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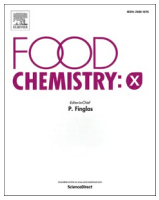

Modification approaches of walnut proteins to improve their structural and functional properties: A review

Min Yang, Yunkun Zhu, Jiangxia Xu, Zhongkai Zhao, Liang Wang, Jie Yang, Minwei Zhang *

Xinjiang Key Laboratory of Biological Resources and Genetic Engineering, College of Life Science & Technology, Xinjiang University, Urumqi 830046, China

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ABSTRACT

Walnut protein has a high gluten content and compact structure, which limits its water solubility and affects its applications. Therefore, improving the sustainability of walnut proteins is an urgent issue that must be addressed. Physical modification can directly alter the structure of walnut proteins, leading to enhanced functional properties. Chemical modifications typically involve the introduction of exogenous substances that react with walnut proteins to obtain novel products with improved processing attributes. As a highly specific modification technique, biomodification uses enzymes or microorganisms to break down walnut proteins into small peptide molecules or cross-link them to form soluble polymers, thereby enhancing their functional properties and bioactivity. This review presents various methods for modifying walnut proteins and their effects on the structure and functional properties of walnut proteins. The challenges associated with the application and development of these unique technologies are also discussed.

1. Introduction

Walnut protein, as a plant-based protein, are typically extracted as a byproduct of walnut oil, with a content of >50 % (Luo et al., 2017). The composition of the walnut proteins is heterogeneous, including gluten (72.06 %), globulin (15.67 %), albumin (7.54 %), and prolamin (4.73 %) (Mao, Hua, & Chen, 2014. The balanced presence of various amino acids in walnut protein, characterised by high arginine and glutamic acid contents, underscores its significant nutritional value and has attracted increasing attention (Li, Guo, Chi, & Ma, 2020). However, owing to the pronounced heterogeneity of walnut proteins and the substantial proportion of glutelin, its compact structure and suboptimal water solubility further impede its digestive characteristics. Moreover, the allergenicity of walnut proteins restricts its use in the food industry (Han, Zhao, Wu, Mao, & Zhang, 2023). Solubility is the most fundamental physical property of walnut proteins and a prerequisite for their other functional properties. It confers the interaction capacity between walnut proteins and water molecules, with stronger hydration ability resulting in improved solubility of walnut proteins (Wu, Fitzpatrick, Cronin, & Miao, 2019). Therefore, poor solubility further affects the manifestation of other functional properties of walnut proteins, including their water- and oil-holding capacity, foaming, and emulsifying properties (Foegeding & Davis, 2011).

Currently, it is possible to use physical modification, chemical modification, and biological modification impact on the aggregation of peptide chains, exposure of amino acid residues, and extent of protein unfolding within walnut proteins to improve their hierarchical structure of walnut proteins and confer them with more desirable functional properties (Akharume, Aluko, & Adedeji, 2021). Physical modification techniques for walnut proteins typically involve the application of external forces, such as heat, mechanical forces, and irradiation, to induce changes in their structure of walnut proteins (Dolganyuk et al., 2023). This typically leads to stretching of the secondary and tertiary structures of walnut proteins and enhancement of their functional properties. To improve modification efficiency, chemical modifications characterised by high efficiency and rapidity have been used to modify walnut proteins (Sahil, Prabhakar, & Kumar, 2024). These methods involve the introduction of new exogenous substances, such as carbohydrates, phenols, phosphates, and oxidising agents into walnut proteins. Through grafting, coupling, or phosphorylation, specific amino acid residues on walnut proteins bind to specific groups present in the chemical substances, thereby generating new products that enhance the functional properties of walnut proteins (Liu, Sun, & Guo, 2022). Biological modification methods are widely used to modify walnut proteins because of their strong specificity. These methods often involve the introduction of enzymes or fermentation to break down walnut proteins

* Corresponding author at: College of Life Science & Technology, Xinjiang University, Xinjiang 830017, China.
E-mail address: zhang78089680@sina.com (M. Zhang).

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into small peptide segments. In addition, enzymes can be used to crosslink walnut protein peptide chains, leading to the formation of soluble aggregates. Products obtained using these methods often exhibit enhanced bioactivity and are suitable for health food production (Zhou et al., 2022). Furthermore, the functional properties of walnut proteins can be optimised by combining the three major types of modification techniques in pairs, such that the modifications can compensate for each other, thus enhancing the functional properties of walnut proteins. In conclusion, a comparative analysis of walnut protein modification techniques using various approaches and exploration of novel walnut protein modification techniques are crucial.

This review systematically summarises the principles, conditions, and results of various modification techniques aimed at enhancing the functional properties of walnut proteins (Fig. 1). The primary focus is on the effects of different modification methods on the hierarchical structure, surface properties, and modification of functional groups in walnut proteins to enhance their functional properties. In addition, the operational conditions of the existing modification techniques and potential barriers are addressed, while predicting future trends in the modification and application of walnut proteins. Collectively, this review aims to provide theoretical guidance for the rational use of walnut proteins and the innovation and development of modification technologies.

2. Physical modification

The secondary and tertiary structures of walnut proteins are sensitive to heat, mechanical forces, and radiation (Cai et al., 2018). These external factors can lead to the depolymerisation of the peptide chain of walnut proteins and stretching of their subunits. This interference can also cause transformation of the secondary structure from an ordered state to a disordered state, resulting in structural unfolding or reorganisation. Thus, the exposure of polar and hydrophobic groups assists in improving hydration, which in turn leads to improved dispersion and solubility of walnut proteins (Liu et al., 2023). The effects of the physical modifications on walnut proteins are shown in Table. 1.

2.1. Mechanical effects

In the natural structure of walnut proteins, hydrophilic groups are located on the outer layer, whereas hydrophobic groups tend to aggregate in the internal regions, forming a dense and rigid internal structure. This configuration is conducive to maintaining the conformation of walnut proteins but may limit their functional properties (Bernard, Regnault, Gendreau, Charbonneau, & Relkin, 2011). Through mechanical treatments such as ultrasound, shear, high pressure, or grinding, the secondary structure of walnut proteins can transition from α-helical and β-sheet tendencies to β-turns and random coil structures. This modification can alter the interactions between protein molecules, including van der Waals forces, hydrophobic interactions, hydrogen bonds, and disulfide bonds (Baskıncı & Gul, 2023). Notably, mechanical treatments typically do not affect the primary structure of walnut proteins. Currently, mechanical methods used for walnut protein modification include ultrasonic treatment (UT), high-speed shear treatment (HST), high-pressure treatment (HPT), and superfine grinding (SG) which discussed in the following sections.

2.1.1. Ultrasonic treatment (UT)

Walnut proteins undergo changes in their internal secondary and tertiary structures upon exposure to ultrasound waves. During the UT, the alternating high-pressure and low-pressure ultrasound waves cause

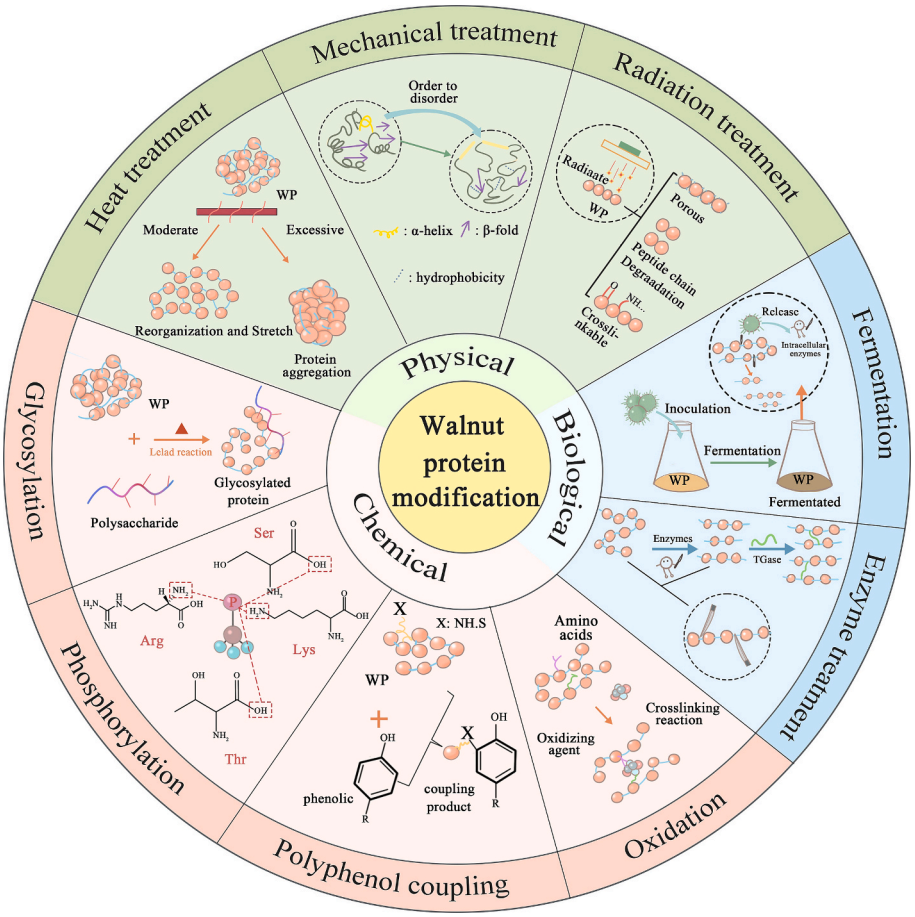

Fig. 1. Classification of walnut proteins modification methods and schematic diagram of principles.

Table 1
Effects of different physical modifications on the properties of walnut proteins.

Modifications	Processing conditions	Structure changes	Functional characteristics							Ref.
			SO	WHC	OHC	FC	FS	EAI	ESI	
UT	400/600 W 15 min/30 min	A (20.61 %) → B, C and D Disulfide bond breakage H_0 enhancement	+0.22	/	/	/	/	+0.26	+0.41	(Zhu et al., 2018)
	400w 30 min	A (33.94 %) → B, C and D Hydrogen bond breaking H_0 enhancement	/	/	/	/	/	+1.57	+2.67	(Shi et al., 2023)
SG	15-45 Hz	B → C; hydrogen bonding is weakened aggregation is reduced.	+0.19	−0.59	−0.45	+0.92	−0.24	+0.11	+0.40	(Zhang et al., 2023)
HPT	400 MPa 20 min	Peptide chain defolding Sulfhydryl group exposure H_0 enhancement	−0.10	/	/	/	+0.36	−1.08	/	(Qin et al., 2013)
	180 MPa treatment twice	A (13.79 %) → C and D H_0 enhancement	+3.60	/	/	+0.18	+0.15	+0.95	+2.90	(Li et al., 2023)
HST	15,000 rpm 5 min	/	+1.58	/	/	/	/	/	/	(Kong et al., 2019)
HT	100 °C 15-30 min	Molecular weight increases	/	/	/	/	/	/	/	(Jiang et al., 2018)
	130 °C 30 min	Disulfide bonds reconnect to form aggregates	−0.16	−0.12	+0.22	+0.48	/	/	−0.49	(Shang, 2022)
RT	0–15.0 kGy 0.5 kGy/h	The particle size becomes larger and the surface becomes porous	/	/	/	/	/	/	/	(Zhao et al., 2017)

Note: "+"indicates the lifting multiplier, "-"indicates the falling multiplier. A: α-helical, B: β-sheet, C: β-turn, D: random coil. Solubility (SO), Water Holding Capacity (WHC), Oil Holding Capacity (OHC), Foaming Capacity (FC), Foaming Stability (FS), Emulsifying Activity (EAI), Emulsifying Stability (ESI), Surface Hydrophobicity (H_0), "/" indicates not studied in the literature.

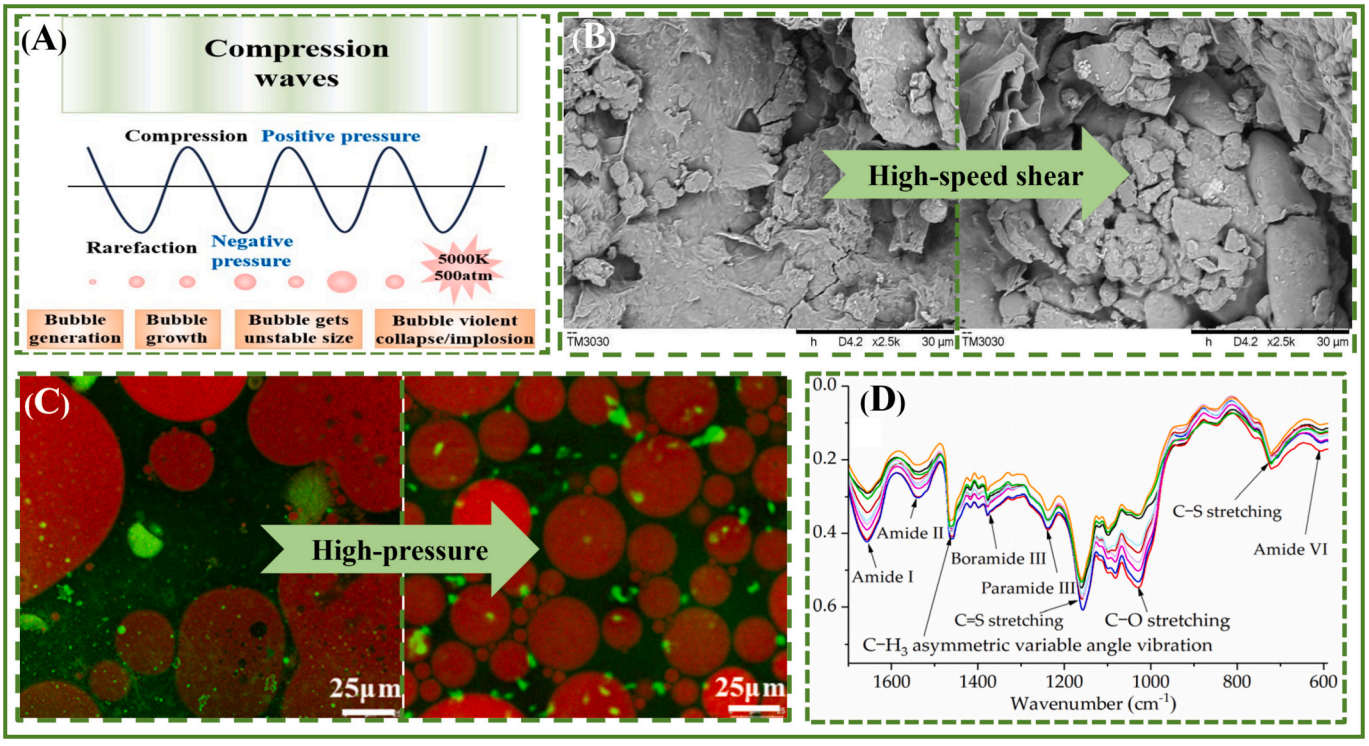

Fig. 2. (A) Ultrasonic cavitation, (B) SEM of walnut proteins before and after HST (Kong et al., 2019), (C) microstructure of emulsions prepared from walnut proteins (Li et al., 2023), (D) FTIR spectra of WPI after HPT (Zhao et al., 2022).

compression and expansion (rarefaction) cycles in the medium (Toledo Guimarães, Silva, Freitas, Almeida Meireles, & Cruz, 2018). Cavitation leads to the implosion and collapse of small air bubbles formed in the walnut protein solution, generating extreme temperatures (5000 K) and pressures (500 atm). During this process, the ultrasonic waves create intense fluid dynamics and shear forces within the solution, simultaneously releasing a substantial amount of energy (Fig. 2A) (Huang, Ding, Dai, & Ma, 2017). In this environment, walnut protein subunits can aggregate or dissociate, further altering their functional properties (Zhao et al., 2022).

UT of walnut protein solutions revealed that at power levels of 400–600w for 15–30 min, the peptide chains of the walnut protein became more extended due to UT. Subsequently, there was a significant decrease in the α-helix content in the secondary structure, indicating that hydrogen bonds within the walnut protein were disrupted due to the ultrasonic effect (Zhu et al., 2018). The dense structure of walnut proteins loosened during sonication, and the interactions between protein molecules and surface charges were significantly altered (Fig. 2D) (Zhao et al., 2022). Walnut proteins with this extended structure expose the hydrophobic groups on their surface more fully, leading to a surface hydrophobicity increase in approximately 3.64-fold. After ultrasound modification, the solubility, emulsifying activity, and emulsion stability of walnut proteins increased by approximately 22 %, 26 %, and 41 %, respectively (Zhu et al., 2018). In addition, the prepared emulsion exhibits improved thermal stability and resistance to lipid oxidation (Shi et al., 2023). Notably, protein concentration can influence UT the effectiveness. Lower protein concentrations may cause protein molecules to avoid cavitation and bubble formation, ehereas higher protein concentrations can increase solution viscosity, affecting the efficiency of ultrasound (Liu et al., 2023).

2.1.2. High-speed shear treatment (HST)

The principle of HST involves the generation of intense shear forces between the internal rotor and external stator through high-speed rotation to blend walnut proteins solution (Ali, Kikumoto, Cui, Ciantia, & Previtali, 2023). High-speed rotation also induces turbulent flow, resulting in strong shear, stretching, and compression interactions among walnut protein particles. Taking into consideration the simplicity, environmental friendliness, and ease of operation of high-speed shearing (Zou, Nguyen, Biers, & Sun, 2019), subjecting walnut proteins to HST under alkaline conditions at a speed of 15,000 rpm for 5 min could break down large flake-like structures into smaller particles (Fig. 2B). This process resulted in a 71.4 % reduction in particle size and a 158.1 % increase in solubility (Kong et al., 2019). These improvements can be attributed to the shear effects during processing, which increase the electrostatic repulsion between protein molecules, thereby enhancing the water solubility of the walnut protein. In addition, shear stress does not affect the amino acid composition of walnut proteins, thus maximising the preservation of their inherent nutritional properties (Wang, He, Fu, Luo, & Huang, 2015).

2.1.3. High-pressure treatment (HPT)

HPT can be used to alter the conformation of walnut proteins and enhance their functional properties by compressing the fluids. Walnut proteins are typically placed in a high-pressure chamber, with water often chosen as the pressure-transmitting medium, with pressure levels typically ranging from 100 to 1000 MPa (Högg, Boguslawski, Sevenich, Rawel, & Rauh, 2024). The primary structure of walnut proteins remains unaffected within the pressure range currently achieved, whereas the secondary structure may undergo slight changes. A significant impact is observed on the tertiary and quaternary structures of the walnut protein (Rivero-Pino, Espejo-Carpio, & Guadix, 2020). This is due to the compression of the protein within a confined space during processing, leading to the breaking of non-covalent bonds such as hydrogen bonds, or the exposure of hydrophobic groups (Yordanov & Angelova, 2010). For instance, after treatment with 400 MPa HPT, the foaming capacity and foam stability of walnut protein could be enhanced (39.3 % and 52.77 %), wheres the emulsifying capacity increased by 36.36 % (Qin et al., 2013). Furthermore, HPT can effectively reduce the allergenic activity of walnuts by altering the conformation of allergens, consequently decreasing their allergenicity (Yang et al., 2017). In addition, HPT can increase the solubility and surface hydrophobicity of walnut proteins by 3.6 and 2.04-fold, respectively, and increase the foaming and emulsifying properties by 17.27 % and 95.50 %, respectively, resulting in improve dispersion of the prepared emulsion (Fig. 2C) (Li et al., 2023). After the HPT, the secondary structure of walnut proteins becomes disordered, disulfide bonds are broken, and the exposed hydrophobic tails of the protein form stronger interactions at the gas-water/oil-water interface, resulting in a more stable foam and emulsion.

Typically, pressure treatment below 200 MPa causes protein unfolding and denaturation, enabling HPT to improve emulsion stability by exposing hydrophobic groups. This results in smaller droplet size and changes in protein flexibility, leading to a more uniform distribution of polar and non-polar amino acid residues, thereby enhancing emulsifying performance (Högg et al., 2024). In addition, HPT minimally affects the sensory and nutritional properties of the final product, allowing the original flavour and nutritional content of walnut proteins to be maintained (Baskıncı & Gul, 2023). However, pressures exceeding 400 MPa may prompt reassociation of the exposed thiol groups of walnut proteins into disulfide bonds, resulting in larger aggregates and reduced solubility, further affecting other functional properties (Qin et al., 2013).

2.1.4. Superfine grinding (SG)

Air separation and shear effects in the SG can pulverise walnut proteins and increase their surface area and porosity. This can improve the dispersibility and solubility of walnut proteins while providing them with a more extended structure and greater flexibility (Gao, Chen, Wang, & Meng, 2020).

Benefiting from SG at 15–45 Hz, the solubility of walnut proteins can increase by approximately 19 %, and their foaming capacity and emulsion stability can increase 1.92- and 1.4-fold, respectively (Zhang et al., 2023). This indicates that SG disrupts the spatial structure of the walnut proteins, transforming the ordered β-fold structure in the secondary structure into an unordered β-turn structure. The extension of the structure leads to the exposure of hydrophobic groups, enhancing the stability of walnut proteins at the gas-water or oil-water interface (Janssen, Lambrechts, Pynket, & Wouters, 2023).

Although SG has a positive effect on improving the water solubility and emulsifying properties of walnut proteins, its powerful crushing action may generate high temperatures owing to strong friction forces, leading to the disruption of the intermolecular forces between walnut protein molecules and protein denaturation (Liu, Liu, et al., 2023; Zhou et al., 2016). Therefore, for practical production, temperature control should be carefully managed, and intermittent grinding methods should be considered for the modification process.

2.2. Heat treatment (HT)

In addition to using mechanical action to improve the structure and properties of walnut proteins, HT can have an impact on the structure and functional properties of walnut proteins using methods such as moist or dry heat (Wu, Chen, Van Damme, De Meulenaer, & Van der Meeren, 2023). During heating, the original secondary and tertiary structures of walnut proteins undergo disruption or recombination, often accompanied by conformational changes in the amino acid side chains within the protein molecules (Li, Tian, Liu, Dou, & Diao, 2023). Modification of conformational sites affects the antigenic stability of walnut proteins, subsequently diminishing their sensitising properties (Anna & Alessandro, 2009).Compared to mechanical processing, this method offers advantages such as rapid implementation and ease of large-scale production. After subjecting walnut proteins to moist HT in a boiling water bath, the antigenicity of these proteins was observed,

which resulted in a beneficial effect of reducing the sensitization to walnut proteins (Jiang, Zhao, & Han, 2018). This is because HT causes changes in the solubility and immunoreactivity of walnut proteins, thereby reducing their allergenicity (Ma, Tong, Chen, Li, & Long, 2024). However, when dry HT was applied to walnut kernels at a high temperature of 120 °C, the solubility and emulsifying activity of the walnut proteins notably decreased (Shang, 2022). This is due to the higher temperature of dry HT causing the peptide chains of natural walnut proteins to unfold and recombine through hydrophobic interactions and disulfide bonding, leading to the formation of larger, less soluble aggregates, thereby affecting water solubility (Van de Vondel, Lambrecht, & Delcour, 2022). Although HT is more feasible for industrial implementation than conventional mechanical and physical modification methods, further in-depth research is required to explore the specific improvement mechanisms related to the choice of HT temperature and its impact on the functional properties of walnut proteins.

2.3. Radiation treatment (RT)

Walnut proteins can directly absorb radiation energy, leading to structural alterations. Concurrently, protein denaturation yields hydroxyl groups and other free radicals (Lalande et al., 2019). Therefore, RT essentially involves using the effect of electromagnetic waves to induce energy absorption in the polar groups of walnut proteins, resulting in surface structural modifications accompanied by peptide chain degradation and stretching (Han, Cai, Cheng, & Sun, 2018). Moreover, generation of superoxide anion radicals during radiation may alter the primary structure of walnut proteins (Farkas & Mohácsi-Farkas, 2011).

As a form of electromagnetic wave, γ radiation possesses formidable penetrating power and can induce the degradation of walnut protein peptide chains, thereby enhancing antigen stability and effectively reducing the allergenicity of walnut proteins (Su, Venkatachalam, Teuber, Roux, & Sathe, 2004). Electron beam irradiation (EBI) significantly affects the surface structure of walnut proteins, and increasing the irradiation dose leads to a loose, porous protein surface, thereby enhancing thermal stability (Zhao et al., 2017). In addition, walnut protein obtained through RT has a porous structure that easily forms cross-links with active substances (Tang, Li, Huang, & Yan, 2021). In comparison to heat and mechanical treatments, RT avoids the production of radioactive substances, and offers advantages such as cost-effectiveness and high efficiency (Tran, Nguyen, & Tran, 2022). These properties can be used to improve the walnut protein performance. However, excessive RT may lead to the aggregation and crosslinking of walnut proteins, potentially affecting their emulsifying and water/oil-holding capacities (Zhao et al., 2017). Therefore, strict control of the irradiation dosage and duration is advisable during practical operations.

Physical modification is the most widely used method for modifying walnut proteins because of their lack of chemical introduction. However, physical modification techniques have limited effectiveness in promoting walnut protein digestion and improve nutritional properties. In addition, physical modifications often require excessive energy consumption, leading to increased modification and maintenance costs at the laboratory stage. Furthermore, the equipment used for physical modification is sensitive to environmental factors, and the rational adjustment of protein processing concentration, equipment operating power, and duration are important factors that need to be explored to achieve industrial-scale production. Therefore, combining the physical modification of walnut protein with other modification techniques would be beneficial for addressing these challenges.

3. Chemical modification

In addition to modification methods based on physical processes, the chemical modification of walnut proteins has been widely used because of its efficiency and rapidity. This method is commonly achieved by adding new functional components or eliminating specific elements from the protein structure (Liu et al., 2022). The side chains of walnut proteins, including amino, carboxyl, sulfhydryl, and carboxyl groups, can form new groups through covalent binding with carbohydrates, phenols, phosphates, oxidising agents, leading to changes in protein solubility, emulsifying properties, and other functional characteristics (Glusac & Fishman, 2021; Shi, Huang, Yu, Lee, & Huang, 2011). Currently, the commonly used chemical modification methods for walnut proteins include glycosylation, phosphorylation, polyphenol coupling, and oxidative modifications. The modification methods and their effects on the performance improvement are outlined in Table. 2.

3.1. Glycosylation

In general, the Maillard reaction can lead to the formation of new conjugate products by covalently binding lysine residues in walnut proteins with the hydroxyl groups of carbohydrate molecules (such as inulin, dextran, and glucose) (Jiménez-Castaño, Villamiel, & López-Fandiño, 2007). These products, known as glycosylation products, typically exhibit a more extended structure, higher solubility, and improved stability than singular walnut proteins (Eichler, 2019).

The glycosylation product obtained by the reaction of walnut proteins with inulin at a 0.5 mass ratio at 89 °C for 75 min showed a maximum increase in solubility of up to 47 %, with a improvement of 49 % and 23 % in foamability and foam stability, respectively (Chen et al., 2023). When glucose and walnut proteins were heated at 95 °C for 3 h, the product contained formations of C═O, C═N, and C—N, and its microscopic morphology revealed the formation of smaller particles. The enhanced specific surface area endowed it with excellent antioxidative properties and resulted in a 52.14 % improvement in its emulsifying activity and a 54.31 % enhancement in emulsion stability (Ullah et al., 2019). Further studies are required to improve the digestive characteristics and allergenicity of walnut proteins after glycosylation. Additionally, it is imperative to consider the potential generation of harmful substances during glycosylation. The Maillard reaction during glycosylation typically involves prolonged high-temperature heating, which may lead to the formation of heterocyclic amines and acrylamides, potentially influencing their conjugate action and resulting in undesirable colour formation. In such scenarios, decolourisation may be necessary (Gupta et al., 2018).

3.2. Phosphorylation

Phosphorylation has garnered attention as a walnut protein modification method that does not require supplementary heat energy and can occur spontaneously (Xiong, Zhang, & Ma, 2016). The hydroxyl groups on serine and threonine or the free amino groups on lysine and arginine in walnut protein can interact covalently with phosphate groups in substances such as sodium trimetaphosphate (STMP) to form phosphorylated walnut protein products (Wang, Yang, Fan, Zhang, & Chen, 2019; Xiong et al., 2016). Thus the electronegativity of the phosphorylated walnut proteins molecules is altered, lowering the isoelectric point of the protein and leading to changes in its secondary and tertiary structures (Zhang, Hou, et al., 2023). This results in enhanced solubility and other functional properties. In addition, the phosphorylation reaction does not yield toxic or harmful by-products, indicating its significant potential for application in the food industry (Hadidi, Jafarzadeh, & Ibarz, 2021).

Furthermore, phosphorylation modification using STMP resulted in a more disordered secondary structure of walnut proteins compared to the original form. This altered structure has led to approximately 3.29-, 3.5-, and 5.7-fold increases in solubility, emulsifying activity, and emulsion stability, respectively (Yan & Zhou, 2021). The emulsion prepared using phosphorylated walnut proteins exhibited smaller particle size and higher stability. This is attributed to the higher surface hydrophobicity and surface charge of phosphorylated walnut proteins, which effectively

Table 2
Effect of chemical modification on the functional properties of walnut proteins.

Modifications	Processing conditions	Structure changes	Functional characteristics							Ref.
			SO	WHC	OHC	FC	FS	EAI	ESI	
Glycosylation	WP + inulin (△) 89 °C, 75 min	Tertiary structure changed.	+0.47	/	/	+0.49	+0.23	/	+0.17	(Chen et al., 2023)
	WP + glucose (△) 95 °C, 3 h.	C=O, C═N, and C—N were generated and the H_0 was reduced.	/	/	/	/	/	+0.52	+0.54	(Ullah et al., 2019)
	WP + Dextran (△)	A is disrupted and the protein structure is stretched.	+4.58	+1.13	−0.26	+0.27	+0.05	/	/	(Zhang et al., 2020)
Phosphorylation	WP + STMP adjust pH for reaction	Protein surface is rough and porous; H_0 enhancement; A → B.	+3.29	/	/	/	/	+3.50	+5.70	(Yan et al., 2021)
Polyphenol coupling	WP + WFP adjust PH to 9	B → C; tertiary structural changes.	+0.9	/	/	/	/	/	/	(Kong et al., 2023)
	WP + JP adjust PH to 11	A → B, H_0 was reduced and antioxidant activity was increased	+1.20	+0.35	+0.09	+0.60	+2.6	+0.12	+0.55	(Wei, 2024)
Oxidative modification	WP +Fe/H_2O_2/ASC 4 °C,24 h	Functional groups producing aldehydes and ketones; protein aggregation; increased porosity.	−0.45	+1.37	+1.46	+0.43	+0.89	+1.09	+0.53	(Zhang et al., 2021)
	WP + APPH 37 °C, 24 h.	A, B → D; tryptophan degradation; tertiary structure altered.	/	+0.21	+0.28	+0.61	+0.15	/	/	(Zhao et al., 2022; Zhang et al., 2022)
	WP + MDA Protected from light, 24 h	A, B → D; Tryptophan buried, tertiary structure changed.	/	/	/	+0.14	+0.18	−0.44	+0.09	(Sun et al., 2022)

Note: "△" indicates the process of heating, "+"indicates the lifting multiplier, "-"indicates the falling multiplier. A: α-helical, B: β-sheet, C: β-turn, D: random coil. Solubility (SO), Water Holding Capacity (WHC), Oil Holding Capacity (OHC), Foaming Capacity (FC), Foaming Stability (FS), Emulsifying Activity (EAI), Emulsifying Stability (ESI), Surface Hydrophobicity (H_0), "/" indicates not studied in the literature.

mitigate protein aggregation and precipitation, leading to an increased adsorption capability at the oil-water interface (Moure, Sineiro, Domínguez, & Parajó, 2006). Treating walnut protein with sodium trimetaphosphate (STMP) enhances the repulsion between walnut protein molecules as well as increases antioxidant activity, thereby improving its nutritional value. As a result, phosphorylated walnut proteins with high antioxidant activity have been used to deliver curcumin, enhancing its solubility and stability (Ling et al., 2022).

3.3. Polyphenol coupling

Walnut protein chains contain nucleophilic amino acid residues that can form covalent cross-links with quinones formed by the oxidation of polyphenols through C—N or C—S bonds, thus creating cross-linked proteins or high-molecular-weight polymers (Ozdal, Capanoglu, & Altay, 2013; Zou et al., 2019). The interaction of walnut protein with polyphenols, as the natural active component, can also endow walnut protein with enhanced nutritional attributes and is of significant importance in reducing its allergenicity (Parolia et al., 2022). This interaction confers excellent antioxidant activity to walnut proteins (Keppler, Schwarz, & Vander Goot, 2020). Polyphenols commonly used for coupling reactions with walnut proteins include walnut film polyphenols (WFP), ellagic acid (EA), epigallocatechin-3-gallate (EGCG), and jujube polyphenols (JP).

Under different pH conditions, WFP covalently coupled with lysine residues in walnut proteins to form larger soluble polymers. This process alters the secondary and tertiary structures of walnut proteins and transforms the local environment of chromophores from relatively hydrophobic protein domains to a more hydrophilic overall structure, resulting in a solubility increase of approximately 90 % (Kong et al., 2023). By modulating the concentrations of EA and EGCG, it was observed that the α-helix content in walnut proteins was positively correlated with the polyphenol addition level. This validates the close association between the polyphenol type, concentration, and walnut protein structure (Huang et al., 2022). Moreover, the conjugation of EGCG with walnut protein can promote the balance of allergy cell factors to alleviate allergy-induced intestinal barrier damage, therby ameliorating allergic symptoms (Vencia et al., 2018). This phenomenon has also been observed in the covalent coupling of JP with walnut proteins (Wei, 2024). Furthermore, polyphenols obstruct positively charged lysine residues, resulting in an increased protein surface charge and reduced hydrophobicity, thereby imparting favourable emulsification, thermal stability and antioxidant capacity to walnut proteins (Fig. 3) (Huang et al., 2022).

3.4. Oxidative modification

During oxidation, the intrinsic covalent structure of walnut proteins is altered, accompanied by intermolecular linkages and peptide chain breakage, which may lead to the deterioration of sensory and functional properties (Wang, Wu, Mao, & Zhang, 2024). However, moderate oxidative stress can regulate the structure and functional properties of walnut proteins by altering the internal redox state of the protein molecules, including the oxidation of side chain groups, fragmentation of the protein skeleton, unfolding, degradation, and aggregation (Duan et al., 2018). Typical oxidising agents used for the oxidative modification of walnut proteins include hydrogen peroxide (H_2O_2) and malondialdehyde (MDA).

By establishing a hydroxyl radical oxidation system, it was observed that, with an increase in the concentration of H_2O_2 within the oxidation system, the porosity of walnut proteins increased. In addition, hydroxyl radicals attack the primary peptide or side chains of walnut proteins, leading to the formation of carbon-centred radicals and the subsequent generation of carbonyl derivatives such as aldehydes and ketones (Zhang et al., 2021). Owing to protein aggregation, the solubility of walnut proteins significantly decrease. However, the foaming, water-holding, and oil-holding capacities demonstrated a gradually increasing trend. In addition, structural changes caused by the oxidative

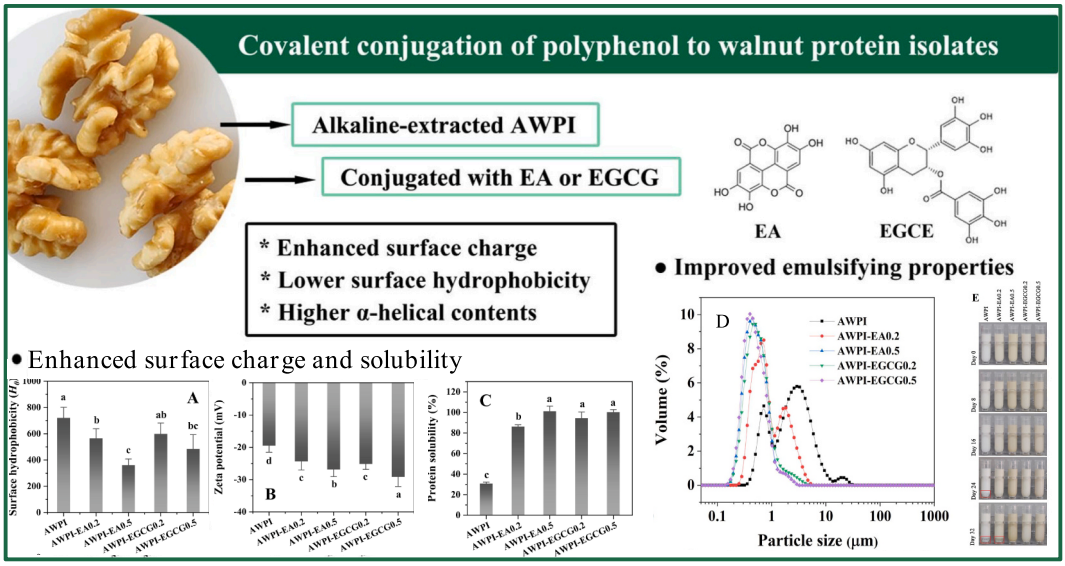

Fig. 3. Effect of walnut proteins in combination with polyphenols on proteolysis, antioxidant activity and emulsification (Huang et al., 2022).

modification of walnut protein lead to resistance during the gastric digestion phase and promote its tendency to be digested in the small intestine (Zhao et al., 2022). Oxidation induced by low concentrations of MDA causes the secondary structure of walnut proteins to become disordered, burying tryptophan residues in the tertiary structure, and resulting in approximately 1.4- and 1.8-fold enhancement in emulsion stability, foaming capacity, and foam stability, respectively (Sun, Wu, & Mao, 2022). Moderate oxidative modification can enhance the conformation of walnut proteins and improve their functional and digestive properties. These findings provide crucial theoretical support for the optimalstorage and processing of walnut proteins.

The effectiveness of chemical modification is often significantly influenced by the type of carbohydrate, polyphenol, phosphate, and the reaction conditions. Moreover, critical reaction sites between walnut proteins and these chemical substances are often overlooked. Therefore, a thorough understanding of the reaction sites between the substances introduced during chemical modification of walnut proteins is crucial. In addition, the chemical modification process may lead to the accumulation of harmful byproducts, potentially causing environmental pollution and impacting the sustainability and environmental friendliness of processing. Therefore, strict risk assessmenst and regulations are required before practical application to ensure food safety.

4. Biological modification

In contrast to chemical and physical modifications, the biological modification approach has high sensitivity and reproducibility received significant research attention (Arte et al., 2015). Currently, the primary techniques for biologically modifying walnut proteins involve enzymatic methods such as enzyme hydrolysis and enzyme cross-linking, as well as microbial fermentation. These methods have the potential to enhance the functional properties and digestibility of walnut proteins as well as offer significant advantages in mitigating the impact of allergenic components (Schlegel, Lidzba, Ueberham, Eisner, & Schweiggert-Weisz, 2021). The treatment conditions and performance improvements of these methods are listes in Table. 3.

Table 3
Effect of Biological modification on the functional properties of walnut proteins.

Modification	Enzyme / strains	Reaction site/conditions	Modified characteristics	Ref.
Enzymatic hydrolysis	Neutral protease	Carboxy-terminal peptide bonds of hydrophobic amino acids	Preparation of Fe^{2+} chelating peptides with high antioxidant activity	(Lv et al., 2017)
	Alkaline protease	Carboxyl side chains of hydrophobic and aromatic amino acids	Enhances antioxidant activity and improves memory	(Fan et al., 2022; Wei et al., 2024)
	Pepsin	Peptide bonds of aromatic amino acids at the amino or carboxyl terminus	Acquisition of ACE-inhibiting peptides with high antioxidant activity	(Wang et al., 2016)
	Trypsin	Peptide bond on the carboxyl side of arginine or lysine	SO (+0.74), H_0 (+1.2) Enhanced EAI, ESI and FC, hypotensive activity	(Jin et al., 2020; Wang er al., 2015)
	Papain	Peptide bonds at the carboxyl terminus of lysine, arginine and glycine residues	Free radical scavenging activity	(Liu et al., 2016)
Enzymatic cross-linking	TGase	ε-amino group on lysine, γ-hydroxylamino group on glutamic acid	SO (+4.61), -SH (−0.39), H_0 (−0.32)	(Shi et al., 2018)
Microbial fermentation	*Bacillus subtilis*	Inoculum 10.40 %, 37 °C, 82 h	An ACE-inhibitory peptide was prepared	(Zheng et al., 2018b)
	Aspergillus nige	Inoculum 5.50 %, 33.5 °C, 84 h	Enhanced antioxidant activity and Fe^{2+} chelating activity	(Wu et al., 2013)
	Bacillus natto	Inoculum 11 %, 33 °C, 84 h	Hydrolysis up to 37.5 %	(Gao, Yan, Yue, Lei, & Xv, 2016)
	Lacticaseibacillus	Inoculum 13 %, 37 °C, 12 h	Enhanced antioxidant activity	(He & Chen, 2022)

Note: "+"indicates the lifting multiplier, "-"indicates the falling multiplier. Solubility (SO), Foaming Capacity (FC), Emulsifying Activity (EAI), Emulsifying Stability (ESI), Surface Hydrophobicity (H_0).

4.1. Enzymatic modification

4.1.1. Enzymatic hydrolysis

The robust structure of walnut proteins can be enzymatically cleaved, resulting in the breakdown of peptide bonds between proteins and generation of peptide fragments (Shi et al., 2018a). These peptides have the potential to interact with each other or with water molecules, ultimately enhancing walnut protein properties. Enzymes can catalyze and break down the long chains of walnut proteins through targeted action, and through the process of separation and purification, bioactive peptides can be obtained (Wouters, Rombouts, Fierens, Brijs, & Delcour, 2016). They often exhibit distinctive biological activities, including antioxidative, antimicrobial, hypoglycemic, immunomodulatory, and neuroregulatory effects (Zhang, Luo, Sun, & Ma, 2021). The commonly used enzymes include neutral proteases, alkaline proteases, pepsin, pancreatin, and papain.

The carboxyl-terminal peptide bonds of hydrophobic amino acids in walnut proteins can be hydrolysed by neutral proteases, leading to the transformation of walnut proteins into low-molecular-weight peptides (Koirala, Prathumpai, & Anal, 2021). Therefore, the hydrolytic action of neutral proteases facilitates the separation and identification of iron-chelating peptides with specific sequences from walnut proteins, enabling improved iron use and metabolism within the organism (Lv et al., 2017). This is crucial for maintaining the balance of nutritional elements in the body and for supporting normal physiological functioning. Furthermore, they also hold the potential to enhance memory and could be used for the development of novel functional foods (Ren et al., 2018). However, the hydrolytic specificity of neutral proteases is not sufficiently strong; thus, a combined approach with other enzymes could achieve improved modification effects.

Alkaline protease can act on the carboxyl side chains of hydrophobic and aromatic amino acids in walnut proteins, catalysing peptide bond hydrolysis to generate peptides with smaller molecular weights (Chen et al., 2022). The use of alkaline proteases for hydrolysis significantly alters the tertiary structure and antioxidant activity of walnut proteins (Fig. 4) (Fan, Mao, & Wu, 2022). This is because the amino acid residues in the hydrolysates being able to interact with ABTS through hydrogen bonding and hydrophobic interactions, thereby exhibiting antioxidant effects. Research has also confirmed that alkaline protease hydrolysis of walnut proteins can impart potential memory-enhancing effects (Wei et al., 2024).

Peptide bonds involving amino- or carboxyl-terminal aromatic amino acids in walnut proteins can undergo cleavage pepsin-catalysed cleavage (Ashaolu et al., 2023). Pepsin exhibits strong specificity; hence, walnut proteins hydrolysed by pepsin show higher antioxidant activity than those hydrolysed by neutral and alkaline proteases. This pepsin hydrolysis process allows for the scavenging of hydroxyl radicals, displaying strong reducing power and inhibiting lipid peroxidation (Chen, Yang, Sun, Niu, & Liu, 2012). By using gastric proteases to hydrolyse walnut proteins, it is possible to obtain angiotensin I-converting enzyme (ACE) inhibitory peptides. These peptides maintain good stability in the gastrointestinal environment and play a crucial role in lowering blood pressure and protecting the cardiovascular system (Wang, Yin, Regenstein, & Wang, 2016).

Trypsin significantly enhances the functional properties of walnut proteins. It catalyzes the cleavage of peptide bonds on the carboxyl side of arginine or lysine in walnut proteins, yielding peptide segments with arginine or lysine as the C-terminal residue (Zhao et al., 2021). After hydrolysis with trypsin, the hydrophobicity of the walnut protein surface increases 1.2-fold, solubility under neutral conditions increased by approximately 74 % (Jin et al., 2020). In addition, trypsin-induced walnut peptides exhibit good antihypertensive activities (Wang, Chen, Li, Zhou, & Xu, 2015).

Papain can enzymatically hydrolyse peptide bonds at the carboxyl-terminus of lysine, arginine, and glycine residues in walnut proteins. This enzyme exhibits strong adaptability as it can hydrolyse of walnut proteins under acidic, neutral, and alkaline conditions (Qazanfarzadeh et al., 2021). Furthermore, research has demonstrated that using papain for the hydrolysis of walnut proteins yields smaller peptides than gastric proteases, leading to further enhancement of free radical scavenging activity (Liu et al., 2016).

4.1.2. Enzymatic cross-linking

Enzymatic cross-linking modification of walnut proteins involves the enzymatic action that forms internal or intermolecular covalent bonds between peptide segments (Glusac, Isaschar-Ovdat, Kukavica, & Fishman, 2017). Enzymatic cross-linking has the potential to enhance the functional properties of proteins while preserving their nutritional characteristics. Transferases and oxidases are among the most commonly used enzymes in the food industry, particularly emphasis placed on transglutaminase (TGase).

The ε-amino group of lysine in walnut proteins and the γ-glutamyl

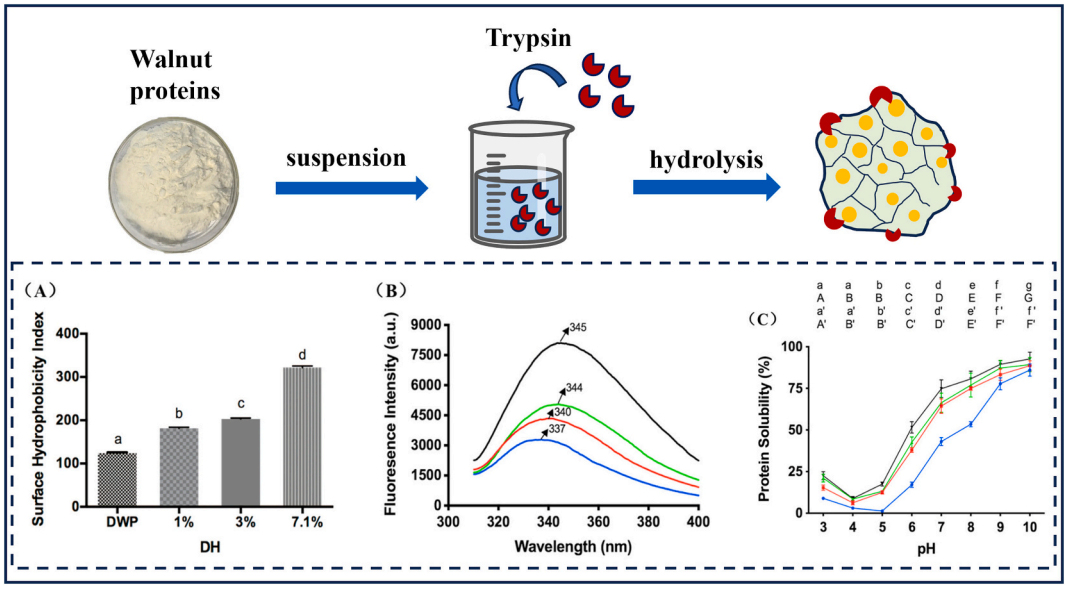

Fig. 4. (A) Surface hydrophobicity, (B) endogenous fluorescence intensity, and (C) proteolysis of WP with different hydrolysis degrees. (In Fig. B and C, different colored lines represent different degrees of hydrolysis) (Jin, Wang, Tang, Regenstein, & Wang, 2020).

amine group can undergo a combined reaction catalysed by TGase, leading to the cross-linking of peptides and the formation of covalent polymers (Yokoyama et al., 2003). TGase treatment selectively cross-links partial peptides of walnut proteins into high-molecular-weight soluble aggregates, resulting in a 4.61-fold increase in solubility and improved thermal stability (Shi et al., 2018). Moreover, the gel properties of walnut proteins were slightly enhanced by TGase treatment.

However, the effect of enzymes on the modification of walnut proteins remains unclear. In addition to endowing walnut proteins with enhanced bioactivity, they confer outstanding nutritional functionality. Therefore, they are important in the formulation of functional foods, nutritional supplements, and pharmaceuticals, and contributed to human health and disease prevention (Olatunde et al., 2023; Shuai et al., 2022). However, it is important to note that the cost of commercial enzymes is high, and the road to developing them into functional products suitable for consumption remains challenging.

4.2. Microbial fermentation

In addition to commercial enzymes, microorganisms can degrade large-molecule walnut proteins through the production of enzymes. Although this mechanism relies on enzymatic action, it differs from purely enzyme-mediated methods. It leverages microbial metabolic byproducts to degrade protein chains and transform them into smaller peptides (Valdés-Velasco et al., 2022). Compared to direct enzymatic hydrolysis, this method reduces modification costs and helps avoid the generation of toxic and bitter compounds during the fermentation process (Zhou et al., 2022). Currently, the bacteria commonly used for the microbial modification of walnut proteins are probiotics, such as *Bacillus subtilis*, *Lacticaseibacillus* and *Bacillus natto*, whereas fungi primarily use *Aspergillus niger* for fermentation.

Using *B. subtilis* for the solid-state fermentation of walnut proteins, a peptide yield as high as 62.20 % was achieved, displaying strong ACE-inhibitory activity (Zheng, Li, & Ding, 2018). In contrast, the fermentation of walnut protein using *Lacticaseibacillus* resulted in a walnut peptide yield of 12.84 mg/g with strong free radical scavenging and reducing abilities (He & Chen, 2022). In addition, fermentation by *A. niger* results in a product with strong in vitro antioxidant activity, making it a promising natural antioxidant for food preservation (Wu et al., 2014). Furthermore, using *B. natto* for liquid-state fermentation of walnut proteins led to peptide content and hydrolysis reaching 2.58 mg/mL and 37.5 %, respectively (Gao et al., 2016).

These studies have confirmed that fermentation is a potential method for enhancing the functional properties of walnut proteins, especially with the use of probiotics which typically have beneficial effects on the human gut. Probiotics can positively affect human health by improving the balance of the gut microbiota, increasing the number of beneficial bacteria, and inhibiting the growth of harmful bacteria. Although microbial fermentation is more economically efficient than enzymatic treatment, current research on modifying walnut proteins through microbial fermentation is limited, and the diversity of microorganisms used is insufficient. Furthermore, careful optimisation based on actual production is necessary during the fermentation process to ensure product stability and cost-effectiveness.

5. Combined modification

However, traditional physical and chemical modifications have certain limitations. For instance, the sole reliance on physical modification may result in incomplete unfolding of the walnut protein structure, leading to limited enhancement of its functional properties (Jin et al., 2020; Li et al., 2023; Sun et al., 2022). Single chemical or enzymatic modifications may not effectively react with external substances due to the compact structure of walnut proteins. To overcome these limitations, the composite modification of walnut proteins have been assessed using two or more methods, whether similar or different, using physical, chemical, and biological approaches. This improves the conformation of walnut proteins and further enhances their functional properties to meet the diverse requirements of different products (Xia, Liu, Dong, Shen, & Wang, 2024). Presently, high-pressure-heat treatment, ultrasound-enzymatic hydrolysis, glycosylation-acylation, enzyme digestion-glycosylation, and other combinations of modification methods have been applied to modify walnut proteins.

Compared with individual modification methods, the combined use of HPT and HT can effectively reduce allergic reactions to walnut proteins in mice. This indicates that such a combined approach holds promise as a method to mitigate allergic immune responses to walnut proteins (Yang et al., 2017). Notably, this may be attributed to the synergistic effects of high pressure and heat, which can significantly alter the conformation of walnut proteins, making them less recognisable by the immune system, thus reducing the likelihood of triggering allergic reactions.

When UT is combined with enzymatic modification, the hydrolysis level of walnut protein by pancreatin can be significantly increased by approximately 85 % compared to a group that only underwent ultrasound pretreatment (Golly et al., 2019). This is due to the ability of ultrasound treatment to unfold the structure of walnut proteins in advance, allowing for rapid binding with enzymes and consequently enhancing their enzymatic activity (Munir et al., 2019). Upon using an ultrasound-assisted alkaline protease for walnut protein extraction, changes in the secondary and tertiary structures of the walnut protein were observed, leading to an increase in the exposed hydrophobic and thiol groups. Compared to the control group, which underwent only UT, the solubility and emulsifying activity of walnut protein were enhanced by approximately 70.77 % and 120.56 m^2/g (Wen et al., 2020). These findings indicate that UT promotes interactions between walnut proteins and enzymes, thereby synergistically improving the functional properties of walnut proteins.

Combining glycosylation with acylation treatment (Wang et al., 2021) was shown to enhance the flexibility of walnut proteins, leading to a reduction in particle size approximately 122-fold, and an increase in water-holding capacity and emulsifying properties 3.41- and 8.46-fold, respectively (Fig. 5). This is attributed to the substitution of the positively charged ε-amino groups during the acylation reaction, resulting in an increase in the electronegativity and electrostatic repulsion of walnut proteins, promoting structure unfolding, reducing aggregation, and providing favourable conditions for glycosylation (Basak & Singhal, 2022). In addition, acylated walnut proteins exhibited an increase in apparent viscosity compared to single walnut proteins. After acylation combined with glycosylation, the apparent viscosity further increased as an appropriate amount of polysaccharides enhanced the water-binding capacity of the protein based on acylation modification, thus increasing the viscosity of the composite system.

Combined enzymatic and glycosylation treatments has been shown to enhance the sensitivity of walnut proteins to acids, alkalis, and heat (Oliveira, dos Coimbra, Oliveira, Zuñiga, & Rojas, 2016). PPeptides from walnut proteins treated with an alkaline protease, followed by glycosylation with chitosan, result in reactions between the hydroxyl (OH) or amino (NH_2) groups of chitosan and amino acid residues in the peptide chain. This disrupts the hydrogen bonds and forms new chemical bonds, significantly altering the secondary structure. These structural changes can increase the solubility of walnut proteins near the isoelectric point by approximately 14.52 %, which cannot be achieved using a single modification method. Moreover, after the combined treatment, the solubility of walnut proteins near the isoelectric point increased by approximately 14.52 %. Their emulsifying ability and stability, compared to single-enzyme modification, can be increased by 10.16 m2/g and 52.73 min, respectively, indicating an enhancement in processing characteristics (Wang et al., 2024).

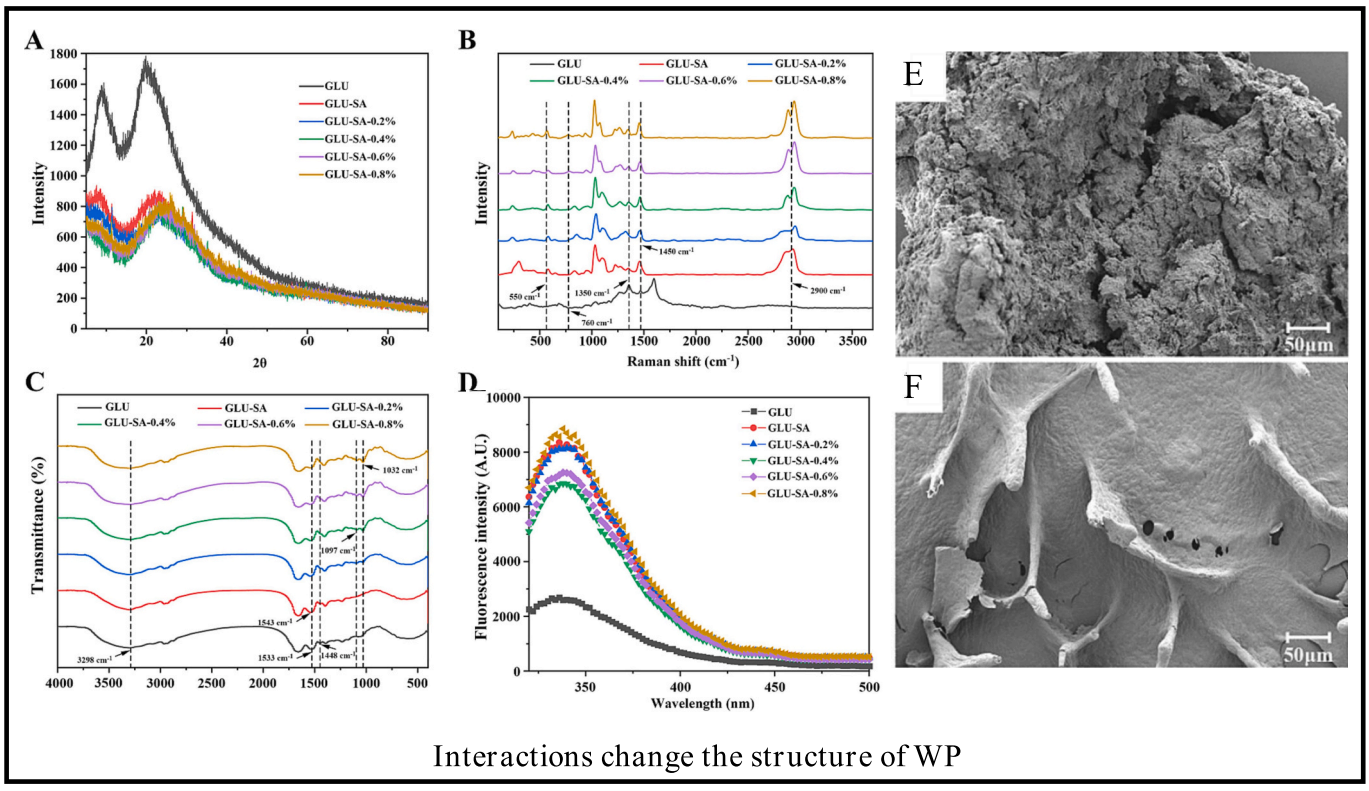

Fig. 5. XRD (A), RS (B), FTIR (C), endogenous fluorescence spectra (E) and SEM of walnut proteins after different treatments (Wang et al., 2024).

6. Challenges and future trends

Based on the current research, there are still gaps and deficiencies in the industrial application of modern walnut protein modification technologies. Most modification techniques are still in the early stages of development and an in-depth understanding of the corresponding mechanisms should still be required. In the realm of physical modification techniques, the extended operation of instruments during mechanical processing (UT, HSH, HPT, and SG) leads to sustained heat exchange throughout the process, resulting in high energy consumption. The incomplete absorption of heat generated by HT leads to its release through heat transfer, leading to control difficulties and safety risks. Moreover, prolonged direct contact between the heating plate and walnut proteins may affect food quality and safety. Regarding RT, there is currently insufficient evidence to indicate that irradiation-modified proteins are hazardous. Potential issues include the formation of azo compounds, oxides, and other compounds, and prolonged exposure may lead to protein degradation or generation of unexpected substances. Compared with physical modification techniques, chemical modification is widely recognised for its effectiveness. However, it is crucial to ensure that the chemicals and methods used for chemical modification of walnut proteins do not compromise their functionality and activity. Thorough testing and evaluation of products are essential to ensure their safety. Currently, biological modification is the safest and most effective method for modifying walnut proteins. However, optimisation of the enzyme and microbial types is required to ensure effective completion of the modification process in the production environment. In addition, research on the use of biological modifications to enhance the functionality of walnut proteins is still limited because of the low solubility of walnut proteins, which is a primary obstacle in creating the reaction conditions required for enzyme activity.

To advance our understanding of the various modification techniques for walnut proteins and their impact on their functional properties, further research on the mechanisms underlying these modification techniques is required. In particular, it is necessary to establish the quantitative relationships between the parameters applied in these techniques and the structure and properties of walnut proteins. These findings provide opportunities for the precise manipulation of the functional, digestibility, and nutritional properties of walnut proteins. Moreover, in addition to traditional physical, chemical, and biological modification techniques, innovative technologies such as low-temperature plasma and pulsed electric fields hold promise for integration into research on walnut protein modification. Notably, combined modification and biological modification, particularly the use of probiotics for the fermentation of walnut proteins, is considered the most promising mode of modification. Therefore, exploring different combined modification methods and using diverse strains of probiotics for fermentation to investigate the relationship between these technical parameters and the functional properties of walnut proteins will be a focal point for future walnut protein modifications. Considering the various processing requirements, a key goal is the long-term preservation of the nutritional components of walnut proteins. Therefore, future developments may increasingly emphasise the development of efficient and specific modification techniques, such as nanotechnology and biotechnology, to achieve targeted improvements in walnut proteins while preserving their original nutritional value and functional properties.

7. Conclusions

Owing to the widespread cultivation of walnuts and their high nutritional value and functionality, walnuts serve as an excellent source of protein. To meet the demand for improved quality and functional proteins, it is necessary to use chemical, physical, or biological modification. These methods can alter the structure, chemistry, surface activity, and biological properties of plant proteins, thereby serving as a panacea to enhance their digestibility, functionality, sensory characteristics, and allergenicity.

In conclusion, ongoing efforts should focus on developing more environmentally friendly and sustainable modification technologies for

walnut proteins and on conducting in-depth research on their modification mechanisms to explore broader application areas. Furthermore, it is crucial to integrate the modification technologies into actual production while maintaining the principles of ecological protection, thereby promoting the industrial application of walnut protein modification technologies to the greatest extent. Overall, this review provides a theoretical basis for the innovation and development of walnut protein-modification technologies.

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CRediT authorship contribution statement

Yunkun Zhu: Writing – review & editing. **Jiangxia Xu:** Writing – review & editing. **Zhongkai Zhao:** Investigation, Conceptualization. **Liang Wang:** Supervision, Investigation. **Jie Yang:** Investigation, Conceptualization. **Minwei Zhang:** Writing – original draft, Visualization, Investigation, Formal analysis.

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.

Data availability

Data will be made available on request.

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