Current Research in Structural Biology 7 (2024) 100135

Contents lists available at ScienceDirect

Current Research in Structural Biology

journal homepage: www.journals.elsevier.com/current-research-in-structural-biology

Research Article

Molecular forces driving protein complexation of lentil and whey proteins: Structure-function relationships of trehalose-conjugated protein complexes on protein digestibility and solubility

Mohammad Alrosan [a,b,j,k,*], Ali Madi Almajwal [c], Ali Al-Qaisi [d], Sana Gammoh [e], Muhammad H. Alu'datt [e,f], Farah R. Al Qudsi [g], Thuan-Chew Tan [a], Ammar A. Razzak Mahmood [h], Sofyan Maghaydah [e,i]

[a] Food Technology Division, School of Industrial Technology, Universiti Sains Malaysia, 11800, USM, Penang, Malaysia
[b] Applied Science Research Center, Applied Science Private University, Al-Arab St. 21, Amman, 11931, Jordan
[c] Department of Community Health Sciences, College of Applied Medical Sciences, King Saud University, P.O. Box 10219, Riyadh, 11433, Saudi Arabia
[d] Department of Agricultural Biotechnology, Faculty of Agricultural Sciences and Technology, Palestine Technical University-Kadoorie (PTUK), Jaffa Street, Tulkarm P. O. Box 7, Palestine
[e] Department of Nutrition and Food Technology, Faculty of Agriculture, Jordan University of Science and Technology, P.O. Box 3030, Irbid, 22110, Jordan
[f] Department of Food Science & Nutrition, College of Life Sciences, Kuwait University, P.O. Box. 5969, Safat, 13060, Kuwait
[g] Department of Food Science, University of Guelph, Guelph, Ontario, N1G 2W1, Canada
[h] Department of Pharmaceutical Chemistry, College of Pharmacy-University of Baghdad, Baghdad, Bab-Al-Mouadam, 10001, Iraq
[i] Department of Human Nutrition and Dietetics, College of Health Sciences, Abu Dhabi University, Zayed City, Abu Dhabi, P.O. Box 59911, United Arab Emirates
[j] Department of Nutrition and Food Science, Faculty of Agriculture, Jerash University, Jerash, Jordan
[k] College of Health Science, QU Health, Qatar University, Doha P.O. Box 2713, Qatar

ARTICLE INFO

Handling Editor: Dr A Wlodawer

Keywords:
Whey proteins
Lentil proteins
Digestibility
Protein structure
Disaccharide

ABSTRACT

Plant-based proteins are often associated with a range of health benefits. Most research primarily investigates pea and soy proteins, while lentil proteins received minimal attention. This study evaluates the effect of protein complexation (using the pH-shifting technique) coupled with trehalose conjugation on lentil and whey proteins. The protein structures after the modification were analysed using spectroscopic methods: Fourier-transform infrared, ultraviolet spectra, and fluorescence spectra. The amide group I, conformation protein, and tertiary structure of the trehalose-conjugated lentil-whey protein complexes (T-LWPs) showed significant changes ($P < 0.05$). Moreover, the surface properties (surface hydrophobicity and charges) of T-LWPs were significantly modified ($P < 0.05$), from 457 to 324 a.u and from 36 to −40 mV, respectively. Due to these modifications on the protein structures, the protein digestibility (80–86%) and water solubility (90–94.5%) of T-LWPs increased significantly ($P < 0.05$) with the increase in the trehalose concentration, from 0 (control) to 5% (w/w), respectively. This study suggested that coupling protein complexation and trehalose conjugation can enhance the overall properties of lentil-based protein complexes. With this enhancement, more opportunities in the utilisation of lentils are to be expected.

1. Introduction

The structural and functional modifications of dairy proteins, specifically whey and casein proteins (Alrosan et al., 2024), and plant-based proteins, such as lentil proteins, have been investigated following various treatments (Alrosan et al., 2023a; Miranda et al., 2023). The protein content of lentils is between the range of 20–26% (Jarpa-Parra et al., 2014). The main fraction of globulin is around 80% of the total fraction of lentil proteins (Jarpa-Parra, 2018), resulting in lower solubility (~68%) and digestibility (~68%) (Alrosan et al., 2023a), contributing to the rigid structure of lentil proteins. The shape, molecular weight, surface properties, and secondary and tertiary structures of proteins can play a major role in the functional properties of proteins (Al-Qaisi et al., 2024; Miranda et al., 2023). Whey proteins are

* Corresponding author. Food Technology Division, School of Industrial Technology, Universiti Sains Malaysia, 11800, USM, Penang, Malaysia
E-mail addresses: mohammad.alrosan@hotmail.com, m.alrosan@qu.edu.qa (M. Alrosan).

https://doi.org/10.1016/j.crstbi.2024.100135
Received 13 November 2023; Received in revised form 20 February 2024; Accepted 22 February 2024
Available online 23 February 2024

frequently considered the most prevalent protein utilised in the food industry (i.e., protein bars, shakes, and other sport nutrition products), mainly due to their excellent solubility (~89%) and digestibility (~88%) (Alrosan et al., 2023b). Whey protein's desirable characteristics have made it a preferred selection for various dietary uses, including sports nutrition items and protein supplements. Moreover, whey proteins are abundant in crucial amino acids, rendering them a perfect protein source for enhancing muscle development and facilitating recovery.

The pH-shifting technique has been well investigated to prove the functional properties of protein-protein interaction to synthesise the soluble protein composite (Alrosan et al., 2023a; Ren et al., 2024). This technique involves altering the pH of the solution to induce conformational changes in the proteins, allowing them to interact and form a stable complex. By studying these interactions, researchers can gain insights into the structure and function of the protein composite, which can have various applications in fields such as biotechnology and medicine. A study by Alrosan et al. (2023b) that the method of complexation between casein and lentil protein using the pH-shifting technique resulted in a significant increase in water solubility, from 68 to 91%. This improvement can be attributed to alterations in the surface characteristics of the protein.

Plant-based proteins can form interactions with additional molecules, such as polysaccharides (Bhat et al., 2023; Han et al., 2023; Jiang et al., 2022) and other proteins (Alrosan et al., 2023a; Wang et al., 2019). Non-covalent bonds, including hydrophobic and electrostatic interactions, and hydrogen bonds, can drive polysaccharides-protein interactions. These molecular forces have an essential effect on the alterations of protein structure, which may alter the water solubility and digestibility of protein (Alrosan et al., 2023a; Liu et al., 2023; Al-Qaisi et al., 2024). It was reported by Jiang et al. (2022) that the water solubility of pea protein rose by 1.05 times after being conjugated with inulin utilizing the pH-shifting and ultrasonication methods. In addition, Wang et al. (2023) demonstrated that disaccharides and maltodextrin can interact with egg yolk using the microwave strategy, resulting in significant effects on the surface characteristics, particle size, and functional features of the egg yolk.

It has been suggested that disaccharides can establish strong hydrogen bonds with hydroxyl groups between molecules, enhancing protein structure stability (Butreddy et al., 2021). Trehalose has hydroxyl groups on its molecular structure, facilitating hydrogen bonding activation. The formation of hydrogen bonds between trehalose and proteins plays a crucial role in stabilizing the tertiary and quaternary structures of the protein. Additionally, Electrostatic interaction may be beneficial in the creation of conjugates during protein interactions in an alkaline environment (Alrosan et al., 2023a).

The main objective of this study is to determine the impact of various concentrations of trehalose on the protein complex formed by whey and lentil proteins on the surface properties, particle size, secondary and tertiary protein structures, and their conformation, as well as the water solubility and protein digestibility.

2. Materials and methods

2.1. Raw materials and chemicals

Whey protein isolates (WPIs, protein content 86%) and lentil seeds were obtained from a local market in Penang, Malaysia. An alkaline technique used to produce lentil proteins (LPs, protein content 64.8% ± 1.65) from the lentil seeds was based on the procedure described by Alrosan et al. (2023a, b). complex protein of LPs and WPIs was performed using the method described by Alrosan et al. (2021). Trehalose (with a molecular weight of 378.33) and materials utilized in the present research of reagent quality and procured from Sigma-Aldrich (California, USA).

2.2. Preparation of the trehalose-conjugated lentil-whey protein complexes (T-LWPs)

A phosphate-buffered solution was used to dissolve a trehalose solution with a concentration of 1, 2, 3, and 5% (w/v) and placed on the stirred for 2 h. The designations used were 0-T, 1-T, 2-T, 2-T, and 5-T, respectively. Subsequently, LWP (1 g) and sodium azide (0.002%, w/v) were dissolved in each of the trehalose solutions to prepare 1T-LWP, 2T-LWP, 3T-LWP, and 5T-LWP, respectively. The suspensions were pH-adjusted to 7.0 using a 0.1 M HCL solution and then placed on a magnetic stirrer for 60 min. The mixtures were left incubated overnight at 4 °C. After that, the pH of the mixtures was adjusted to pH 12.0 using 1 M NaOH and placed on the stirrer for 60 min (Alrosan et al., 2021). Following that, the pH of the solutions was adjusted to neutral (pH 7.0) and left overnight. The supernatants were collected after the centrifugation at 7000×*g* for 10 min. The supernatants were then dried using a freeze-dryer to yield lyophilised T-LWPs. The control sample (0T-LWP) underwent the same treatments previously described but without trehalose.

2.3. Water solubility

The water solubility of control (0T-LWP) and T-LWPs was determined at pH 7.0 using the procedure described by Alrosan et al. (2023a). Distilled water (18 g) was used to dissolve the sample (200 mg) at pH 7.0 and stirred for 60 min before adjusting the weight to 1% with distilled water. After that, the samples were transferred to 50 mL centrifuge tubes and centrifuged at 7000×*g* for 10 min. The nitrogen content was determined based on the AOAC Kjeldahl method (AOAC Method 930.29) (AOAC, 2012) according to Eq. (1).

$$\text{Water solubility } (\%) = [(N_S - N_B)/N_T] \times 100\% \quad \text{(Eq. 1)}$$

where, N_B is the nitrogen content in the blank sample, N_S is the nitrogen content in the supernatant, and N_T is the nitrogen content in the sample.

2.4. Fourier-transform infrared (FTIR)

The control (0T-LWP) and T-LWPs were analysed using an FTIR spectrophotometer (Shimadzu, IRAffinity-1S, Kyoto, Japan) according to the procedure described by Alrosan et al. (2021). The absorption bands of amide group I (1600–1699 cm^{-1}) represent the percentage of the secondary protein structures.

2.5. Particle size

The particle size distribution of the control (0T-LWP) and T-LWPs (1 mg/mL, pH 7.0) was determined using the Zetasizer Nano-ZS instrument (Malvern Panalytical, Mastersizer, 2000; Malvern, UK) based on the method described Wang et al. (2023). Deionised water, which has a refractive index of 1.33, was utilised as the continuous stage, and protein, which has a refractive value of 1.45.

2.6. Surface charge

The surface charge of control (0T-LWP) and T-LWPs was determined based on the procedure described by Alrosan et al. (2023a), employing the Zetasizer Nano-ZS instrument (Malvern Panalytical, Mastersizer, 2000; Malvern, UK), sample (1%, w/v) dissolved with distilled water at pH 7.0.

2.7. Surface hydrophobicity

Surface hydrophobicity of control (0T-LWP) and T-LWPs were determined using a fluorescent probe spectrophotometer (Agilent, Cary Eclipse, Santa Clara, USA), based on the procedure conducted by

Johnston et al. (2015) with slight modifications. The samples were produced using a phosphate buffer solution at a pH of 7.0 to achieve a concentration of 0.1% (w/v) and were stirred for 2 h at 1000 rpm. A calibration curve (0.01–0.1%) was used to determine the concentration of a sample by comparing its absorbance to the absorbance values obtained from the diluted sample, which employed a phosphate-buffered solution. This technique allowed for accurately quantifying surface hydrophobicity within the specified concentration range. Diluted samples (0.1%, w/v) were prepared by dissolving the sample (4 mL) and 20 μL of 8 mM 8-anilino-1-naphthalenesulfonic acid (ANS) dye at pH 7.0 and placed in the dark for 15 min prior to scanning. The emission and excitation of the fluorescence spectrophotometer were set at 390 and 470 nm, respectively, and 1 nm is the slit width. The slit width is used to control the amount of light passing through the sample. The concentration of the samples is a variable in the experiment. The relationship between protein concentration and fluorescence intensity is used to infer information about surface hydrophobicity. The slope of the graph is analysed. The slope provides information about the change in relative fluorescence intensity per unit change in protein concentration. The slope may serve as an indicator of the surface hydrophobicity of the protein and trehalose conjugates.

2.8. Intrinsic fluorescence

The measurement of control (0T-LWP) and T-LWPs intensity was carried out by utilizing the intrinsic fluorescence of tryptophan following the procedure described by Wang et al. (2019) with slight modifications. Each sample (0.001% w/v) was dissolved in distilled water at pH 7.0, at a temperature of 21 °C. The emission and excitation of the Cary Eclipse fluorescent probe spectrophotometer were placed at 280 and 300–450 nm, respectively (Alrosan et al., 2021).

2.9. UV-spectro

The absorbance of the control (0T-LWP) and T-LWPs was determined using a UV–vis spectrophotometer (Shimadzu, UV-3600, Kyoto, Japan) based on the procedure conducted by Alrosan et al. (2021). The protein samples (0.01, w/v) were solubilised in distilled water at a pH of 7.0. The samples were submitted to scanning within the wavelength range of 190–350 nm (Alrosan et al., 2023a, b). This wavelength range was determined to record the absorption and emission characteristics of the sample (Liu et al., 2023; Alrosan et al., 2021).

2.10. Molecular forces governing interactions

NaCl, thiourea, and sodium dodecyl sulphate were used to determine non-covalent bonds using the Cary Eclipse fluorescent probe spectrophotometer according to the procedure described by Alrosan et al. (2021). Each of 10 mM NaCl, thiourea, and sodium dodecyl sulphate was added during the interaction between LWP and trehalose (alkaline environment) to measure the electrostatic interaction, hydrogen bonds, and hydrophobic interactions, respectively (Wang et al., 2019).

2.11. Differential scanning calorimetry

The differential scanning calorimeter (DSC) is a commonly used instrument to study the thermal behaviour of control (0T-LWP) and T-LWPs (Mettler-Toledo, DSC 3, Greifensee, Switzerland) based on the method described by Wang et al. (2023). The samples (5 mg) were placed and sealed in DSC pans. The samples were allowed to balance out at a temperature of 25 °C, and the equilibration time continued for 3 min. Substantially, the samples were subjected to a cooling process, reducing their temperature to −70 °C, which continued for 20 min. Then, the samples were subjected to heat, increasing their temperature to 150 °C at an average rate of 20 °C/min. The denaturation temperature (T_d) of the samples was determined from the thermogram obtained.

2.12. Protein digestibility

The protein digestibility was determined using the method Almeida et al. (2015) described with slight modifications. Samples (250 mg) were combined with 1.5 mg/mL of pepsin and 15 mL of 0.1 M HCl solution. The mixtures were then heated to 37 °C for 3 h using a water bath (Memmert, WB22, Schwabach, Germany). The mixtures were added with 7.5 mL of 0.5 M NaOH, 10 mg of pancreatin, 1 mL of 0.005 M sodium azide, and 10 mL of 0.2 M of phosphate buffer (pH 8.0). The mixtures were then incubated overnight at 37 °C in the WB22 water bath. After the protein digestion, 1 mL of 10% trichloroacetic acid was added to the mixtures before centrifugation at 7000×g for 20 min. The protein digestibility of the samples was calculated using Eq. (1) based on the percentage of nitrogen content determined using the Kjeldahl AOAC method (AOAC Method 930.29) (AOAC, 2012).

2.13. Statistical analysis

The statistical analysis of the study's results was conducted using SPSS version 23.0, one-way analysis of variance (ANOVA) with Duncan's multiple range test. A significance level of $P < 0.05$ was employed to determine whether the differences between the means were statistically significant.

3. Results and discussion

3.1. Surface properties

3.1.1. Surface charge

Protein surface charge can have a significant impact on their capacity to attach and engage with other molecules (Ren et al., 2024; Amiratashani et al., 2024), with the surface charge of LWP around −36.1 mV (Fig. 1A). Our previous study showed that LWP had a surface charge of ~36 mV (Alrosan et al., 2021). The surface charge of T-LWP showed a substantial rise after its interaction with trehalose. Hence, the enhanced surface charges around protein molecules generated significant electrical repellent forces (Wang et al., 2019; Dai et al., 2022; Ren et al., 2024; Amiratashani et al., 2024), preventing their aggregation and improving the solubility of LWP when immersed in water. This enhancement indicates that the non-covalent bonds have a major role in increasing the surface charge of the protein (Wang et al., 2018; Dai et al., 2022; Ren et al., 2024; Amiratashani et al., 2024), resulting in an increased negative charge of T-LWPs. T-LWPs form hydrogen bonds, hydrophobic and ionic interactions, and undergo neutralisation. In the end (pH 7.0), molecules develop into established conjugates with charged groups exposed to the water solvent (Dai et al., 2022; Ren et al., 2024; Amiratashani et al., 2024). Thus, T-LWPs can maintain stability in water by creating adequate self-repulsive exteriors. Jiang et al. (2022) conducted a study showing that the relationship between inulin and chickpea protein occurs through electrostatic interaction. The researchers found that the electrostatic interaction between inulin and chickpea protein enhanced stability and improved protein functionality. Additionally, this study observed that the alkaline environment (over pH 11.0) played a crucial role in facilitating this interaction by altering the charges on both molecules (Alrosan et al., 2021; Ren et al., 2024), leading to stronger binding. On the other hand, the surface charge of 1T-LWP is not significantly different ($P > 0.05$) from that of the control. Hence, conjugation with 1% (w/w) may not be suitable to enhance the properties of LWP.

3.1.2. Surface hydrophobicity

The surface hydrophobicity of proteins can impact their overall physical stability (Al-Qaisi et al., 2024). Proteins with higher surface hydrophobicity are generally more prone to unfolding and aggregation, leading to reduced stability (Alrosan et al., 2021; Al-Qaisi et al., 2024). The function of proteins is primarily influenced by the surface

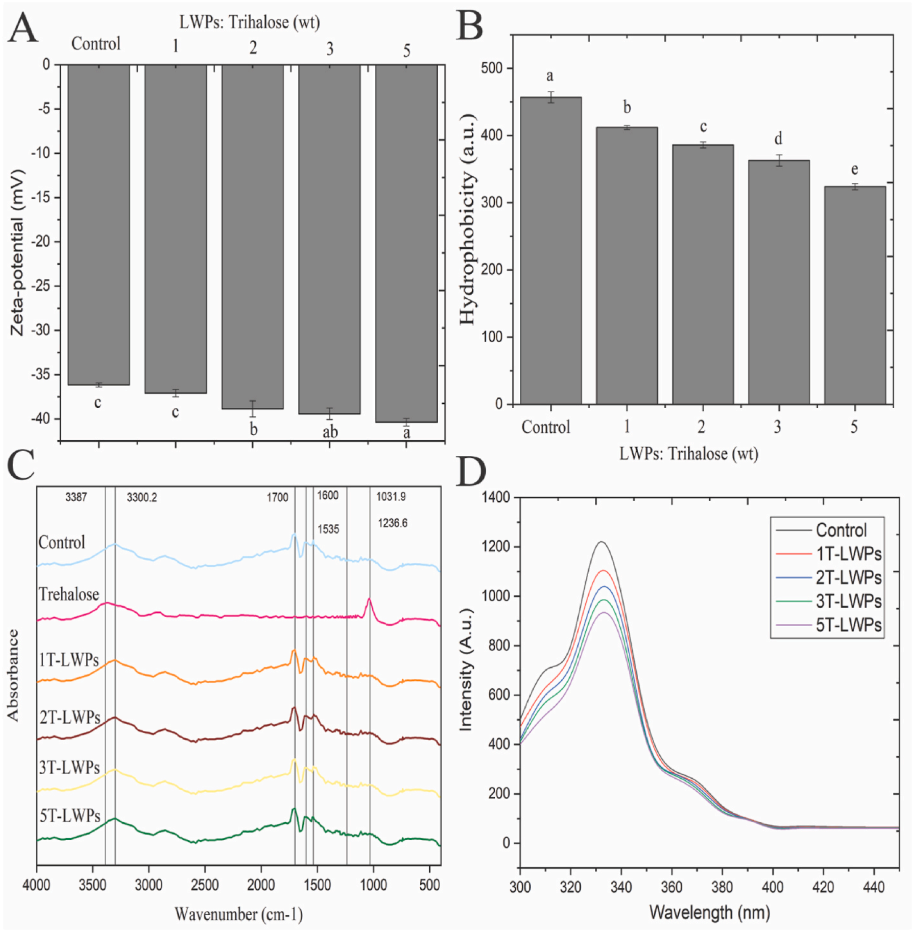

Fig. 1. Changes in the (A) water solubility, (B) protein digestibility, (C) fluorescence intensity, and (D) UV absorbance of lentil-whey protein complexes (LWPs) after trehalose conjugation. Control, 1T-LWPs, 2T-LWPs, 3T-LWPs, and 5T-LWPs represent LWPs conjugation with trehalose at 0, 1, 2, 3, and 5% (w/w), respectively.

hydrophobicity of the structures (Yi et al., 2024), which can be more significant than the total hydrophobicity due to the macromolecular nature of proteins. Hydrophobic fluorescent dyes can be used to evaluate the surface hydrophobicity of both denatured and native proteins. The surface hydrophobicity of LWP is around ~450 a.u. The surface hydrophobicity of LWP dropped significantly ($P < 0.05$) to ~412, following conjugation with 1% (w/w) trehalose. Subsequently, the hydrophobicity decreased dramatically (324 a.u.) as the amount of trehalose in LWP increased (Fig. 1B). It was demonstrated by Wang et al. (2023) that trehalose can decrease the surface hydrophobicity of proteins after conjugation. The interaction between trehalose and the hydrophobic residues protects them from exposure to the surrounding water, reducing the surface's hydrophobic nature.

3.2. Protein structures

3.2.1. Secondary protein structure

FT-IR spectroscopy was employed to examine the structural characteristics of LWP and T-LWPs. The unique peaks confirm its molecular shape at 400 to 4000 cm^{-1} (Fig. 1C) (Al-Qaisi et al., 2024). The secondary protein structure (β-sheet, RC, α-helix, and β-turn) of LWP and T-LWPs (Table 1). The results of this study indicate that trehalose plays a substantial effect in the secondary structure of T-LWP. The percentage of β-Turn was around 46.75% in the 0T-LWP (control) and decreased significantly ($P < 0.05$) to approximately 37.89%. A β-turn usually consists of four amino acid residues and is characterized by a sharp turn in the protein backbone (Fig. 3). The percentage of α-helix, RC, and β-sheet in the increased significantly ($P < 0.05$) in LPW after interaction with trehalose from 9.68 ± 0.08 to 10.44 ± 0.05, 13.77 ± 0.08 to 17.43 ± 0.20, and 29.80 ± 0.11 to 34.26 ± 0.11, respectively (Table 1). The finding of this result shows that trehalose has a major effect on the amide group I band 1600–1700 cm^{-1}, indicating that modification occurred after the interactions between trehalose and LWP. FT-IR spectroscopy

Table 1
The proportion of secondary protein components detected in complex protein structures derived from lentil and whey proteins that have been conjugated with trehalose (T-LWP) at different ratios, ranging from 0% to 5%.

Secondary Protein Components	Concentration of trehalose					*P*-Value
	0T-LWP	1T-LWP	2T-LWP	3T-LWP	5T-LWP	
β-Sheet (Σ)	29.80 ± 0.11^{a}	30.70 ± 0.10^{b}	32.01 ± 0.10^{c}	33.94 ± 0.17^{d}	34.26 ± 0.11^{e}	<0.05
RC (Σ)	13.77 ± 0.08^{d}	13.96 ± 0.03^{d}	15.38 ± 0.04^{c}	16.49 ± 0.09^{b}	17.43 ± 0.20^{a}	<0.05
α-Helix (Σ)	9.68 ± 0.08^{c}	9.77 ± 0.05^{bc}	9.88 ± 0.07^{b}	10.42 ± 0.08^{a}	10.44 ± 0.05^{a}	<0.05
β-Turn (Σ)	46.75 ± 0.35^{a}	45.46 ± 0.40^{b}	42.84 ± 0.40^{c}	39.13 ± 0.40^{d}	37.89 ± 0.27^{e}	<0.05
T_d	95.5 ± 0.40^{d}	97.6 ± 0.11^{c}	98.4 ± 0.20^{b}	98.7 ± 0.28^{b}	99.6 ± 0.15^{a}	<0.05

Means (n = 3) with different superscripts in the same row differ significantly ($P < 0.05$). Control (0T-LWP) represents the absence of trehalose conjugation with the QPs. Meanwhile, 1T- LWP, 2T- LWP, 3T- LWP, and 5T- LWP represent LWP conjugated with trehalose at 1, 2, 3, and 5% (w/w), respectively. Denaturation temperature (T_d).

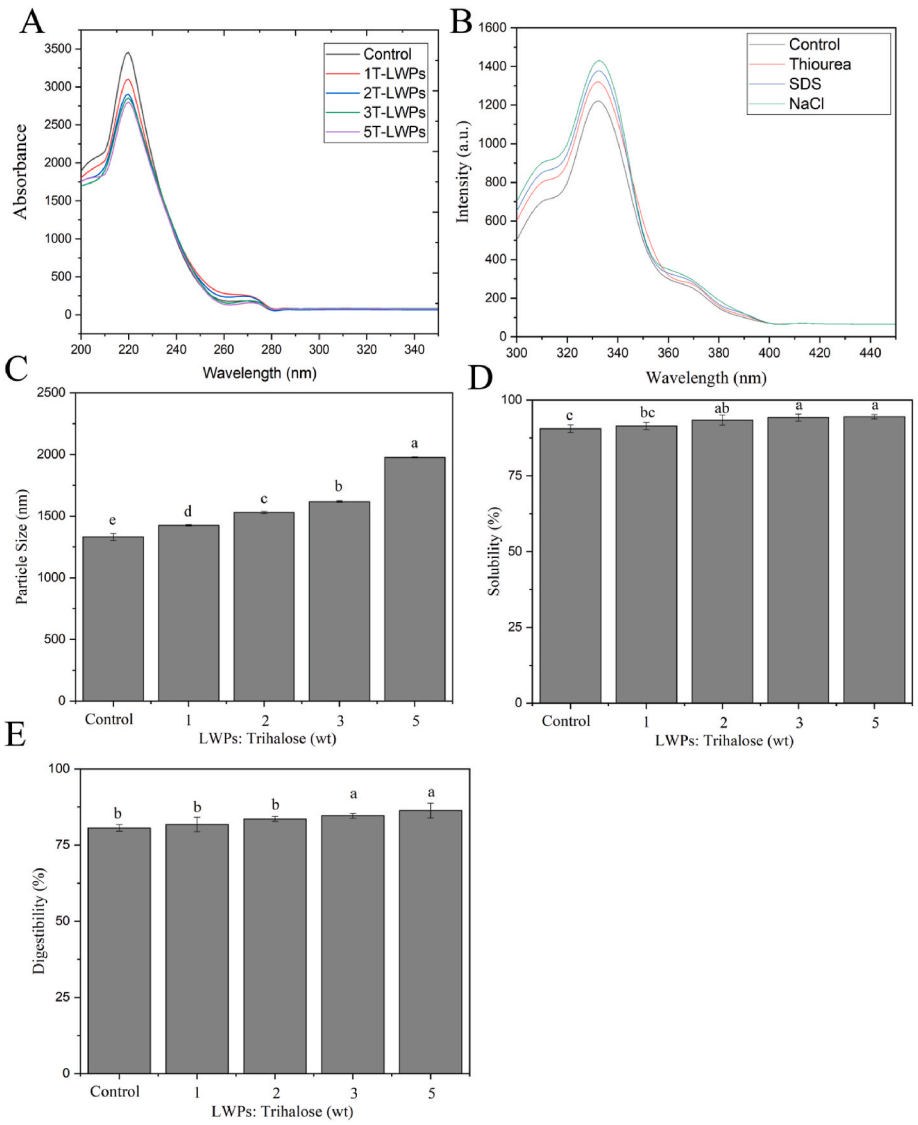

Fig. 2. Changes in the (A) FTIR spectra, (B) molecular forces via fluorescence intensity, (C) surface charges, (D) surface hydrophobicity, and (E) particle size of lentil-whey protein complexes (LWPs) after trehalose conjugation. Control, 1T-LWPs, 2T-LWPs, 3T-LWPs, and 5T-LWPs represent LWPs conjugation with trehalose at 0, 1, 2, 3, and 5% (w/w), respectively.

can determine the nature of a range of chemical modifications resulting from this method. Additionality, which can be detected in the changes in amide group II, the peak at 1535.3 cm^{-1} could provide information on the functional groups, including C–N stretching and N–H bending, and amide group III band (C–N stretching and N–H bending) at peak 1236 cm^{-1} (Jiang et al., 2022), In addition, the hydroxyl group can also be detected at absorbance 3300.2 cm^{-1} (Dai et al., 2022). Fig. 1 C shows that absorbance changed in LPW after conjugation with trehalose. Changes in the stretching vibrations of C–H, C–C, and C–O bonds in LPW were observed (Dai et al., 2022). This peak indicates alterations in the molecular structure of LPW, specifically related to carbohydrates.

The absorption peaks at 3300.4 and 3388 cm^{-1} are reported for the conjugates of LWP and T-LWPs. These peaks likely correspond to specific vibrational modes associated with functional groups, such as O–H stretching vibrations in hydroxyl groups or N–H stretching vibrations in amide groups. The shift to 3308 cm^{-1} upon incorporation and the reported absorption peaks at 3300.4 and 3388 cm^{-1}. These change of absorption peaks indicate a modification or interaction between the LWPs, T-LWP, and trehalose. Changes in the molecular environment, bonding, or interactions can alter vibrational frequencies, resulting in shifts in absorption peaks. It was reported by Chen et al. (2019) that the degree of interactions between carbohydrates and proteins depends on the shifting of the amide II and amide I bands.

3.2.2. *Tertiary protein structure*

Fluorescence analysis can determine modifications to the tertiary structure of proteins by examining the fluorescence produced by naturally fluorescent amino acids, such as tryptophan, tyrosine, and phenylalanine (Alrosan et al., 2021; Dai et al., 2022; Al-Qaisi et al., 2024). Fig. 1D revealed that the intensity of intrinsic fluorescence (339 nm) of 1T-LPW was reduced compared with the control (0T-LPW). The hypothesis suggests that trehalose may cause changes in the tertiary structure of proteins, which is demonstrated by a decrease in intensity following the interaction between trehalose and LWP. Moreover, the intensity at the peak wavelength of intrinsic fluorescence (339 nm) (Dai et al., 2022;Amiratashani et al., 2024) decreases as the amount of trehalose increases in the LPW (Fig. 1D). It was reported by Miranda et al. (2023) that the interaction between pectin and lentil protein might lead to alterations in the protein structure, resulting in changes in functional properties, specifically solubility. This study has demonstrated that a decrease in intrinsic fluorescence intensity plays a significant role in protein unfolding and reduces aggregation. In addition, the

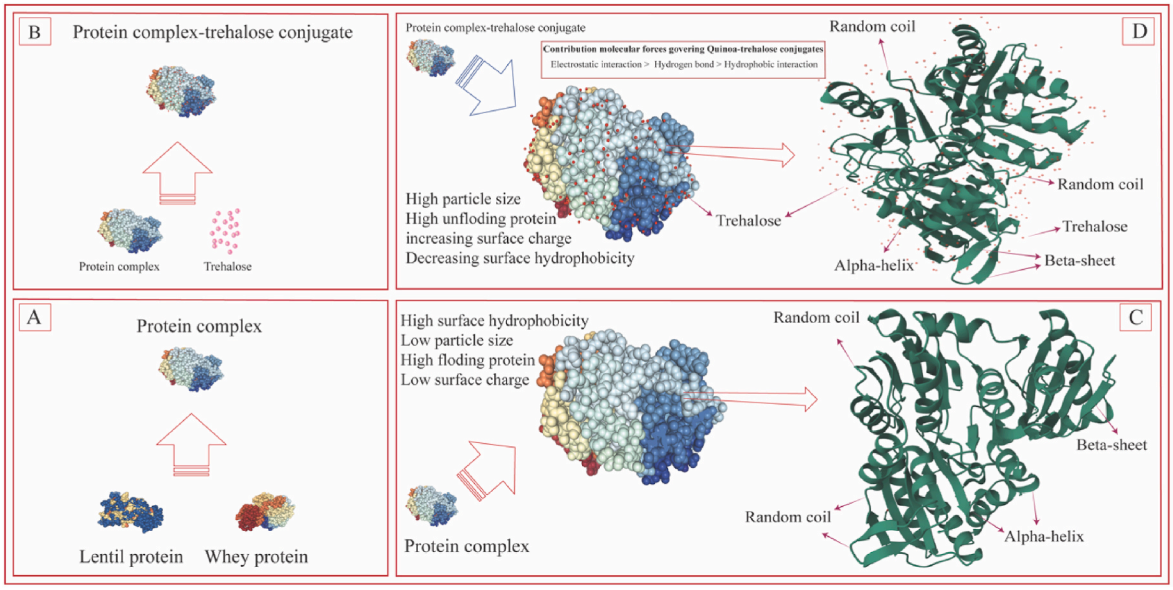

Fig. 3. An illustration to depict the changes in the secondary and tertiary protein structures upon (A) protein complexation of lentil and whey proteins and (B) trehalose conjugation of the lentil-whey protein complex.

findings suggest that monitoring changes in intrinsic fluorescence could be a valuable tool for studying protein stability and aggregation kinetics (Amiratashani et al., 2024; Dai et al., 2022; Al-Qaisi et al., 2024).

Additionally, this study highlights the importance of understanding the relationship between protein structure and fluorescence intensity for various biomedical applications. Protein aggregation may influence the intensity of tryptophan residues' fluorescence (Wang et al., 2019; Alrosan et al., 2021; Liu et al., 2023). Protein aggregation can also impact the fluorescence intensity of tryptophan residues (Dai et al., 2022; Amiratashani et al., 2024; Al-Qaisi et al., 2024). When proteins aggregate, the local environment of tryptophan residues can change, influencing the fluorescent property. A study by Wang et al. (2023) found a reduction in the fluorescence intensity related to tryptophan residue and alterations in tertiary protein structure.

3.3. Conformation of proteins

The ultraviolet (UV) spectrum can detect variations in the structure of proteins inside a protein solution (Alrosan et al., 2021; Dai et al., 2022). The increase in absorbance at wavelength 230 nm can be attributed to the formation of aromatic amino acid residues (folding), such as tryptophan and tyrosine, which have strong absorption properties in this range. Conformational protein folding also plays a crucial role in determining the protein's stability and functionality. As shown in Fig. 2A, the absorbance (A_{230}) of 1T-LWP was decreased compared with 0T-LWP (control), indicating that the folding of proteins reduces after the interaction between LWP and trehalose. A study by Liu et al. (2023) showed that absorption at A_{230} changes in chickpea protein after the treatment with fermentation, which led to an increase in water solubility; absorbance at 230 nm decreased, which led to the unfolding of the protein.

The drop in absorbance at 230 nm was caused by the increasing levels of trehalose in LWP, which led to an increase in the unfolding of proteins (Fig. 2A) and was reflected in the increase in water solubility. Several studies demonstrated that a decrease in the wavelength at 230 nm led to a rise in the unfolding of proteins, suggesting an increase in the water solubility of proteins (Wang et al., 2019; Alrosan et al., 2021, 2023a; Liu et al., 2023). The presence of this peak may reveal information regarding the surrounding environment and activities involving phenylalanine residues inside the protein (Liu et al., 2023). The absorbance at peak 260 nm increased whenever trehalose levels increased in the LWP (Fig. 2A). It is evident that an interaction occurred between trehalose and LWP, resulting in a modification in the conformation proteins.

3.4. Molecular forces

The impact of trehalose conjugation on the intermolecular forces of protein complexes was determined by using SDS, NaCl, and thiourea as represented for hydrophobic interaction, hydrogen bonding, and electrostatic interaction, respectively, using a fluorescence probe (Alrosan et al., 2021; Dai et al., 2022; Al-Qaisi et al., 2024). The results presented in Fig. 2B demonstrate that the absorption of trehalose at its peak wavelength of >339 nm increases with the addition of NaCl, SDS, and thiourea, suggesting that electrostatic interactions, hydrophobic interaction, and hydrogen bonds play a significant role in the interaction between trehalose and LPW. Electrostatic interactions contribute to the formation of the conjugates between trehalose and LWP. The hydrogen bonds have less to contribute compared to the electrostatic interaction and hydrogen bonds (Fig. 2B). It was reported by Liu et al. (2023) that the protein structure could be altered by hydrophobic interactions incorporating water.

On the other hand, the digestibility and water solubility of plant protein might be influenced by activated electrostatic interaction and hydrogen bonds (Liu et al., 2023; Alrosan et al., 2023a). Meanwhile, hydrogen bonds are weak electrostatic attractions between a hydrogen atom bonded to a highly electronegative atom (such as nitrogen or oxygen) and another electronegative atom in a different molecule. Hydrogen bonds form between the carbonyl oxygen of one amino acid residue and the amide hydrogen of an amino acid residue four positions ahead in the sequence, stabilizing the helical structure in the α-helix. Hydrogen bonds are crucial for holding adjacent strands together in a β-sheet, forming a stable secondary structure in β-Sheet (Wang et al., 2019; Alrosan et al., 2021). Trehalose, a disaccharide sugar consisting of two glucose molecules, can form hydrogen bonds with water molecules and interact with various biological compounds, including proteins and lipids. With its hydroxyl groups, trehalose can form hydrogen bonds that can interact with the surface of proteins. The formation of hydrogen bonds between trehalose and specific amino acid residues on the protein surface can stabilize the protein's native structure. (Butreddy et al., 2021).

3.5. Thermal stability

Regarding the effects of trehalose conjugation on the thermal stability of LWPs (Table 1), DSC was extensively employed for observing the alterations in the heat properties of proteins during thermal denaturation or unfolding (Lee et al., 2024). The denaturation of the quaternary, tertiary, and secondary protein structures can change protein conformations (Zhang et al., 2024; Al-Qaisi et al., 2024). LWP and conjugated trehalose range thermal stability is between 95.5 and 99.6 °C. The thermal stability of LWP was measured with a value of around 95.5 ± 0.40. Nevertheless, following conjugating with trehalose, the thermal stability of 5T-LWP was enhanced significantly to 99.60 ± 0.15. These findings indicate that adding trehalose has a beneficial effect on the thermal stability of LWP, leading to an increase in water solubility. In recently studies reported by Wang et al. (2023) and Jiang et al. (2022) that interaction between the disaccharides and protein can enhance the stability of the protein. Disaccharides can replace water molecules in the hydration shell around proteins (Jiang et al., 2022). This water displacement can help protect the protein structure by reducing water-mediated interactions that may lead to denaturation (Wang et al., 2023). Glycosylation adds bulky carbohydrate groups to the protein molecule (Schneider et al., 2023). These bulky groups can provide steric hindrance, preventing or inhibiting the unfolding of the protein structure (Ke & Li, 2023; Higa & Nickerson, 2023). Large glycan moieties may obstruct or limit the accessibility of some areas of the protein, making it more resistant to unfolding or denaturation (Bilardo et al., 2022). Moreover, this glycosylation process might have formed a shielding layer over the proteins, inhibiting protein denaturation. The results of this study indicate that T-LWP interaction and glycosylation could have an essential role in enhancing the thermal stability of LWP.

3.6. Particle size

The results of the study on the interaction between the trehalose and LWP with different amounts of trehalose showed that trehalose could enhance the creation of small particles and greatly improve the stability of T-LWP (Fig. 2C). The particle size of LWP increased significantly ($P < 0.05$) from 1331.3 to reach 1977.7 nm. This increase demonstrates that polysaccharides are crucial in synthesising conjugates between the protein and trehalose (Zhao et al., 2023). Previous investigation has suggested that particle size increased after the interactions between proteins and polysaccharides/disaccharides could potentially result in an enlargement of the particle size of complex substances (Han et al., 2023; Wang et al., 2023). An increase in particle size can substantially impact the functionality and stability of conjugated proteins (Siddiquy et al., 2023). Moreover, the binding of polysaccharides or disaccharides to proteins can have a significant effect on the solubility and bioavailability of the resulting complexes (Wang et al., 2023). Adding polysaccharides or disaccharides can enhance the solubility of proteins, mainly if the proteins are prone to aggregation or precipitation. The carbohydrates may shield hydrophobic regions on the protein surface (Deng et al., 2024), reducing interactions that lead to aggregation and promoting overall solubility.

3.7. Water solubility

Protein solubility is a crucial functional characteristic of a protein (Wang et al., 2019; Alrosan et al., 2022; Amiratashani et al., 2024); and understanding the water solubility of proteins is essential in the field of structural biology investigation (Alrosan et al., 2023a). The water solubility of LWPs is ~90% (Fig. 2D). This finding is consistent with the outcomes described by Alrosan et al. (2021), which are attributed to the amino acid composition of whey proteins, including a significant proportion of hydrophilic (water-attracting) residues, such as serine and threonine. These residues form hydrogen bonds with water molecules, enhancing solubility. The water solubility of 2T-LWP improved significantly ($P < 0.05$) following conjugation at a ratio of 2% (w/w), clear evidence that trehalose enhances the water solubility after the interaction with LWP. The solubility of 5T-LWP rose significantly ($P < 0.05$) at 5% (w/w), reaching around 94.51%. Trehalose's hydroxyl groups can replace water molecules, establishing stable hydrogen bonds with proteins, preserving the proteins' inherent structure and safeguarding them from aggregation and denaturation. Trehalose's ability to form stable hydrogen bonds with proteins is crucial in various biological processes (Butreddy et al., 2021). This unique property of trehalose also protects proteins from harsh environmental conditions, such as extreme temperatures or high concentrations of solutes.

3.8. Protein digestibility

Protein is essential for various physiological functions in the human body, including the synthesis and repair of tissues, enzyme production, and immune function (Yang et al., 2023; Alrosan et al., 2024; Rivero Meza et al., 2023). However, the body can only benefit from protein if it is effectively broken down into constituent amino acids during digestion. The study revealed that the digestibility of control (0T-LWP) was around 80.65%, with the lower digestibility attributed to 45% of the lentil proteins of LWP. Lentil proteins contain certain factors that may affect their digestibility, primarily related to anti-nutritional factors, including tannins, protease inhibitors, and lectins (Arbab Sakandar et al., 2023). These compounds can interfere with the digestion and absorption of nutrients in the human digestive system (Rivero Meza et al., 2023). The digestibility of 5T-LWP increased significantly after the interaction with trehalose to reach around ~86.34%. On other hand, no significant difference ($P > 0.05$) was observed between 2T-LWP and 3T-LWP. Trehalose can modify the protein structure of plant-based proteins (Zhu et al., 2023; Wang et al., 2023), as it results in the improved digestibility of protein. In a recent study by Miranda et al. (2023) found that the protein structure acquired alterations due to the interaction between lentil protein and polysaccharides, specifically pectin, following the application of ohmic heating treatment. The hypothesis suggests that the presence of proteins combined with trehalose is mostly attributed to electrostatic interactions, hydrophobicity, and hydrogen bonding. The hydrogen bonds play a critical role in forming the protein structure during the interaction of plant-based proteins with polyols (Han et al., 2023). Hydrogen bonds play a crucial role in shaping the secondary and tertiary structures of proteins, which form between the carbonyl oxygen of one amino acid and the amide hydrogen of an amino acid, three or four residues down the chain in α-helix and β-sheet. Hydrogen bonds are formed between neighbouring strands. The carbonyl oxygen of one strand forms a hydrogen bond with the amide hydrogen of an adjacent strand.

4. Conclusion

Trehalose conjugation was successfully applied on LWPs to form a stable T-LWP. In this study, suitable concentrations of trehalose were evaluated to enhance the water solubility and protein digestibility of LWP by modifying the protein structures and surface properties. The secondary, tertiary, and conformation proteins were altered after being conjugated with trehalose. As a result of the conjugation, the water solubility and protein digestibility of LWP improved. The interaction between trehalose and LWPs was found to be governed by various molecular forces, including electrostatic interaction, hydrogen bonds, and hydrophobic interactions. These findings highlight the potential of trehalose to enhance the quality and nutritional value of plant-based protein complexes.

CRediT authorship contribution statement

Mohammad Alrosan: collected test data and drafted the manuscript, designed the study, interpreted the results, and revised the

manuscript. **Ali Madi Almajwal:** contributed equally to this work. **Ali Al-Qaisi:** analysed the samples. **Sana Gammoh:** contributed equally to this work. **Muhammad H. Alu'datt:** contributed equally to this work. **Farah R. Al Qudsi:** analysed the samples. **Thuan-Chew Tan:** designed the study, interpreted the results, and revised the manuscript. **Ammar A. Razzak Mahmood:** contributed equally to this work. **Sofyan Maghaydah:** contributed equally to this work.

Declaration of competing interest

We confirm that there is no found conflict of interest in association with the following study, along with that there no also no financial support for this publication in order to influence any outcome with this source.

We assure that this study has been read and approved by the authors being named in this publication and no other person/s (Except named in this study) satisfies the criteria for claiming the authorship for this study. Along with that the order of names of authors has been approved by all the authors of this study.

It is also confirmed that for the protection of intellectual property association, the required consideration has been given including the publication timing. For that purpose, we assure that the regulations have been followed as set by the institution for intellectual property.

All the authors understand that the corresponding author is the sole contact person for editing procedure which includes direct communication with the office and editorial management. We provide the consent over that corresponding author bears the responsibility of communicating all the upgradation, progress, revisions and final approval of proofs to all the authors of this publication. It is confirmed that the provided information (email address) has been verified and is accessible to the corresponding authors and has been configured to accept email from mohammad.alrosan@hotmail.com and m.alrosan@qu.edu.qa.

Data availability

Data will be made available on request.

Acknowledgment

This research was funded by the Researchers Supporting Project number (019032), Jordan University of Science and Technology Irbid, Jordan. In addition, the authors express their gratitude to Universiti Sains Malaysia, Penang, Malaysia for granting permission to use its facilities. We gratefully acknowledge and sincerely appreciate the unidentified referees for their insightful comments and suggestions for improving the content of the manuscript.

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