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Review

Food protein aggregation and its application

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ABSTRACT

The phenomenon of protein aggregation in food science is very common, but the formation mechanism is unclear. The formation of protein aggregates (PAs) is influenced by various factors, and aggregates with different sizes and shapes play an important role in food systems. Although there have been many reports on food PAs, many challenges still need to be addressed, and a systematic literature review is lacking. Thus, hypotheses about the PAs formation mechanism were summarized, and the fibrosis aggregates formation mechanism was described. The main findings of this paper indicate that the forces driving protein aggregation are covalent and non-covalent cross-linking interactions. The determination of PAs is mainly based on protein particle size combined with multispectral methods. PAs are not only associated with protein functional properties (such as emulsion and gel) but also related to harmful substances (such as advanced glycation end products, AGEs) formation. Finally, the applications of PAs in food science were summarized. Outlook and challenges were described from the perspectives of food processing conditions and parameters, food components and their interactions, food nutrition and health relationships, etc. This review will attract more food scientists to participate in related research on protein aggregation in the future.

1. Introduction

The phenomenon of protein aggregation occurs in our daily life, such as blood coagulation, making tofu, boiling eggs, cooking cheese, etc. However, protein aggregation in biological processes can also be associated with undesired processes or severe diseases. For example, the phenomenon of protein aggregates (PAs) can be traced back to 1907 following the report of Alois Alzheimer (German psychiatrist, 1864–1915) that proteinaceous amyloid plaques were found in his demented patients' postmortem brains (Lansbury & Lashuel, 2006). After>100 years of investigation and debate, PAs (such as amyloid fibrosis aggregates) associated with neurodegenerative diseases including Parkinson's, Alzheimer's, Huntington's, prion-related disorders, etc., has eventually been recognized (Amani, Shamsi, Rabbani, & Naim, 2014; Wu & Du, 2021; Zhang et al., 2019). Fig. 1 (A) illustrates the number of publications with the keyword "protein aggregates" obtained from the "Web of Science Core Collection" database from 2010 to 2021. The increase in PAs-related publications annually indicates that this research direction has become a potential hot topic.

Take the rapid development of PAs in material science in the past 20 years as an example, when the phenomenon that aggregation-induced emission (AIE) was unintentionally developed and systematically reported by Benzhong Tang and his co-workers in 2001 (Tang et al., 2021), PAs have gradually developed into an independent research discipline marked by the launch of "Aggregate" journal (Online ISSN: 2692–4560) in 2020. However, though the discovery time of food-related PAs is similar to AIE's reporting time, the overall development speed remains a big gap compared with other disciplines. Fig. 1 (B) exhibits the top ten research directions obtained by searching "protein aggregates" in the topic of the "Web of Science Core Collection" database, with the years ranging from 1985 to 2022. It was found that many papers and reviews on PAs have been reported in medicine and life science (Schramm et al., 2020; Amani, Shamsi, Rabbani, & Naim, 2014). However, the PAs review in food science is very limited.

More important, since Fandrich et al. (2001) firstly reported the formation of aggregates in myoglobin, an integral protein present in

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meat muscle to store oxygen and responsible for meat color, PAs has been reported in many foods, including meat (Ebert et al., 2021; Li et al., 2021), milk (Bashir et al., 2021; Gaspard et al., 2017; Wang & Roberts, 2018), soy (Dong et al., 2013; Fang et al., 2013), wine (Jaeckels et al., 2016), eggs (Guo et al., 2020), etc. However, the research on PAs in food science mainly focuses on the aggregation phenomenon, but the mechanism of aggregates formation is relatively lacking. In addition, it is worth noting that food proteins differ from other classes of proteins in terms of composition and overall structure. Food materials are complex systems, and the presence of other components (such as hydrocolloids and active compounds) largely affects the aggregation behavior of proteins (Liang, Ma, Yan, Liu, & Liu, 2019).

Moreover, the protein aggregation should be contextualized. For instance, in the supramolecular chemistry, the protein assembling, or aggregation, concerns the biological systems as self-assembling (cell physiology and biochemistry, milk, meat and other raw matter production) and food processing as the induced-assembling where different physico-chemical conditions determine the final products (Yousuf et al., 2022; Ebert et al., 2021; Li et al., 2021). Many external factors have been reported to affect PAs formation, such as temperature, processing time, pH, oxidation, cooking methods, etc. (Angioloni & Collar, 2013; Ma et al., 2020; Yang et al., 2018; Yousuf et al., 2022; Zhou et al., 2013). However, different external conditions may have synergistic effects on the PAs formation, and the effects between factors are still unclear (Axelrod et al., 2021; Vilotte et al., 2021; Zhao et al., 2020).

Many methods have been applied to determine PAs, such as dynamic light scattering (DLS) (Sun & Zhao et al., 2011), microscope observation (Zhao et al., 2020), low-field nuclear magnetic resonance (LF NMR) (Indrawati et al., 2007), high performance liquid chromatography (HPLC) (Williams et al., 2017), turbidimeter (LaClair & Etzel, 2009), electrophoresis (Promeyrat & Gatellier et al., 2010), rheology (Chen et al., 2018; Maticorena et al., 2018), etc. However, different methods vary in characterization. Hence, collating formation mechanisms under different protein sources and conditions is challenging (Vilotte et al., 2021; Wu et al., 2021).

From the application point of view, Fig. 1 (C) shows the top ten food journals obtained with searching "food protein aggregates" in the topic of the "Web of Science Core Collection" database with the years ranging from 1985 to 2022. The results show that the top two journals on PAs research published in food science are "Food Hydrocolloids" and "Food Chemistry". It's not difficult to find out that the application of PAs is mainly focused on the phenomenon and mechanism of protein gelation and emulsification by looking for research papers on PAs in these two journals. But many other research directions such as food engineering, food safety, food nutrition, etc., lack theoretical guidance and deep investigation.

Hence, to provide comprehensive research progress on PAs in food science, the present work aims to summarize the following points: (1) the PAs formation mechanism; (2) factors affecting the PAs formation; (3) PAs detection methods; and (4) PAs application in food science. The

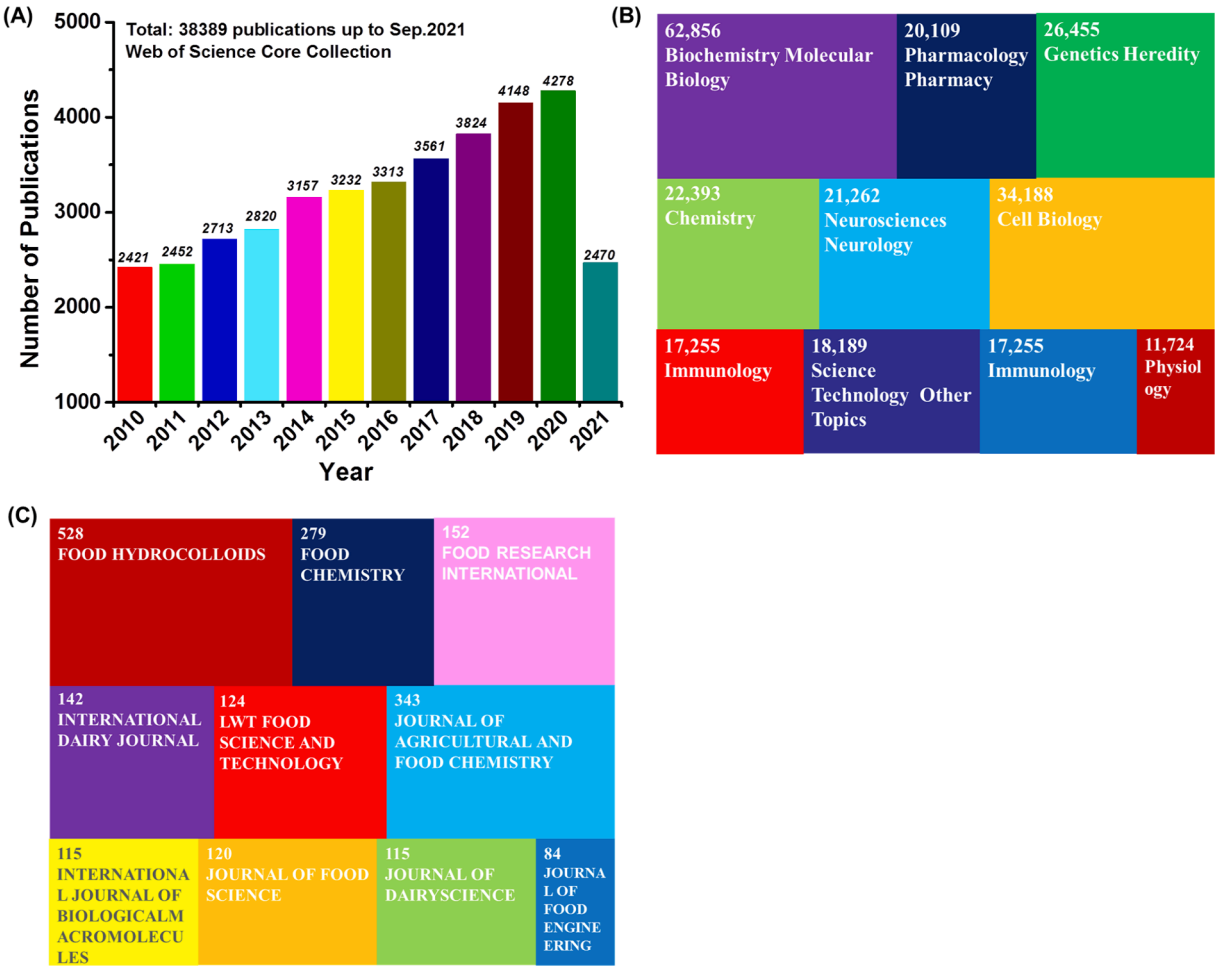

Fig. 1. (A) Number of publications was obtained by searching "protein aggregates" in the "Web of Science Core Collection" database with the years ranging from 2010 to 2021. (B) Top ten research directions were obtained by searching "protein aggregates" in the "Web of Science Core Collection" database ranging from 1985 to 2022. (C) Top ten food journals were obtained by searching "food protein aggregates" in the "Web of Science Core Collection" database database ranging from 1985 to 2022.

ultimate goal is to provide some scientific insights to promote PAs research in food science in the future.

2. The PAs formation mechanism

2.1. Mechanism hypothesis

PAs can be categorized into different structures and sizes, such as oligomers, protofibrils, amyloid fibrosis, spherulites, particulates, amorphous aggregates, etc., and can also be classified as soluble and insoluble (Cox et al., 2020; Jung et al., 2008). Specifically, the amount of soluble aggregates is small (5–10 %), with a low molecular mass and reversible nature. When insoluble aggregates are larger than soluble aggregates, correlating with the size of particulates upon reconstitution (Pham & Meng, 2020). Roberts, (2014) asserted that PAs mediated by unfolded or partially unfolded monomers induce different effects on macroscopic aggregates (>100 nm) and phase separation, which involves the formation of protein-enriched droplets or macromolecules mediated by protein–protein interaction. The correlation between particle size and concentration is very complicated during protein aggregation. It was reported that gaps were found between the aggregates/particles' size and concentration under different aggregation mechanisms (Amin et al., 2014). Therefore, factors, such as the size range and particle concentration should be considered when evaluating the formation of PAs.

The molecular mechanisms of PAs have been discussed extensively in the medical, biological, and colloid fields. These theories provide references for studying the formation mechanism of food PAs. These hypotheses include (1) "protein fibrosis theory", a feature of high level of β-sheet structures mediated by improper folding or misfolding *in vivo* and *in vitro* (Lansbury & Lashuel, 2006). However, this theory applies to the formation of fibrosis aggregates, which is difficult to explain for other shape aggregates (such as spherulites, particulates, and amorphous aggregates); (2) "balanced-imbalanced theory" following research on proteome mislocalization and aggregation in neurodegenerative disease (Moscatelli, 2018). Typically, once the balance in the protein control system is disrupted under stress conditions, the aberrant PAs would lose their original functions, resulting in cytotoxicity (Wu & Du, 2021). In other words, proteins immobilized on surfaces without losing activity (balance status) in normal solutions but aggregate in highly concentrated crowded solutions (imbalance status) (Cox et al., 2020). This theory is applicable to protein aggregation under physiological conditions, but many proteins in foods are *in vitro*; (3) "structure and function theory" is also recognized. Specific proteins with definite structures can perform their specific functions. Otherwise, their biological functions and activities will be impaired (Wu & Du, 2021). This indicates that PAs are impacted by a protein's structural characteristic *in vitro*. According to Ebert et al. (2021), surface hydrophobicity, the amount of charge at the protein's surface, oxidation and glycation modification, covalent and non-covalent interactions of protein or peptide side-chains can influence or modulate PAs. Most peptides achieve their functional or reach their native state by folding into a specific three-dimensional structure. However, once the non-covalent interactions within the protein were disrupted under stress conditions, the advanced (secondary or tertiary) structure would be lost, generating a PAs structure (Schramm et al., 2020). This theory may match the situation of protein aggregation in food gel system, but the relationship between function and other structures, such as one- (native monomers) and two- (pre-existing aggregates), is worthy of further consideration; (4) Other theories, including "phase transitions theory" and "DLVO theory (very classical theory to naturally folded globular proteins from Derjaguin, Landau, Verwey and Overbeek)" are proposed at soft matter colloid field (Mezzenga & Fischer, 2013). These theories consider the aggregation of spherical molecules, ambient temperature, surface charge, ionic strength and other factors (Mezzenga & Fischer, 2013). Meanwhile, the possibility of forming aggregates at the interface of solid–liquid, liquid–liquid, gas–liquid, etc., are also considered (Roberts, 2014).

The limitations of research on the formation mechanism of food protein aggregation are summarized as follows:

- The protein folding and aggregation literatures generally rely on pure protein model systems. However, in proteinaceous food systems (e.g. dairy products, baked goods, meats), it is rare to encounter highly purified protein systems.
- Instead, heterogeneous protein mixtures (often in combination with a non-protein component such as fats and sugars) are used, adding more complexity for deciphering underlying molecular mechanisms.

Therefore, food PAs based on research models have certain limitations compared with existing complex food systems. From the perspective of the formation mechanism, we should start from the phenomenon, establish a model, and then return to the specific food system for verification. Overall, the formation mechanism should be combined with the actual situation of specific food or biological macromolecular systems to summarize its unique molecular mechanism, rather than a unified mechanism.

2.2. Fibrosis aggregates

Because PAs are derivatives of multiple reactions, including native monomers, denatured proteins, and pre-existing aggregates, the formation mechanism of PAs in food science is complicated (Jung et al., 2008). The general order of aggregation includes unfolding of the folded proteins yields misfolded intermediates, then monomers or oligomers evolve into insoluble aggregates such as amorphous aggregates or fibrosis. Moreover, monomers or denatured native proteins can also integrate into oligomers (Tang et al., 2021).

The formation mechanism of fibrosis protein aggregation in food science is relatively clear (Meng et al., 2022). Thus, this work summarized the fibrosis aggregation mechanism in foods. To begin with, understanding the kinetics of self-assembly of monomeric proteins into fibrosis aggregates is essential to illustrate the possible mechanism of fibrosis PAs (Ramamoorthy, 2018). Fig. 2 (A) exhibited the self-template aggregation based on the "growth through nucleation theory" (Knowles et al., 2009). This process includes three main stages: (1) elongation, (2) fragmentation, and (3) leading to sigmoidal kinetic curves for the mass concentration of fibrosis as a function of time. Specifically, the *S-curve* has three periods: induction, growth and stationary. Aggregates are formed following the accumulation of deposits in the critical nucleus, as protein naturally cannot solely initiate the process. This process, also named "primary nucleation theory", usually shows a lag period, during which no apparent deposits appear for a long time. The smaller primary nucleation, also known as oligomerization or secondary nucleation, is the new nucleus and has a stronger tendency to aggregate (Michaels et al., 2019).

Secondly, "docking-locking" was also proposed to elucidate fibrosis PAs formation (Fig. 2 (B)). Typically, when a free chain approaches the aggregation core area, its molecule will be attracted to the area. During this period, the free chain is yet to conform to β-sheet structure. This process is called docking. As the captured free chain undergoes a series of conformations to form aggregates, the process is named locking (Straub & Thirumalai, 2011).

Tertiary, "hydrolysis and assembly" was also proposed to elucidate fibrosis PAs formation. For example, β-lactoglobulin (β-lg), an integral component of whey protein isolate (WPI) could produce fibrosis aggregates through hydrolysis and assembly (Fig. 2 (C)). The β-lg is acidically hydrolyzed (pH = 2, 80 °C) into peptides with rate k_n. With high hydrophobicity and capacity to form β-sheets, these peptides could be self-assembled into fibrosis with rate k_2 (Ardy Kroes-Nijboer et al., 2012).

The limitations or future trends of food protein fibrosis aggregation

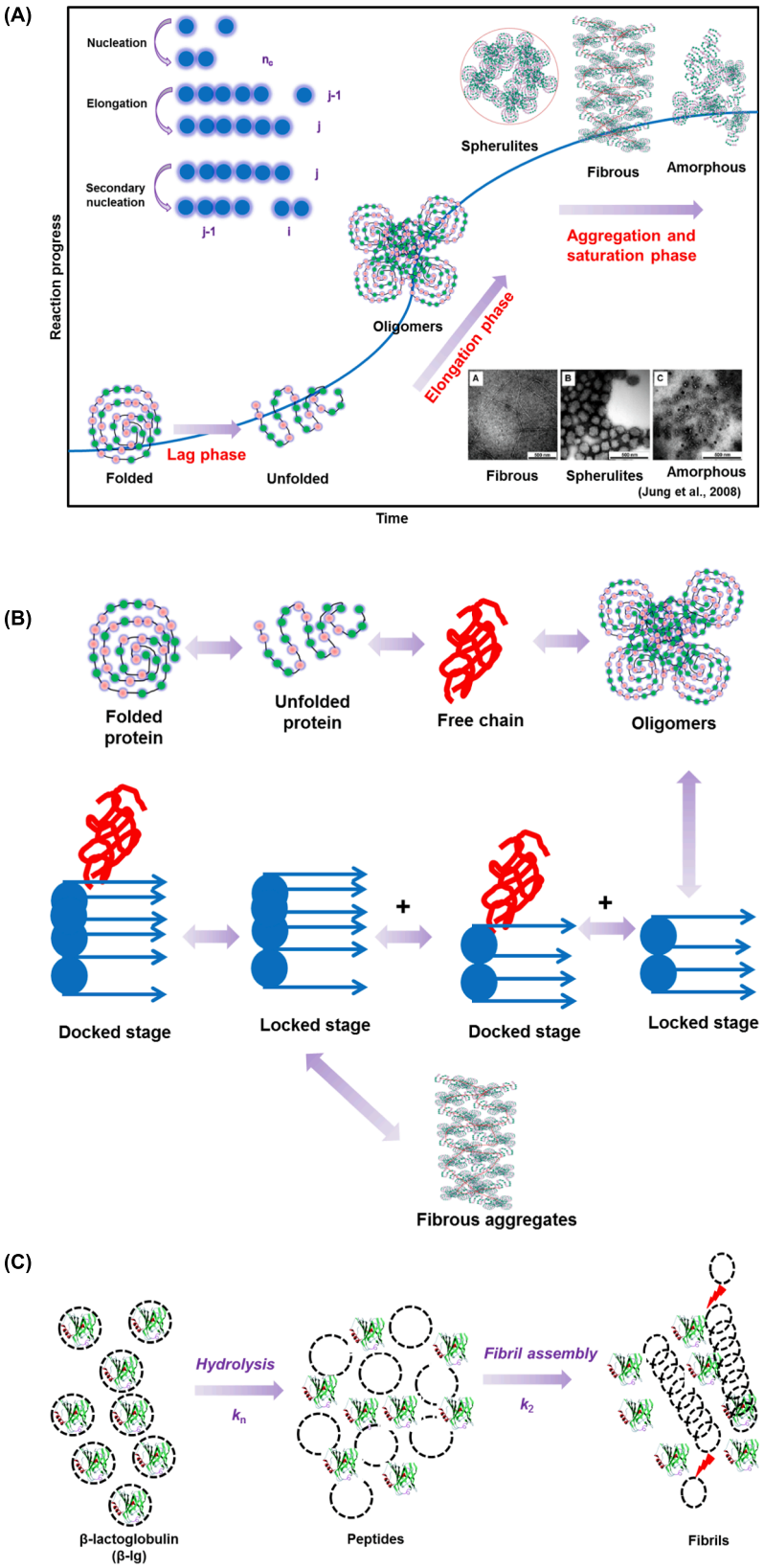

Fig. 2. (A) The "growth through nucleation theory" mechanism of protein fibrosis aggregation. (B) The "docking-locking" mechanism of protein fibrosis aggregation. (C) The "hydrolysis and assembly" mechanism of protein fibrosis aggregation. (D) The PAs formation mechanism of egg white protein (ovotransferrin, ovalbumin, and lysozyme) under the pulsed electric field (PEF) condition (25 kV/cm, 800 μs). (E) and (F) changes in protein folding, unfolding, and aggregation activation energy.

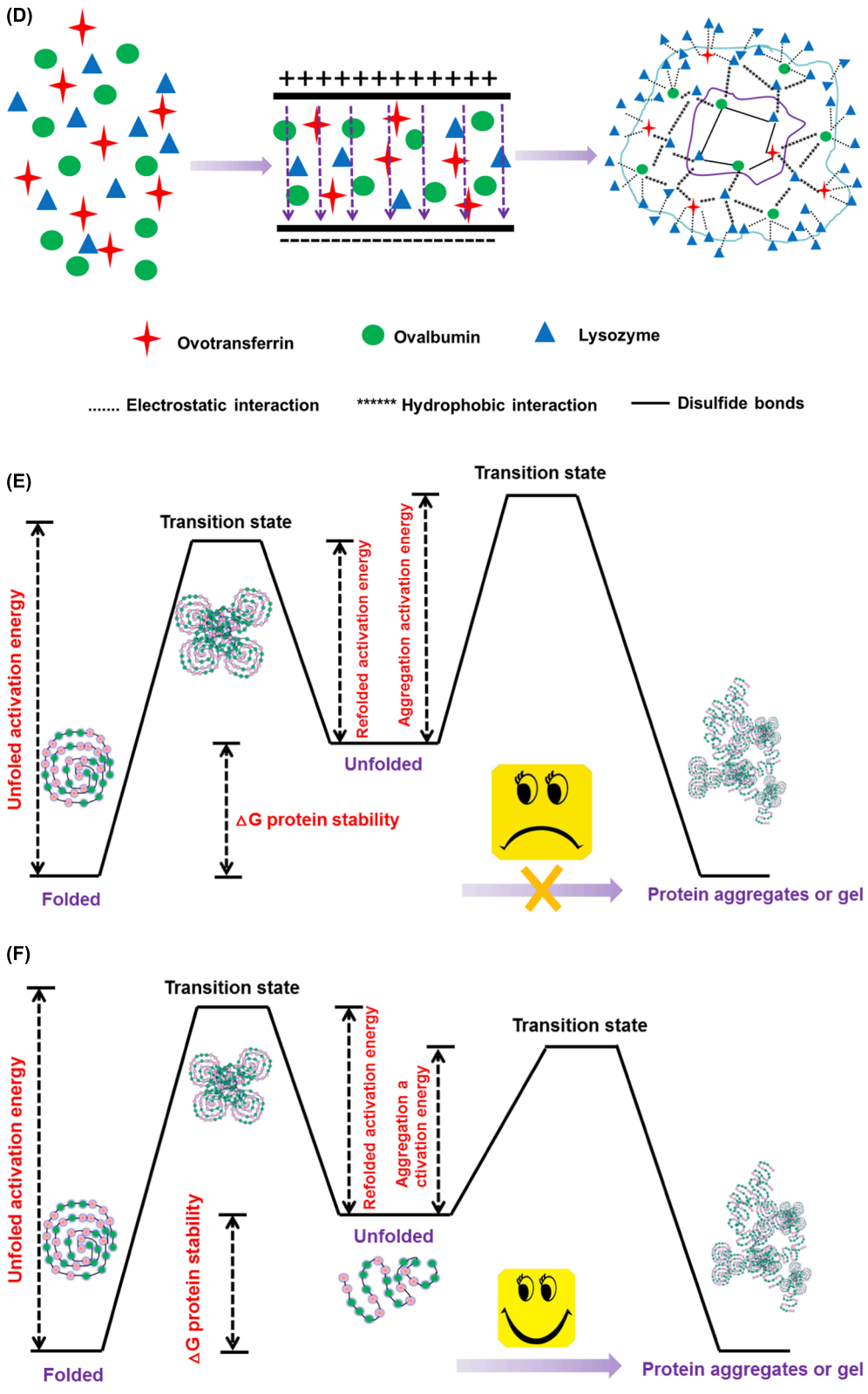

Fig. 2. (*continued*).

are summarized as follows:

- Following the diversity of food materials (meat, dairy, eggs, grains, etc.), the molecular mechanisms of the different food proteins fibrosis aggregation, such as the self-assembly of proteins from different sources and their interactions, require further in-depth research.
- Many protein fibrosis aggregates have been used as protective agents for food colorants (Zhu & Huang et al., 2020). Whether consumers can accept the flavor and quality of their products is still a research direction to be considered. Hence, the need for further research.

- Protein fibrosis has been widely studied as delivery systems of nutritional components (Du et al., 2022; Liang et al., 2019). The fibrosis structures can be used to create a variety of encapsulating materials owing to their excellent gelation and emulsification capabilities. However, there is still a lack of relevant research on the encapsulation of two or more active ingredients with protein fibrils as loading agents, which is also the goal of our future work.

2.3. Driving forces

The fundamental forces and interactions that drive PAs (protein folding) include *van der Waals* and hydrophobic attractions between side-chain and backbone atoms, minimizing steric clashes and energetically unfavorable bond torsional angles, maximizing hydrogen bonding, minimizing (maximizing) electrostatic repulsions (attractions), and minimizing unfavorable interactions between amino acids and the solvent (water) and its co-solutes (Roberts, 2014). These interactions that occur between amino acids within the protein also exist between amino acids in neighboring proteins (Roberts, 2014). Although low molecular weights oligomers with non-covalent linkages can still revert to their native states, the unstable state becomes irreversible once their sizes increase to a certain extent (Tang et al., 2021). This process can be mediated by charge-charge interactions, covalent linkages *via* hydrophilic-hydrophobic residues, or protein conformation dynamic change (Pham & Meng, 2020). Importantly, the interaction between disulfide bonds, hydrophobic and electrostatic interactions is important in PAs formation. For example, Fig. 2 (D) exhibited the PAs formation mechanism of egg white protein (ovotransferrin, ovalbumin, and lysozyme) aggregates under the pulsed electric field (PEF) condition (25 kV/cm, 800 μs) (Li et al., 2015). (1) Under the PEF condition, the protein partially unfolds and results in the exposure of sulfhydryl groups; (2) The inner core is formed by disulfide bonds between ovalbumin, lysozyme and ovotransferrin; (3) The second layer is formed through hydrophobic interactions between lysozyme, ovotransferrin and ovalbumin; (4) The outermost precipitate is formed through lysozyme connection with the second layer by electrostatic interactions.

Understanding the effect of protein aggregation on gels at the molecular level has also been an important topic in food protein research. It is noted that proteins can assume different conformations (from native to aggregate), ranging from unfolded to folded forms. Each conformation has an inherent *Gibbs* energy in all closed protein systems, which can be regarded as the thermodynamic driving process. The lowest *Gibbs energy* corresponds to the highest stable conformations during the complex protein energy landscape, as illustrated in Fig. 2 (E) and **2** (F) (Leeson et al., 2000; Straub & Thirumalai, 2011). The native fold form is favored under normal physiological conditions, known to have a very low *Gibbs* energy. However, once the protein balance is disturbed by the surrounding stress, the stability of the thermodynamic energy landscape will be broken, inducing its transfer to an energy minima form, thereby resulting in aggregation (Hartl & Hayer-Hartl, 2009).

Furthermore, protein aggregation can prevent the hydrophobic group from directly encountering water, thereby returning the system to a stable state. Protein unfolding also results in the exposure of thiol/disulfide bonds within the molecule leading to increased reactivity. The thermodynamic stability of the folded protein determines the degree of hydrophobic group exposure and the possibility of thiol/disulfide exchange reactions (Visschers & de Jongh, 2005). Whether the unfolded protein can aggregate depends on the activation energy and refolding activation energy. When the protein aggregation activation energy is greater than that of refolding, the unfolded protein cannot undergo aggregation and cannot form protein gel when it returns to the folded state (Fig. 2 (E)). Conversely, when the protein aggregation activation energy is less than the refolding activation energy, the unfolded protein can form aggregates, and gels can also be formed (Fig. 2 (F)) (Leeson et al., 2000).

The research limitations or future trends of food protein aggregation driving forces are summarized as follows:

- For the theoretical research of aggregation of globular proteins in the native state, some of the models (such as “phase transitions theory” and “DLVO theory”) have been applied to understand protein aggregation and association, with varying degrees of success. However, other interactions (such as hydrophobic interactions, hydrogen bonds, hydrophilic interaction, and charge residues complementarity) play an important role in the phase behavior of proteins should be further developed.
- For the aggregation of hydrolyzed proteins (this aggregation route is typically found in globular proteins), it has been long debated whether the fibrosis structures found in food proteins only share structural similarities with pathological amyloid fibrosis or whether the self-assembly scheme is general and common to this two shapes (globular and fibrosis) PAs.
- A considerably different type of interaction in aggregating proteins is mediated by covalent bridges (thiol/disulfide bonds). In food proteins, this reaction can be thermally activated and promoted by the unfolding of proteins and the subsequent exposure of cysteine residues, which were protected by the folded tertiary structure prior to thermal unfolding. The effect of the cross-linking drive of disulfide bridges on the different sizes and shapes of PAs remains to be further investigated.

3. Factors affecting PAs formation

The process of PAs formation is complicated as it depends on numerous variables, including pH, oxygen, temperature, metal ions, pressure, concentration, molecular crowding, mechanical forces, and factors associated with the protein source (Liu et al., 2017; Martínez-Maldonado et al., 2020; Ramamoorthy, 2018; Vilotte et al., 2021). Fig. 3 (A) summarized these factors, which mainly affects the PAs formation through two pathways: One is by changing the protein conformation, such as pressure, mechanical forces, temperature, etc. Another is by changing the driving forces or interaction, such as pH, temperature, protein source, etc. Some studies on affecting PAs formation factors are summarized in Table 1.

3.1. Thermal treatment

Different cooking methods have different effects on the formation of PAs. Sous-vide cooking, a milder thermal processing method with less nutrient loss, has received more and more attention in food processing, especially meat processing. Jiang et al. (2022) investigated Sous-vide cooking (65 °C, 70 °C, 75 °C, 8 ~ 12 h) on the effect of braised pork. They found that this low-temperature and long-term treatment methods increased the degradation of myofibrillar protein into antioxidant peptides, alleviated heat-induced aggregation, and improved the digestibility of myofibrillar protein. Notably, compared with the traditional high-temperature treatment method (110 °C, 150 min), the α-helix content of samples was lower, and β-sheet content was higher. However, some harmful substances formed under thermal treatment are also associated with protein aggregation due to the modification of protein (or amino). For instance, Zhu and Huang et al. (2020) compared the different heat treatment methods (frying, boiling, grilling, and pan-frying) on the AGEs formation in prepared chicken breast. It was found that heat treatment increased the carboxymethylation and carboxyethylation modifications of lysine and arginine.

Additionally, PAs formation is a dynamic process during cooking. For example, the aggregation behavior of myofibrillar protein under different cooking conditions is different. It was reported that intermediate fibrosis aggregates were generated under low temperature or short cooking conditions (50 °C ~ 60 °C ~ 80 °C (<20 min)). However, amorphous aggregates appeared when the cooking conditions increased (80 °C ~ 100 °C ~ 140 °C (>20 min)) (Promeyrat & Gatellier et al.,

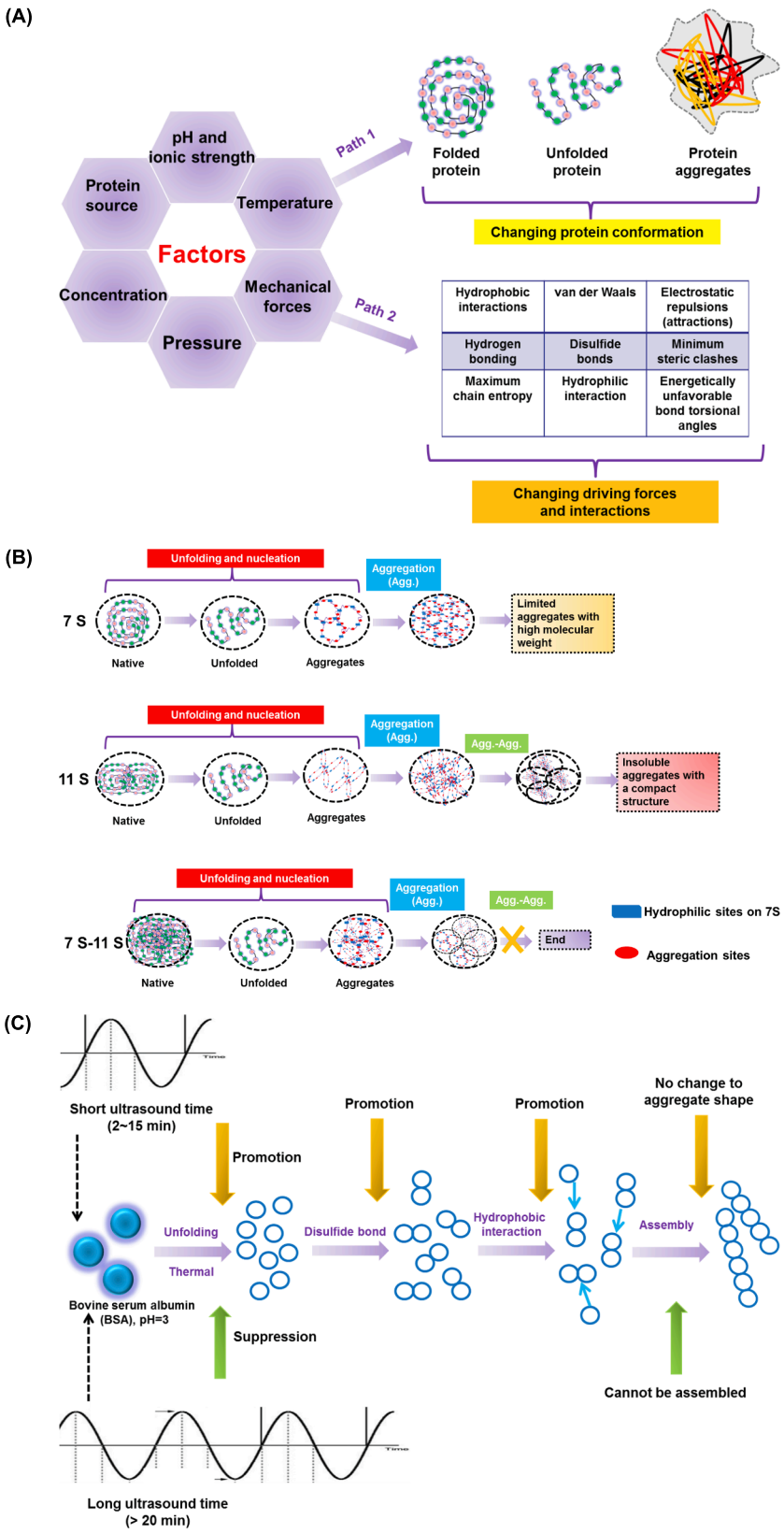

Fig. 3. (A) Factors affecting PAs formation. (B) Aggregation behavior of soybean proteins with different subunits under thermal treatment. (C) The mechanism of ultrasound pretreatment (200 W) on bovine serum albumin (BSA) fiber aggregation behavior. (D) Mechanisms of high-pressure treatment increase the solubilization of insoluble PAs. (E) The relationship between myofibrillar protein oxidation and water holding. (F) The effect of protein oxidation on PAs formation.

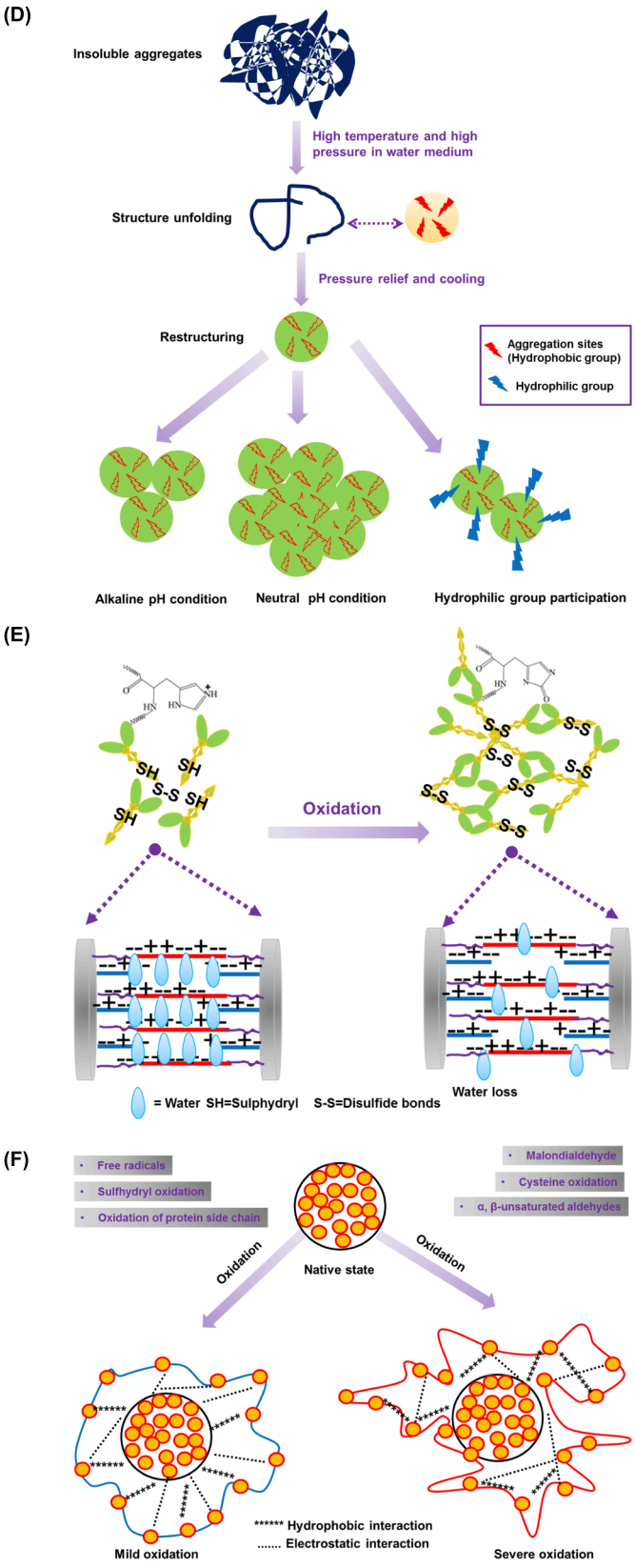

Fig. 3. (*continued*).

2010). Notably, PAs can also be formed *via* hydrophobic interaction of different proteins after heat treatment (Wu et al., 2021; Zhou et al., 2018). For example, the effect of heat-induced β-conglycinin (7S) and glycinin (11S) aggregates in soy proteins has been investigated shown in Fig. 3 (B). The unfolded 7S active sites in soluble aggregates were found to be limited when 7S and 11S were heated individually. The unfolded 11S exposed more active sites and formed larger aggregates due to the hydrophobic interaction. However, aggregation was terminated

Table 1
Factors affecting the protein aggregates formation.

Factors	Protein examples	Protein formulations	Study conditions	Results	References
Temperature	Whey protein isolates (WPI)	4 % w/w WPI, pH = 7.0	22 ~ 92 °C, 20 s ~ 15 min	Nucleation limited the aggregation at 92 °C	(Vilotte et al., 2021)
Microsecond pulsed electric field	WPI	10 % w/w WPI	3 kV cm^{-1}, 10 μs, 40–50 Hz, 64.1 ~ 73.2 °C	Aggregations above 3 μm started appearing at 71.2 ± 0.2 °C (45 Hz)	(Axelrod et al., 2021)
Exogenous crosslinking enzymes	Wheat flour protein	Medium gluten, moisture 144.5 g kg^{-1}, protein 129.8 g kg^{-1}, wet gluten 265 g kg^{-1}	Treated at glucose oxidase (20 mg kg^{-1}), lipoxygenase (20 mg kg^{-1}), xylanase (20 mg kg^{-1}), respectively	Lipoxygenase treatment exhibited the largest protein aggregation	(Ma et al., 2020)
Extreme alkaline pH	Pale, soft, exudative (PSE)-like chicken meat myofibrillar proteins	0.6 M NaCl, 100 mM KH_2PO_4/K_2HPO_4, pH 7.0	pH = 11.0, 11.5, 12.0	More protein aggregates existed at pH 12.0 than pH 11.0 and pH 11.5	(Zhao et al., 2020)
Pulsed electric field	PSE-like chicken breast meat myofibrillar proteins	0.6 M KCl, 20 mM K_2HPO_4/KH_2PO_4, pH 7.0,10 mg/mL	0–28 kV/cm, 0–1000 Hz	When the intensity exceeded 18 kV/cm, aggregates were increasingly formed	(M. Dong et al., 2020)
High-pressure processing (HPP)	Blue crab (*Callinectes sapidus*) myosin	0.5 % NaCl, 0.5 % of microbial transglutaminase and 3 % albumin	100, 300 and 600 MPa/5 min	600 MPa induced the denaturation and aggregation of myosin heavy chain	(Martínez-Maldonado et al., 2020)
Anticaking agents (SiO_2 and $Ca_3(PO_4)_2$)	Whey protein concentrate bars (WPC, containing 75 % protein)	45 g/100 g WPC bars, 48 g/100 g maltose syrup, 4 g/100 g coconut oil, 2 g/100 g glycerol and 1 g/100 g soybean phospholipid	SiO_2 and $Ca_3(PO_4)_2$ at a level of 0.5 g/100 g WPC, heat-sealed and stored at 37 °C	SiO_2 and $Ca_3(PO_4)_2$ prevent Maillard-induced aggregation	(Meng et al., 2019)
Microwave irradiation	Silver carp myosin	1 mM $KHCO_3$ and treatment with $MgCl_2$ at a final concentration of 10 mM	The myosin solution was heated via short-duration microwave (MW) at 40 °C, 60 °C or 90 °C for 5, 10, 15, 20, 30, 40 or 60 min	MW irradiation improves the myosin aggregation	(Cao et al., 2019)
High pressure homogenization	Faba bean proteins	The protein precipitate was neutralized using 1 mol/L NaOH	15,000 psi (103 MPa) and 30,000 psi (207 MPa) for 6 cycles, respectively	High pressure homogenized induced protein aggregation	(Yang et al., 2018)
Sodium chloride, chopping and beating	Pork leg lean meat salt-soluble proteins	0.6 M NaCl and 20 mM phosphate, pH 7.0	1 % NaCl or 2 % NaCl	1 % NaCl enhanced protein aggregation	(Kang et al., 2018)
Non-enzymatic browning	Royal jelly glycoprotein	Royal jelly (0.5 g) was homogenized in 100 mL of ultrapure water, and then 100 μL royal jelly solution was mixed with 2 mL of nitroblue tetrazolium	The royal jelly samples were dispensed into seven sterile air-tight glass bottles and then stored at room temperature (25 ± 3 °C) for one to six months	Major royal jelly protein 1 (MRJP1) monomer gradually aggregated with MRJP1 oligomers into new oligomers of about 440 kDa and 700 kDa	(Qiao et al., 2018)
Temperature	Milk protein (whey proteins and κ-caseins)	13.5 % (w/v) protein in distilled water, and sodium azide (0.05 %, w/v)	90 °C, 25 min, pH 7.2	Whey proteins and caseins combined aggregates show higher heat stability	(Gaspard et al., 2017)
Polysaccharides	Arabinogalactan-protein	Lyophilised Auxerrois wine colloids was concentrated in a Sartocon beta ultrafiltration system	Colloid-polysaccharides-ratios (1:1; 1:0.75; 1:0.5; 1:0.25)	Protein aggregation was influenced by different polysaccharides in the model wine system	(Jaeckels et al., 2016)

following the interaction between 7S and 11S during heating (Wu et al., 2021). Thus, the interaction of different proteins under heat treatment also affects the final PAs formation.

The research limitations or future trends of thermal treatment on food protein aggregation are summarized as follows:

- The size and shape of aggregates formed by heat-induced proteins need to be further investigated from a thermal processing perspective. For example, it is necessary to deeply explore the formation of PAs from various perspectives, such as different heat sources, heat transfer methods, and heat transfer efficiency.
- From the perspective of heat-induced protein structural modification, combining omics techniques, such as proteomics, metabolomics, peptidomics, etc., is necessary to characterize their aggregates.
- From the results of heat-induced aggregation, the functional properties (gel, emulsification, foaming, etc.) of PAs under mild, moderate and severe aggregation need to be further exploitation.

3.2. Non-thermal treatment

Non-thermal processing technologies including ultra-high pressure, high pressure homogenization, ultrasound, PEF, cold plasma, etc. have been widely used in protein modification and sterilization of fruits, vegetables and meat (Olatunde et al., 2021; Zhu & Huang et al., 2020). Unlike thermal treatment, non-thermal treatment has a more complex effect on the formation of PAs, and the reactions involved are also more complex.

As a commonly-used non-thermal technology, PEF has attracted more and more attention, especially in food science. For example, PEF improved the PSE-like chicken breast meat myofibrillar proteins gel properties. A phenomenon that PAs increased formed by mutually attractive polar protein molecules was observed when the PEF intensity exceeded 18 kV/cm (M. Dong et al., 2020). The possible formation mechanism could be asserted that the protein structure was modified *via* electrostatic interactions induced by PEF, resulting in unfolding, aggregation, and gel formation.

Nonetheless, ultrasound associated with the cavitation effect can alter the functional properties of proteins through physical or chemical mechanisms. Notably, ultrasound intensity and time were associated with structural unfolding and exposure of reactive groups (SH and NH_2), making it possible to control the rate of PAs formation (Xu & Xu, 2021). As shown in Fig. 3 (C), the mechanism of ultrasound pretreatment (200 W) on bovine serum albumin (BSA) fibrosis aggregation behavior was elucidated. Short-time (2 ~ 15 min) ultrasound pretreatment can accelerate protein conformational transitions, resulting in faster formation of antiparallel β-sheet structures and disulfide bonds, resulting in a stronger hydrophobic forces and more exposure of hydrophobic

groups after heating without a significant change in morphology. However, long-term (>20 min) ultrasonic pretreatment is not conducive to the antiparallel β-sheet structures formation, eventually resulting in fibrosis aggregates cannot be assembled (Zhao et al., 2018).

High pressure treatment can increase the protein aggregation degree, but under a certain condition, high pressure can improve protein solubility and gel properties (Angioloni & Collar, 2013). It was reported that while dynamic high pressure dissociated large PAs, protein denaturation or aggregation mainly depends on the intensity of mechanical forces (such as intense shear forces leasing to cavitation) and/or temperature (Grácia-Juliá et al., 2008). After high pressure treatment, adjusting different pH can modulate protein aggregation formation. Fig. 3 (D) exhibited the mechanism of high-pressure treatment on the PAs formation. Under the high temperature and high-pressure environment, the structure that maintains the stability of the insoluble PAs is destroyed, and the structure of the insoluble aggregates is unfolded. When the high temperature and high-pressure conditions disappear, the unfolded proteins undergo molecular rearrangement induced by environmental factors. Since the structure of the protein is in an unfolded state at this point, re-aggregation can occur following induction from the exposure of many hydrophobic groups. The solubility of the system is determined by the degree of re-aggregation. Controlling environmental factors, such as increasing the pH of the dispersion, adding adjuvants to limit protein aggregation, etc., can improve the protein surface charge, shield the hydrophobic interaction on the surface of protein molecules, and reduce the protein re-aggregation degree. Finally, solubilization of insoluble PAs is achieved (Martínez-Maldonado et al., 2020; Xue et al., 2017).

The research limitations or future trends of non-thermal treatment on food protein aggregation are summarized as follows:

- Compared with thermal processing, a major advantage of non-thermal processing is that non-thermal processing can alleviate protein aggregation and degradation caused by excessive thermal processing and improves food quality. Hence, the combined effect of different thermal processing methods requires further study.
- Non-thermal processing can promote food nutrition. For example, high-pressure homogenization can achieve solubilization of myofibrillar proteins under low-salt conditions, which provides a new strategy for salt reduction. Therefore, developing nutritious soluble PAs foods based on non-thermal processing needs is imperative.
- Non-thermal processing can promote food safety. Non-thermal processing can alleviate the formation of harmful substances and biofilms. For example, air frying inhibits AGEs, and PEF inhibits biofilm formation. However, the mechanism of remains to be further studied.

3.3. pH and ionic strength

Aggregation involves a combination hydrogen bonding, disulfide bridges, charge-charge interactions, etc. Generally, low ionic strength is responsible for protein self-assembly during the process of nucleated growth, while high ionic strength results in disordered precipitates. Moreover, low pH often induces a stable aggregate (Khan et al., 2014).

The pH has a significant effect on the morphological structure of proteins. pH affects the ionization state of free amino groups and terminal carbonyl groups and the ionic strength of amino acids (such as arginine, histidine, lysine, aspartic acid, glutamic acid, etc.). Furthermore, pH and ionic strength can drive protein aggregation through electrostatic interactions (Amagliani & Schmitt, 2017; Majhi et al., 2006; Weijers et al., 2008; Zhao et al., 2020). For example, the electrostatic potential contours of β-lg were different at pH 5 at various ionic strengths (0.0045 ~ 0.5 M) (Majhi et al., 2006). It was found that electrostatic interactions adjust β-lg aggregation closer but not at its isoelectric point (pI). This is because, under this pH condition, protein precipitates at the pI, resulting in the concentration decrease and a low protein net charge formation. Afterwards, with the growth of aggregates, the excess charge accumulation is limited (Majhi et al., 2006). The study of Weijers et al. (2008) also corroborated this phenomenon. The charge density on the surface of protein molecules played an important role in aggregate morphology in ovalbumin networks. A low net charge led to fibrosis structures and resulted in the formation of turbid systems.

Electrostatic interactions also influence the physicochemical properties of aggregates. For instance, Li, Zhao, & Xu (2022) used pH-shifting strategy to improve the emulsifying properties of myofibrillar PAs extracted from PSE-like chicken. It was found that unfolding-refolding process markedly changed myofibrillar proteins secondary and tertiary structure, and a fibrosis appearance was exhibited at pH 7.0. However, myofibrillar PAs exhibited a spherical shape after pH-shifting (pH 11.0–7.0). During this process, the folding and refolding of protein structures driven by electrostatic interaction forces are an important reason for shaping the appearance of PAs. Another study demonstrated that isoelectric solubilization/precipitation (ISP) treatment could increase the gelling properties of PSE-like chicken and effectively collect PAs (Zhao et al., 2016). It was found that protein isolated from PSE-like chicken can form a well-developed gel network (soluble pH was 11.0) and the PAs was collected at pH 5.5. The underlying mechanism may be that ionic strength-driven electrostatic interactions induce changes in protein sulfhydryl and disulfide bonds.

Last but not least, pH and ionic strength can also alter the shape of PAs. Amagliani & Schmitt (2017) represented the types of aggregates by different morphologies, including spherical particles, flexible strands, and semi-flexible fibrosis, characterized under different pH (from 2.0 to 7.0). Briefly, a spherical particle is collected at the pI of the protein ranging from 50 nm to a few microns. Flexible strands are obtained at high levels of electrostatic repulsion from tens of nanometers to tens of microns. Semi-flexible fibrosis are generated at low ionic strength (pH < 2.5), ranging to microns (Amagliani & Schmitt, 2017).

3.4. Oxidation and glycation

Oxidation is an integral factor responsible for PAs formation occurred more frequently under extreme conditions (such as thermal processing) (Jiang et al., 2022). Studies showed that oxygen may have improved PAs by decreasing the overall crosslinking or by allowing the oxidation of tryptophan (*Trp*), tyrosine (*Tyr*), histidine (*His*), and methionine (*Met*) residues (Bao et al., 2018; Lévy et al., 2019). For example, the relationship between myofibrillar protein oxidation and water holding was illustrated in Fig. 3. (E). It was found that oxidation of myofibrils resulted in the loss of *His* residues and induced larger aggregates resulting the water loss (Bao et al., 2018). It was reported that inter- and intra- covalent di-tyrosine (*di-Tyr*) and di-tryptophan (*di-Trp*) crosslinks induced α- and β-casein aggregation *via Trp*-or *Tyr*-derived oxidation (Lévy et al., 2019). Furthermore, disulfide bonds (S—S) and sulphydryl (SH) oxidation also play a critical role in PAs formation. Typically, the formation rate of S—S of native proteins is very low but can be markedly increased under processing conditions by the exposure of cysteine residues (Visschers & de Jongh, 2005).

The driving forces that initiate protein aggregation during protein oxidation are mainly hydrophobic and electrostatic interactions (Hematyar et al., 2019). Different degrees of oxidation have different effects on the formation of PAs shown in Fig. 3 (F). On the one hand, protein oxidation can affect the hydrophobicity of proteins. For example, *Met* in proteins can be oxidized by free radicals to *Met* sulfoxide, which reduces hydrophobicity. However, lysine, cysteine and *His* can undergo *Michael* addition reaction with α, β-unsaturated aldehydes to attach a long-chain alkane to the protein side chain group, thereby increasing the hydrophobicity. On the other hand, protein oxidation can also change the charged state of proteins. For example, free radicals and reactive aldehydes can oxidise lysine in proteins to form electrically neutral carbonyl derivatives. Protein oxidation mainly forms protein covalent cross-links through the following six ways: (1) Free radicals convert proteins into

carbon-centered free radicals through hydrogen abstraction reactions, and the coupling reaction between carbon-centered free radicals can form protein carbon–carbon cross-links; (2) The sulfhydryl group of protein is oxidized into disulfide bond, forming protein disulfide bond cross-linking product; (3) Free radical converts *Tyr* into *Tyr*-aromatic free radical through hydrogen abstraction reaction, and the coupling reaction between *Tyr*-aromatic free radical can form protein di-*Tyr* cross-linking products; (4) The double bond of α, β-unsaturated aldehyde react with the sulfhydryl group of cysteine residue, the ε-amino group of lysine residue, and the imidazole group of *His* residue through *Michael* addition reaction to form protein carbonyl derivatives. Then protein carbonyl derivatives react with the ε-amino group of lysine residues in another polypeptide chain to form *Schiff* bases; (5) The two carbonyl groups of malondialdehyde (MDA) react with the ε-amino groups of lysine residues in different polypeptide chains simultaneously to form protein *Schiff* base cross-links; (6) The carbonyl group formed by the oxidation of protein side-chain groups react with ε-amino group of lysine residues to form protein *Schiff* base cross-links (Bao et al., 2018; Zhao et al., 2021; Zhu & Fang et al., 2020).

Glycation is another integral factor responsible for PAs formation (Zhao et al., 2018). After glycation, protein and sugar can form covalent interactions, and the products formed by covalent interactions are more stable than complexes formed by non-covalent interactions (Wu et al., 2021). Zhao et al. (2018) studied the effects of glucose, lactose, and maltodextrin on the aggregation behavior of β-lg. They found that the glycation process could decrease the β-sheet content and inhibit the formation and extension of aggregates. Additionally, di-carbonyl compounds, such as methylglyoxal (MGO) and glyoxal (GO), which are side-products of advanced Maillard reaction also induce PAs formation (Zhu & Bassey et al., 2021). Oxidation and glycation interaction by di-carbonyl compounds may also trigger the generation of high molecular mass aggregates *via* protein secondary and tertiary structure modifications (Zhu & Huang et al., 2020).

4. Detection methods

The native protein structure is frequently altered during food processing, and aggregation often occurs during food processing and storage. The aggregation pathway and final aggregate structure may vary depending on the chosen conditions. Different structures, in turn, may impact the functional properties of the resultant protein network. Therefore, we need to adopt appropriate methods to characterize PAs in food processing to meet human needs for different food engineering systems is essential.

There are many ways to measure protein aggregation. These methods include but are not limited to calorimetry (such as differential scanning calorimetry, DSC), chromatography (high performance liquid chromatography, HPLC), electrophoresis (sodium dodecyl sulfate–polyacrylamide gel electrophoresis, SDS-PAGE), rheology (dynamic shear rheometry), spectroscopy (such as dynamic light scattering (DLS) and Fourier transform infrared spectroscopy (FTIR)), etc. (Andreescu & Vasilescu, 2021; Chen et al., 2018; Chernukha et al., 2019; Williams et al., 2017). Some reports on the methods used as PAs determination are summarized in Table 2. It was found that the spectroscopic method was mainly used for the protein size differences.

4.1. Particle size

Protein particles formed through aggregation can span many orders of magnitude from tens of nanometers to several hundred micrometers. Therefore, aggregates size distribution could monitor the aggregation process (Ndoye et al., 2013). *Malvern Panalytical* is the world's leading supplier of particle size analyzers (https://www.malvern.co.uk). This brand is the instrument used by many top universities worldwide to measure particle size. This series has many measuring machines according to the sample particle size range and the measuring function (Fig. 4 (A)). Granulometry measurement can also be used for quantifying PAs, including the number and size. Promeyrat and Gatellier et al. (2010) investigated the effect of cooking strength on the granulometry parameters using a particle image analyzer. Briefly, particle size is

Table 2
Determination methods of the protein aggregates.

Categories	Techniques	Applications	Protein examples	References
Light scattering; Spectroscopy	Light scattering/Fourier transform infrared (FTIR) spectroscopy	ξ-potential and particle size determination	Meat and potato proteins	(Ebert et al., 2021)
Spectroscopy	Synchronous fluorescence spectra	Gelation characterization	Myofbrillar protein	(Chen et al., 2021)
Spectroscopy	Small-angle X-ray scattering (SAXS)	Protein conformation	Whey protein isolates	(Vilotte et al., 2021)
Microscopy; Spectroscopy	Atomic force microscopy (AFM)/Multiangle laser light scattering (MALS)/UV–vis	Changes in the morphology of protein aggregates/particle size	PSE-like chicken meat protein	(Zhao et al., 2020)
Electrophoresis	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and diagonal-PAGE	Aggregation process and mechanistic probing	Pork and beef proteins	(Chernukha et al., 2019)
Spectroscopy; Microscopy	Thioflavin T (ThT) fluorescence assay/Transmission electron microscopy (TEM)/Circular dichroism (CD)/Nuclear magnetic resonance (NMR)	Aggregation and disaggregate process/secondary structures	Like Tau protein fibrils	(Zhang et al., 2019)
Tension method	Dynamic surface tension measurement	Degree of protein aggregation	Whey protein isolate	(Maticorena et al., 2018)
Rheology; Spectroscopy	Dynamic rheological measurement, including temperature ramp tests, frequency sweeps and steady shear/Spectrophotometer/CD	Particle size distribution/turbidity/ secondary structure	Myofibrillar protein	(Chen et al., 2018)
Tension method	Automatic drop tensiometer	Protein interfacial properties	Faba bean protein	(Yang et al., 2018)
Electrophoresis; Chromatography	SDS-PAGE and Native-PAGE analysis/size exclusion chromatography-high performance liquid chromatography (*sEC*-HPLC)	Size distribution of aggregates/ mechanistic probing	Royal jelly protein	(Qiao et al., 2018)
Spectroscopy; Electrophoresis; Microscopy	Ortho-phthaldialdehyde (OPA) and trinitrobenzenesulfonic acid (TNBS) methods/SDS-PAGE/TEM	Shape and size estimation/ Mechanistic probing	Whey protein isolate	(Mulcahy et al., 2017)
Chromatography; Microscopy; Electrophoresis	HPLC/TEM/SDS-PAGE	Size estimation and isolation	Hen egg white protein	(Hong et al., 2017)
Spectroscopy; Calorimetry	Total reflection Xray fluorescence spectroscopy (TXRF)/differential scanning calorimetry (DSC)	Aggregation behavior /denaturation	Wine protein	(Jaeckels et al., 2016)

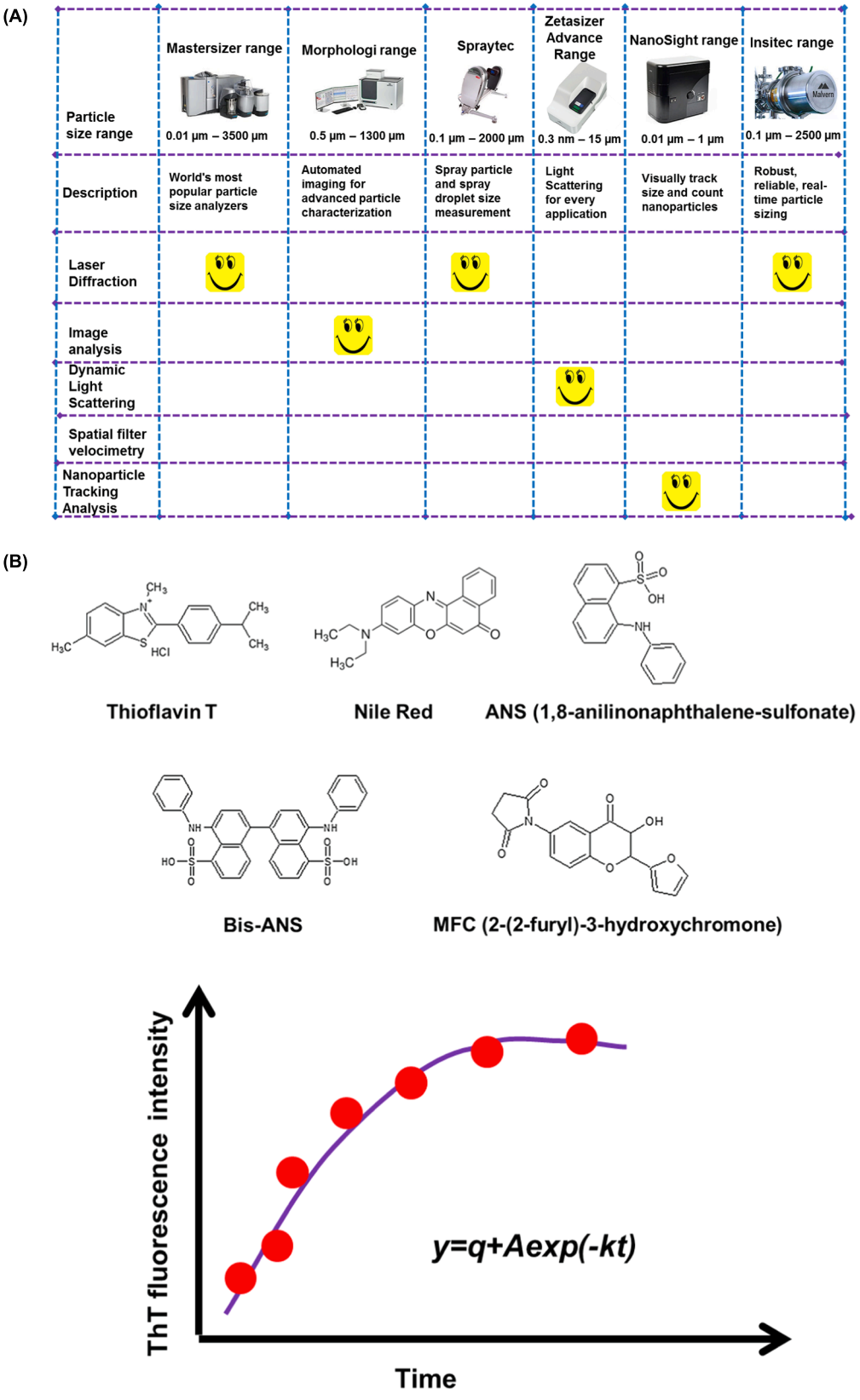

Fig. 4. (A) Several particle size measuring instruments and their characteristics. (B) The chemical structural formulas of several fluorescent probes and the plot of ThT kinetics. (C) The approach of protein unfolding and refolding kinetics.

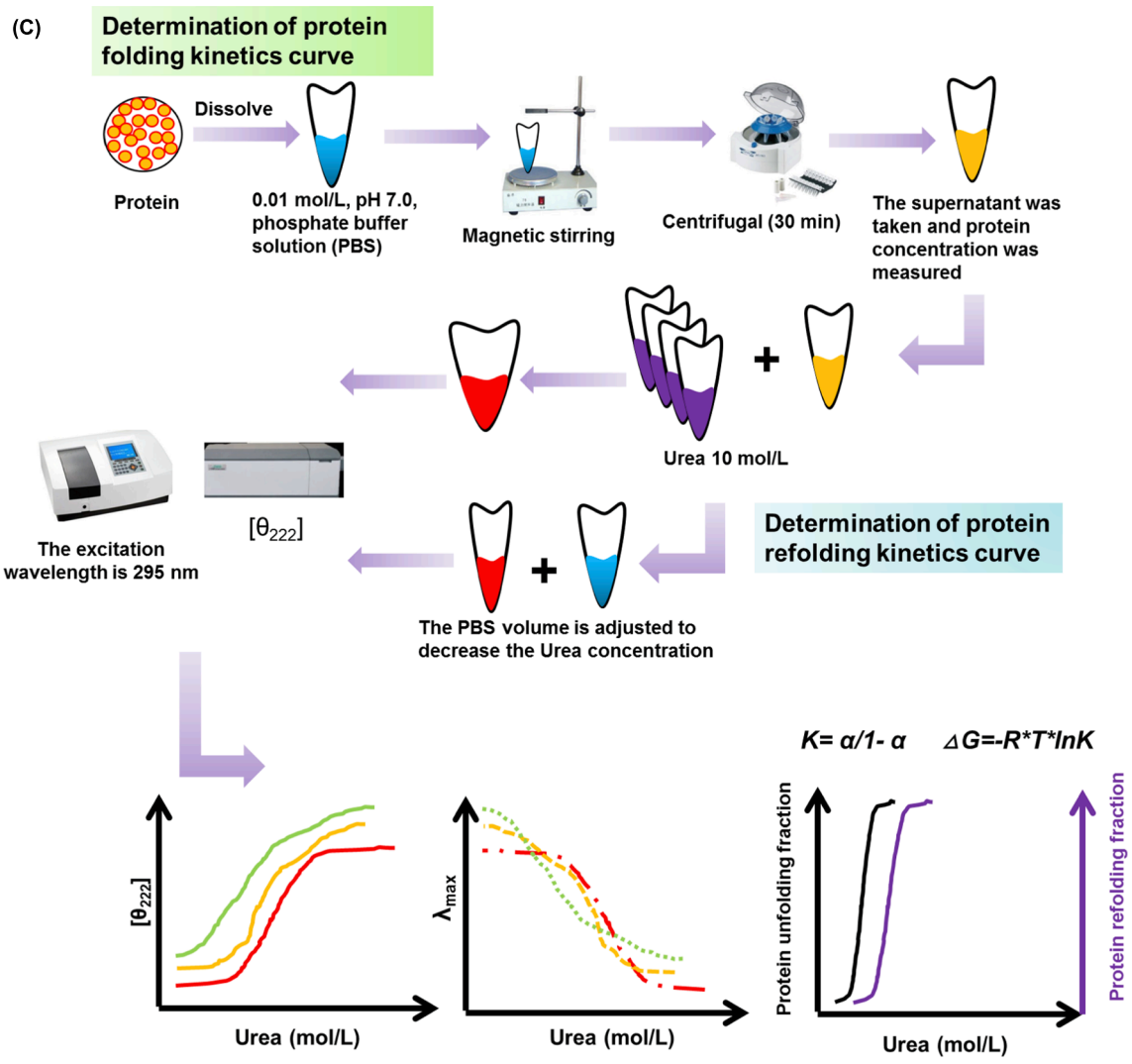

Fig. 4. (*continued*).

evaluated by the equivalent circle diameter ($2\times(S/\pi)^{1/2}$), where S is the area of the particle image. The formula $\theta = 2\times (S \times \pi)^{1/2}/\lambda)$ is used to calculate the particle shape, where θ is circularity and λ is the peripheral length of the projected image (Promeyrat & Gatellier et al., 2010). Another example is that myofibrillar PAs, characterized by the $D_{4,3}$, $D_{3,2}$, $D_{v,0.5}$, $D_{v,0.1}$, and $D_{v,0.9}$ is measured using a laser light scattering instrument ($D_{4,3}$ and $D_{3,2}$ represent the volume and surface mean diameters, respectively). The dispersion index is calculated using the formulation: $(D_{v,0.9}- D_{v,0.1})/ D_{v,0.5}$, where $D_{v,0.5}$, $D_{v,0.1}$, and $D_{v,0.9}$ denote the samples with 50 %, 10 %, and 90 % lower size, respectively (Sun & Zhao et al., 2011). Notably, the measurement range of particle size is different for different models of machines. DLS can be used to determine the size and quantity of polydispersity in aggregation sizes between 1 nm and 1 μm. Nanoparticle tracking analysis can be used to detect the nanoscale between 30 nm and 1 μm, while resonant mass measurement (relying on a mechanically resonant structure that can detect mass changes, increasing or decreasing the sample mass causes the structure's resonant frequency to change) quantifies PAs size ranging from 50 nm to 2 μm. Other techniques to characterize particle size between 2 μm and 10 μm including light obscuration, coulter counter, flow imaging, flow microscopy, etc. (Amin et al., 2014).

4.2. Spectroscopic methods

Spectroscopic methods such as fluorescence spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, circular dichroism (CD), Raman spectroscopy, etc., can be applied to analyze PAs structures (Kumar et al., 2017). However, these methods only give a whole situation of aggregation. In the actual measurement process, a combination of multiple techniques is mostly used to ensure the reliability of the results. Some spectral determination methods and application are shown in Table 2. For example, FTIR, a typical spectroscopic technology, is often used to monitor PAs changes in meat, egg, and milk by determining protein secondary structure (Chae et al., 2022). Calabrò & Magazù, (2020) investigated the PAs formation using FTIR during microwave heating in beef meat. It was found that the β-sheet contents at 1695 cm^{-1} and 1635 cm^{-1} increased with increasing microwave power (700 to 1100 W, 2 min).

Importantly, it is necessary to use some exogenous fluorescent probes such as Thioflavin T (ThT), Nile Red, ANS (1,8-anilinonaphthalenesulfonate), etc., when using spectroscopy to evaluate the PAs degree (Kumar et al., 2017). Understanding and controlling aggregation kinetics is another key aspect to gaining insights into the aggregation mechanism and the resulting final aggregate microstructure. Fig. 4 (B) shows the chemical structural formulas of several fluorescent probes and the study of aggregation ThT kinetics. ThT can specifically bind to PAs

and exhibit significantly enhanced fluorescence absorption at 440 nm (excitation wavelength) and 482 nm (emission wavelength). It has been widely used to identify PAs and characterize the kinetics of formation (Wang & Roberts, 2018). The curve was drawn with the reaction time as the abscissa and the ThT fluorescence intensity as the ordinate. The reaction kinetics were simulated using "$y = q + Aexp(-kt)$", where A is the change in fluorescence absorption during the reaction, q is the maximum fluorescence absorption value at the equilibrium stage of the reaction, and k is the apparent rate constant (Wang & Roberts, 2018).

Likewise, *ortho*-phthaldialdehyde (OPA) and trinitrobenzenesulfonic acid (TNBS) methods have been applied to quantify the available amino acid in the denaturation and aggregation of protein solutions. The basic principle of OPA is that it can react with thiol group in the protein buffer, while TNBS can react specifically with primary amino groups to form colored amino acid derivatives. Both OPA- and TNBS- amino acids derivatives are quantified by measuring their absorbance at 340 nm wavelength. A typical example is the measurement of whey-protein aggregates (Mulcahy et al., 2017). As whey protein isolate solutions with different PAs levels, both methods noticed a marked difference in amino groups in the heated solutions. According to Mulcahy et al. (2017), lower levels measured by the TNBS method than OPA indicated that the measurement of available amino groups by the OPA method was less impacted than by the TNBS method after heat-induced aggregation of whey protein molecules.

In addition, thermodynamic equations could also be established using the mean ellipticity at 222 nm ($[\theta_{222}]$) in the CD spectrum and the fluorescence intensity at the absorption maximum wavelength (λ^{max}) in the endogenous fluorescence spectrum to characterize the changes in secondary and tertiary structure during protein unfolding and refolding (Eftink & Ionescu, 1997). The approach to the kinetics of protein unfolding and refolding is shown in Fig. 4 (C).

Protein unfolding fraction: $\alpha=(F_0-F_n)/(F_d-F_0)$ (1).

where α is the protein unfolding fraction, F_0 is the $[\theta_{222}]$ or λ^{max} without urea, F_d is the $[\theta_{222}]$ or λ^{max} with 8 mol/L urea, F_n is the $[\theta_{222}]$ or λ^{max} with different urea concentration (0 ~ 8 mol/L).

Protein unfolding and refolding equilibrium constants: $K = \alpha/1-\alpha$ (2).

Changes in free energy during protein unfolding: $\triangle G = -R*T*lnK$ (3).

$\triangle G=\triangle H_2O_2-m$ *(urea concentration)* (4).

Where $\triangle H_2O_2$ is the free energy $\triangle G$ without urea, m is the exposure of protein hydrophobic group, R is the thermodynamic equilibrium constant, and T is the thermodynamic temperature. Both R and T are constants in the same reaction.

Therefore, $\triangle G$ under different urea concentrations could be obtained by combining equations (1) to (3). According to equation (4), a straight line is obtained by plotting the urea concentration as the abscissa and ΔG as the ordinate, the opposite number of the slope of the straight line is m, and the intercept of the straight line is $\triangle H_2O_2$. The ratio of $\triangle H_2O_2$ to m is 1/2 of the urea concentration ($[urea]_{1/2}$), which means the corresponding urea concentration when the unfolding fraction is 0.5.

4.3. Other methods

Other methods have been developed to detect PAs. One example is the fully automated two-dimensional high-performance liquid chromatography (2D-HPLC) method. This method utilizes a novel in-line fraction collection device allowing separation by aggregates size exclusion into a single analytical module (Williams et al., 2017). Transmission electron microscopy (TEM) and atomic force microscopy (AFM) may provide accurate qualitative PAs results, but the quantitative information on the aggregation process is limited (Promeyrat & Gatellier et al., 2010). More, low-field nuclear magnetic resonance (L NMR) measurement has been used in protein denaturation and aggregation. That is because protein denaturation induces aggregation and interaction of water and protein molecules under thermal condition. Based on this principle, the spin–lattice (T_1) and spin–spin (T_2) relaxation times are two direct indicators to detect PAs and degradation under different moisture states. Importantly, L NMR can complete the T_2 test within a minute, and sample preparation is non-destructive.

Electrochemistry, an array of biosensor method, can also be applied to detect PAs. Its primary principle relies on the electrochemical oxidation of amino acids in proteins and is generally achieved by using a label-free monitor (Andreescu & Vasilescu, 2021). Proteomic analysis using mass spectrometric (MS) is also remarkable in identifying PAs fractions during meat post-mortem ageing or storage. For example, it was reported that unpacked meat during storage accelerated the high molecular PAs (160–200 kDa), identified as myoglobin and troponin I *via* mass-spectrometric (Chernukha et al., 2019). The formation of PAs was also characterized through the surface and rheological method (Maticorena et al., 2018). The automated contact angle goniometer was often measured to evaluate the changes in the protein surface tension, while dynamic surface tension (DST) measurement (τ) was achieved by the pendant drop method. DST results were derived in terms of surface pressure, defined as the decrease in surface tension of a pure solvent. The relevant expression is "$\Pi = \tau_w - \tau_p$", where Π = surface pressure of the protein dispersion (mN/m), τ_w = surface tension of pure water (72.8 mN/m at 25 °C), and τ_p = surface tension of the protein dispersion (mN/m) at the same temperature. The diffusion rate constant (k_{diff}) was defined as Π against $time^{1/2}$ plots, which also is equal to the slope value of the plot (Maticorena et al., 2018). Another technique to measure PAs degree is the shear rate rheological test. Flow curves of PAs were determined by the power-law model $\sigma = K \times \gamma^n$, where σ = shear stress (Pa), K = consistency index (Pa s^n), γ = shear rate (s^{-1}), and n = flow behavior index (dimensionless). Newtonian dispersion is $n = 1$, while $n < 1$ signifies a shear thinning behavior. For instance, Chen et al. (2018) investigated chicken breast myofibrillar PAs solution and its water-soluble properties at low ionic concentrations prepared by high-pressure homogenization using shear rate rheological test. It was found that after the high-pressure homogenization treatment, the "shear thinning" phenomenon (viscosity decreases with increasing shear rate) appeared.

These measurement methods aforementioned can benefit the fundamental research on PAs and their applications, which are summarized as follows:

- Protein denaturation and aggregation brought about by isothermal incubation is often the desired method to probe the aggregation kinetics. DLS and size exclusion chromatography (*SEC*) are increasingly being utilized in order to perform such measurements and complementary techniques such as analytical centrifugation. Combining these two techniques provides a powerful tool to separate and characterize the high molecular weight aggregates formed during the protein aggregation process and helps provide insights into the aggregation mechanism.
- Common techniques for elucidating structural changes in aggregating proteins include FTIR, circular dichorism (CD), intrinsic fluorescence (FL), and Raman spectroscopy. Raman and FTIR spectroscopy are both based on the vibrational spectra of proteins in solution. CD and FL measurements require orders of magnitude lower protein concentrations than those for Raman spectroscopy. Raman spectroscopy can be carried out for solutions, gels and solids and this has clear advantages in studying aggregation for proteins systems that enter into a gel phase as aggregation progresses.
- The self-assembly and aggregation processes seen in both therapeutic and food protein systems due to thermal or chemical treatments eventually can lead to a liquid–solid transition and to the formation of a gel. The microstructural and morphological changes associated with these processes require utilization of techniques which allow the visualization/ probing of the large length-scale structures associated with these processes. The key techniques which have been

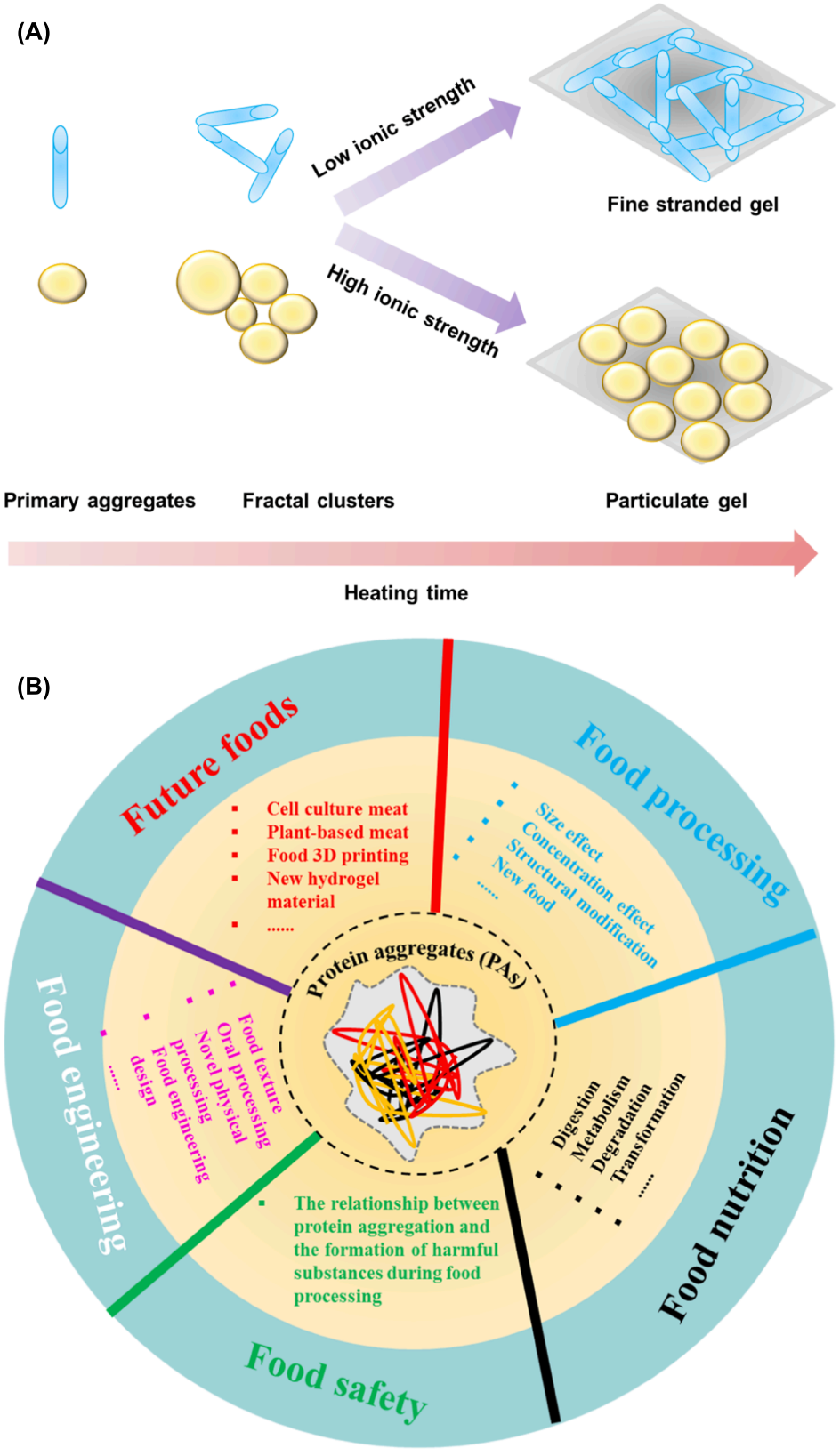

Fig. 5. (A) Schematic of particulate globular protein gels formation during heating. (B) Some applications of PAs in food science. (C) Research trends and challenges of food protein aggregation.

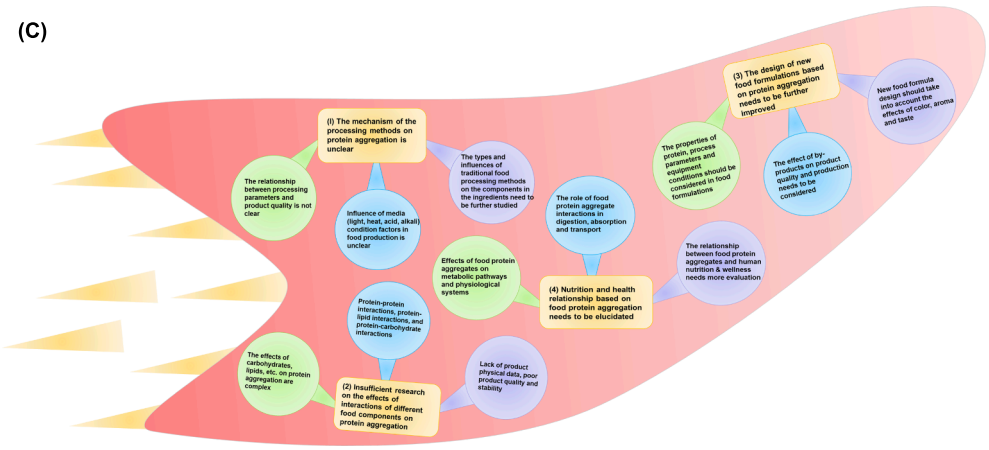

Fig. 5. (*continued*).

utilized extensively in the food protein area have been based on microscopy.

5. Applications

Protein aggregation is a double-edged sword. As mentioned above, PAs are related to neurodegenerative diseases, but some anti-neurodegenerative diseases drugs are also quickly developed due to the exploration of the pathogenesis of amyloid PAs (Ramamoorthy, 2018; Wu & Du, 2021). As a typical form of protein conformation presentation, PAs are closely associated with function properties (such as gelation and emulsification) in food systems (Wu et al., 2021; Xu & Xu, 2021). The increase in protein properties is usually accompanied by the PAs formation, which is the positive side of the coin (Chen et al., 2021; Zhu & Fang et al., 2020; Zhu & Yang et al., 2021).

However, inappropriate PAs formation, resulting in the decrease in the functional properties and quality attributes of foods, which is the negative side of the coin (Amagliani & Schmitt, 2017; Xu & Xu, 2021; Yu et al., 2017). This PAs formation is related to decrease in the food safety and influence protein digestion process. For example, the different digestion and absorption rules of aggregates at different scales (nm ~ μm) are found. A positive correlation between the formation of PAs and harmful substances such as advanced glycation end products (AGEs) during thermal cooking are reported (Dear et al., 2021; Ding et al., 2022; Zhu & Bassey et al., 2021; Zhu & Huang et al., 2020).

5.1. Emulsion

Emulsion, using food proteins as emulsifier, can be divided into water-in-oil (W/O) and oil-in-water (O/W) types (Wu et al., 2021). Aggregates are formed when denatured non-adsorbed protein and those adsorbed at the emulsion droplet surface interact. Droplet-droplet, protein–protein, and droplet-protein are the possible underlying aggregation mechanisms (Euston et al., 2000). Also, lipid oxidation greatly influences on the PAs, which in turn affects the interaction between emulsion particles. For example, it was reported that with the MDA concentration increase, sarcoplasmic protein O/W emulsion flocculation and *Ostwald Ripening* were observed in a confocal laser microscope image, that is because MDA induced an oxidizing environment at the surface of droplets and enhanced protein unfolding and aggregation (Zhu & Fang et al., 2020). PAs also participate in the preparation of high internal phase emulsions (HIPEs), which means a type of emulsion with an internal phase volume fraction>74 %, also known as gel emulsion or super concentrated emulsion (Tao et al., 2022). Food proteins including soy protein isolate, zein, wheat prolamin, whey protein, etc., have hydrophilic and hydrophobic structural groups, no toxic side effects, and rich nutritional properties. So their aggregates are suitable as natural stabilizers for HIPEs (Abdullah et al., 2020; Zhao & Zaaboul et al., 2020).

5.2. Gel

Protein gel is an important functional feature of food protein. A gel is usually produced under the heat promotion. The degraded protein driven by protein thermal degradation and hydrophobic interaction will further aggregation, and the cross-linking of these aggregates is achieved through disulfide bonds and sulfhydryl groups (Visschers & de Jongh, 2005). For example, a phenomenon that octenyl succinic anhydride (OSA) modulated the aggregation of myofibrillar protein, alleviated the deterioration of protein gels at high temperature was reported by Chen et al. (2021). The underlying mechanism is that OSA enhanced the crosslinking of disulfide bonds and improved the conjunction zone hydrophobic interaction (Chen et al., 2021). Like lipid oxidation's effect on emulsion aggregation, protein oxidation also plays a critical role in the protein gel aggregation process. The impact of peroxyl radicals on myofibrillar PAs and their gel formation was investigated in our previous work. It was found that free radicals promoted myofibrillar proteins oxidation and enhanced the Maillard reaction. During this process, disulfide bonds and AGEs are thought to be involved in the modification of protein structure (mainly changes in protein secondary structure) and the gel aggregation network formation (Zhu & Yang et al., 2021).

Globular proteins can also join the gel aggregation network formation under heating (Amagliani & Schmitt, 2017). During this process, electrostatic interactions at different ionic strengths play a critical role in globular protein gels formation. Fig. 5 (A) displays the schematics of particulate globular protein gels formation during heating. It was found that spherical particles formed secondary aggregates at low ionic strength, while secondary aggregates aided phase separation and restructuration at high ionic strength (Nicolai & Durand, 2013). Thus, PAs formation can increase gel and emulsifying properties, which helps to enhance the food quality.

The research limitations or future trends of food protein aggregation on the effect of emulsion and gel are summarized as follows:

- In addition to O/W or W/O traditional emulsions, PAs also play an important role in Pickering emulsions, double or multi-layer emulsions, and HIPEs. How to break the theoretical limitations of the traditional emulsion interface protein film and explore the role of PAs in the new emulsion system will be the task of future research.
- Proteins can form gels after aggregation under different conditions such as pH, ion concentration, oxidation, and glycosylation. However, in addition to traditional gels such as meat gels, starch gels and milk gels, the gelation mechanisms of novel gels such as amino acid

hydrogels, peptide gels, etc. under different conditions need to be further investigated.

- Gel and emulsification are inseparable and interact. However, the association mechanism of PAs in gelation and emulsification remains unclear. For example, in aggregation-induced gel emulsification, the optimal aggregation point for protein gel formation may not be consistent with the optimal point for emulsion stability. Therefore, a balance point needs to be selected in the processing of food (such as emulsified sausage).

5.3. Anti-aggregation

Proteins in food play an important role as nutrients and as structural elements. High protein diets have been described to have a more satiating effect compared to control meals of the same calorie content but a lower content of protein. Moreover, regular and sufficient protein consumption is important for normal growth, especially important for children, pregnant women, the elderly, but also for high intensity athletes. In addition to nutritional improvement of proteinaceous foods it may be desirable to improve structural features. For these two reasons, controlling protein aggregation of food grade proteins is of growing interest to the food industry.

Some harmful substances such as AGEs formed during food thermal processing as well as gel formation during the PAs re-aggregation (H. Dong et al., 2020; Qi et al., 2018; Zhao & Li et al., 2020; Zhao & Xu et al., 2020; Zhu & Huang et al., 2020). Hence, it is necessary to control PAs to reduce their unfavorable substances formation. Recently, plant-derived bioactive compounds (phytochemicals), including carbohydrates, lipids, steroids, polyphenols, alkaloids, terpenes, other nitrogen-containing compounds, etc., have attracted massive attention for their anti-aggregation properties (Zhang et al., 2019). Limanaqi et al. (2020) asserted that the underlying mechanism of phytochemicals to inhibit PAs may be through anti-glycation. Some catechin polyphenols also have been found to inhibit the formation of PAs. The underlying mechanism is that catechin can protect proteins from the aggregation of AGEs and trap free radicals (Zhu & Bassey et al., 2021). Furthermore, curcumin ((1E, 6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) was reported to bind proteins and modulate their conformation and anti-aggregation corroborating the findings of Roy & Kurganov, (2017) that curcumin exhibited a perfect effect on the anti-fibrillation of hen egg globular protein. In addition, amino can also be used as PAs inhibition. For instance, Lyutova et al. (2007) found that arginine, one of the the low molecular weight solution additives, exhibited a marked suppression effect on PAs by enhancing the solubility and refolding of aggregated proteins. Moreover, polysaccharides play an essential role in controlling PAs. It was reported that the aggregation of soy protein was suppressed when the gum arabic added because of their (protein-polysaccharide) electrostatic interaction (Dong et al., 2013).

5.4. Innovative food processing

The process of aggregation is often a desired outcome of food manufacturing processes. However, protein aggregation can also be undesirable, for example in high protein foods where gelation may be unwanted. The concept of deliberately inducing and harnessing protein aggregation stands in stark contrast to the pharmaceutical industry, which aims to eliminate the aggregation of peptide and protein drugs (Wu & Du, 2021). Traditionally, industrial and domestic food processes that influence protein aggregation include heating, pH modifications, salt concentration, and pressure treatments. Further industrial processes include spray drying, extrusion for porous, low moisture products, and spinning for fibrosis formation (Chen, Liang, & Xu, 2020). Despite this versatility in domestic and industrial processes, there is some need for innovative food processes that allow further finetuning of protein matrices. New means of protein denaturation for food manufacturing purposes include high pressure treatment (Chen et al., 2018), high intensity ultrasound (Xu & Xu, 2021), and high pressure homogenization (Chen, Liang, & Xu, 2020). The nature of most of these studies is empirical, providing only vague theoretical models of food networks. Moreover, there is a plethora of protein functionality that is yet to be harnessed by food manufacturers.

5.5. Develop new functional food systems

From a structural perspective, there are two common goals of current food protein research. These include improving food texture and developing new functional food systems, including protein-enriched foods and delivery vehicles for pharmaceuticals and nutraceuticals. The encapsulation of hydrophobic bioactives (such as antioxidants, vitamin, curcumin, and carotene) has been shown to protect the chemical compounds and to facilitate absorption of the active compound by the body (Liang, Ma, Yan, Liu, & Liu, 2019). Proteins with the potential to be used as an encapsulation matrix for sensitive compounds include milk proteins (Bashir et al., 2021; Gaspard et al., 2017; Wang et al., 2018), soy proteins (Dong et al., 2013; Fang et al., 2013), meat proteins (Ebert et al., 2021; Li et al., 2021), and egg white proteins (Guo et al., 2020).

Another process that restructures typical food proteins involves partial hydrolysis of proteins (Ardy Kroes-Nijboer et al., 2012). Under appropriate conditions, the hydrolysis of proteins leads to a weakened gel structure compared to a nonhydrolysed protein, thereby allowing more protein inclusion into a food system. Consequently, this would allow higher concentrations of hydrolysed protein to achieve a similar texture. Therefore, creating protein-rich foods with similar textural properties is possible through partial hydrolysis. Overall, structural studies on protein aggregation in food merits further investigation because it opens up a new field of opportunities for food engineering in the food industry. To harness the full range of possibilities in engineering new food products, it is, therefore, necessary to understand the basic mechanisms that underlie structural changes during food processing.

5.6. Other applications

In addition, fibrosis aggregates can be obtained through electrospinning technology. It is a process in which charged macromolecule protein (collagen, gelatin, zein, etc.) or their solutions are sprayed, stretched, split, solidified or solvent volatilized in a high-voltage electrostatic field (Angel et al., 2022). Protein electrospinning aggregate membranes have also shown great application value in active packaging, enzyme immobilization, tissue engineering, sustained drug release, and membrane processing (Deng et al., 2018). More, using plant-based protein as raw material (such as soybean protein) to produce artificial meat by spiral extrusion into aggregate fibers is also available (Sha & Xiong, 2020). Furthermore, PAs are used to carry active substances (such as curcumin, carotene, etc.) through the self-assembly to achieve sustained release in the gastrointestinal tract (Du et al., 2022; Liang et al., 2019). Importantly, the construction of such aggregates requires some methods such as ultrasound assisted, glycation modification, or building HIPEs as templates to form porous aggregate materials (Gao et al., 2021; Xu & Xu, 2021; Zhao & Zaaboul et al., 2020).

Although some applications have been investigated partially, many problems still have not been studied clearly. These applications are summarized in Fig. 5 (B). (1) For food processing, the correlation mechanism between particle size and concentration, as well as structural modification should be further considered (Amin et al., 2014; Flores et al., 2019; Hirota et al., 2019; Rutigliano et al., 2019; Sobral et al., 2018; Zhao et al., 2021). For example, Rutigliano et al. (2019) investigated the molecular weight distribution of PAs from raw meat and cooked pork products heat treatment using proteomics and found that protein re-organization in heat-induced supramolecular structures

might be responsible for the texture and the structural properties of the final products. Zhao et al. (2021) also reported that changes of pH and ionic strength of the medium represent further chemical-physical factors contributing to protein re-organization, thereby leading to induced"irreversible" assembly of denatured proteins. (2) For food nutrition, studying the changes in digestion and metabolism of aggregates in different phases, including the mouth, stomach, intestines, etc., should be further elucidated (Martinez-Saez et al., 2017; Mulet-Cabero et al., 2020; Zhao & Zhang et al., 2020). (3) For food safety, the correlation mechanism between protein aggregates and AGEs such as N^{ε}-carboxymethyllysine and N^{ε}-carboxyethyllysine should be further explored (Zheng et al., 2021; Zhu & Bassey et al., 2021; Zhu & Huang et al., 2020). (4) For food engineering, applying physical processing methods such as ultrasound, PEF, high-pressure homogenization, electrospinning, etc., should be deeper studied (Axelrod et al., 2021; Cao et al., 2019; Chen et al., 2020; Ma et al., 2020; Martínez-Maldonado et al., 2020; Yang et al., 2018). (5) For future foods, reproducing the methods explored in life science and material science into future food researches such as animal cell culture meat, plant-based meat, food 3D printing, etc., are recommended (Ma & Zhang, 2022; Rubio et al., 2020; Sha & Xiong, 2020; Zhang et al., 2021).

6. Outlooks and challenges

Research trends and challenges on food protein aggregation need to be further in-depth in the following aspects (Fig. 5 (C)):

(1) The mechanism of the processing methods on protein aggregation is unclear. Some new hypotheses of PAs formation mechanism, not limited to fibrosis proteins but other morphological aggregates need to be further explored.

- The relationship between processing parameters (such as temperature, humidity, etc.) and product quality (such as texture, color, etc.) is not clear. Different processing parameters result in different degrees of food protein aggregation, affecting product quality.
- The influence of media (light, heat, acid, alkali) condition factors in food production is unclear. The processing environment affects the stability of PAs, which affects the shelf life of products, such as the oxygen permeability and light transmission of PAs food coating films.
- The types and influences of traditional food processing methods on the components in the ingredients need to be further studied. Traditional food processing methods, such as frying, braising, smoking, etc., may effect PAs in different ingredients. This may be related to the properties of the ingredient protein. For example, meat protein, such as chicken protein, beef protein, duck protein, fish protein, pork protein, etc. may have different degrees of protein aggregation under the same thermal treatment conditions.

(2) Research on the effects of interactions of different food components on protein aggregation is insufficient.

- The content and proportion of food components such as protein, carbohydrates, lipids, etc., are related to the type of food. Even if a food has the same component, these components undergo structural modification during processing. Thus, the effects of carbohydrates, lipids, etc., on protein aggregation are complex.
- Food components interactions such as protein–protein interactions, protein-lipid interactions, and protein-carbohydrate interactions may also influence protein aggregation.
- The lack of physical data on the product, quality, and stability is poor. The component properties of food raw materials need more data to reveal the law of component interaction. This may need to be combined with new technology means such as big pass data, artificial intelligence, data modeling, etc. Furthermore, the interaction of different components to form new PAs may be detrimental to product quality and stability. For example, protein-lipid interactions can aggravate protein oxidative aggregation, decreasing the water holding capacity of emulsified sausages.

(3) The design of new food formulations based on protein aggregation needs to be further improved.

- The properties of protein, process parameters and equipment conditions should be considered in food formulations. For example, ultrasonic assist emulsification can form stable PAs, which can be used to prepare HIPEs for the delivery of active substances. The hydrophobicity, rheological properties, structural properties of the protein, ultrasonic time, power, etc., will affect the delivery effect of the active substance.
- The effect of by-products on product quality and production needs to be considered. Many by-products of animal-derived food, such as chicken blood and duck blood, are high-quality protein sources. These blood proteins can form stable protein gel aggregates under salt and acid conditions.
- New food formula design should take into account the effects of color, aroma and taste. Unfriendly color and unacceptable flavor may be induced by oxidation and Maillard reactions through protein aggregation. For example, soy drinks with added peanut protein may cause aggregate flocculation during preparation and increase the soy odor.

(4) Nutrition and health relationship based on food protein aggregation needs to be elucidated.

- The effects of food PAs on metabolic pathways and physiological systems are still unclear. Elucidating the digestion, absorption, and the bioavailability of food and its influence on human health is imperative.
- During the digestion process of food PAs, after the digestion with enzymes and strong acids, the rigid protein structure may be altered, and active substances such as small molecular functional peptides (such as antioxidant peptides, antihypertensive peptides) are produced. Whether or not these active substances can continue to be absorbed and transported needs to be further explored.
- The relationship between food PAs and human nutrition & wellness needs more evaluation. The relationships between food PAs and human wellness need to be explored not only through "wet experiments" such as cells, animals, clinical trials, etc., but also need to develop some "dry experiment" techniques, such as protein aggregation kinetic simulation, protein–protein interaction prediction, protein aggregation function analysis, etc.

7. Conclusions

The formation mechanism of food PAs is not very clear due to the complex aggregate morphology. It has been recognized that the "docking-locking" and the hydrolysis self-assembly models were two crucial mechanisms for food fibrosis aggregates. More, nucleation growth model has also been explored and confirmed in the formation of fibrosis aggregates. The forces driving protein aggregate formation include covalent (S—S, SH-, etc.) and non-covalent (charge-charge, hydrophobic, hydrogen bonding, etc.) interactions. The testing methods of PAs are mainly based on protein particle size using multispectral technology. PAs have been used in the field of food colloids and shown an important role in many other fields. However, some harmful substances (such as AGEs) can generate with the process of protein thermal aggregation. In general, the research on food PAs is accompanied by many opportunities and challenges, which is bound to attract more scientists in the field of food or biomacromolecules to engage in protein aggregation research and promote the vigorous development of this field.

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CRediT authorship contribution statement

Zongshuai Zhu: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Anthony Pius Bassey:** Writing – review & editing. **Yaqi Cao:** Writing – review & editing. **Yanlan Ma:** Writing – review & editing. **Ming Huang:** Writing – review & editing, Resources, Supervision, Project administration, Funding acquisition. **Hongshun Yang:** Writing – review & editing, Resources, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

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

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