity and oil holding capacity

The water- and oil-holding capacities of native zein, ZH, and TGase-treated ZH were also evaluated and shown in Fig. 4e and Fig. 4f. Native zein had a WAC of 2.58 g/g and an OAC of 7.9 g/g. The results indicated that the modification of zein impacted both WAC and OAC, probably because of the availability of hydrophilic polar side chains (Noman et al., 2018). The Zein-Tg and ZH7 treatment resulted in the highest and lowest WAC (4.47 g/g and 1.79 g/g, respectively), while zein and ZH7-Tg exhibited the highest and lowest OAC values (7.9 g/g and 2.66 g/g, respectively). The ZH showed decreased WHC and OHC ($p < 0.05$) with an increased DH. The increased concentration of polar groups, such as COOH and NH_2, resulting from hydrolysis may cause of the decrease in

water absorption. Moreover, the absorption of oil and water is reduced due to the lower molecular size (Noman et al., 2018). Zhang et al. (2021) reported that soybean protein hydrolysates demonstrated a decrease in WHC and an increase in WOC. This implies that the hydrolysis disrupted the protein network, exposing hydrophobic amino acid groups.

4. Conclusion

The enzymatic modifications by TGase altered the tertiary structure of zein, resulting in changes to its functional properties. Fluorescence and UV–visible spectroscopy confirmed these structural changes, with TGase treatment enhancing fluorescence intensity. FTIR analysis revealed changes in secondary structure, specifically an increase in β-sheet content and a decrease in α-helix content. Surface hydrophobicity rose with increasing DH and TGase treatment, resulting from the exposure of hydrophobic areas. Free amine group content increased with DH but decreased after the TGase reaction. Enzyme modification by Alcalase improved the protein solubility of zein, particularly through one step limit hydrolysis, which produced smaller peptides and disrupted protein interactions. Limited hydrolysis displayed the highest free amine content, emulsifying properties, and solubility, while TGase treatments exhibited the highest H_0 and foam properties, and water holding capacity. The results highlight the key interactions affecting protein solubility, particularly the roles of HBs and disulfide bonds. The findings suggest that enzymatic hydrolysis and subsequent TGase reactions may have modified zein's structure and functionality, introducing disulfide bonds and potentially creating plant-based products with potential applications in food formulation, especially for gluten-free foods.

CRediT authorship contribution statement

Adieh Anvar: Writing – original draft, Methodology, Investigation, Data curation. **Mohammad Hossein Azizi:** Writing – review & editing, Supervision. **Hassan Ahmadi Gavlighi:** Writing – review & editing, Supervision.

Declaration of competing interest

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

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Data availability

Data will be made available on request.

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