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pH-Dependent Behavior and Stability of Protein-Based Particles in Aqueous Media

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Abstract

This review focused on the characteristics of protein particles from different sources, namely whey proteins, sodium caseinate and gelatin, their structural stability and the stability of dispersions at different pH values. To create particles, controlled aggregation and gelation were used in several methods. Different chemical structures of the proteins provide different gelation properties. Whey proteins undergo thermal denaturation above 68°C, therefore heat-set gelatin was often used for particle preparation. When whey protein particles were prepared at the iso-electric point (IEP) of proteins, they became dense and small; whereas at other pH values, particles were soft and spherical due to increased repulsive forces between proteins. Such particles could swell when the pH of the aqueous phase was away from the IEP. Sodium caseinate is more heat stable compared to whey proteins; however, it is pH-sensitive. When sodium caseinate particles were prepared through acidification, particles were stable against disintegration only around the IEP of proteins. More stable caseinate particles could be produced using enzymatic crosslinking. On the other hand, gelatin particles, which were prepared via cold-set gelation, were stable over a wide pH range; however, as they were thermo-sensitive, particles disintegrated above 30°C. This review explained the chemical differences of proteins, preparation of particles using different methods, and stability of particles and their dispersions at different conditions. Such differences in protein particles should be carefully investigated before they are used in food products, which could have complex matrix.

Keywords: Aggregation, gelatin, microstructure, sodium caseinate, whey protein

1. Introduction

Stability of proteins refers to a wide range of area, such as stability against aggregation, against sedimentation or against structural integrity. Native proteins, due to the presence of ionic groups in their structure, are affected by the electrostatic interactions. In this review, we focused on the structure and stability of different proteins (namely whey proteins, caseins and gelatin) in dispersions particularly considering the electrostatic interactions through pH change. These proteins are widely used in food industry and their micro- and nano-particulate forms could give several properties to food products. Therefore, their behaviour at different pH values in aqueous media is important to know. The chosen proteins are from different sources, have different chemical structures, and thereby having all different properties from each other.

Whey proteins are the by-product in cheese production and constitute almost 0.6% (w/v) of the proteins in milk. Of the total protein, nearly 20% (w/w) is whey proteins and 80% (w/w) is caseins. Whey proteins include β-lactoglobulin (BLG), α-lactalbumin (ALAC), bovine serum albumin and immunoglobulin, and therefore it is highly complex [1]. In whey proteins, secondary and tertiary structures, including α-helix and β-sheets, are present [2]. BLG and ALAC have disulfide bonds, which could change the hydrophobicity and thereby the solubility of the protein. Therefore, solubility of whey proteins is also affected by the chemical structure besides physical conditions, such as acidification or heating [3]. Such treatments often cause aggregation of whey proteins that result in increased turbidity or viscosity in aqueous solutions. In addition, heating of whey protein solution with a high concentration could result in gel formation. These changes in liquid food

products including whey proteins are not desired as the product acceptability decreases.

Caseins, another protein from milk source, has a complex structure with little amount of secondary or tertiary structure [4]. Caseins show amphiphilic characteristics and therefore they are highly used in emulsion or foaming systems [5]. Caseins could also give texture and increase the water binding capacity of food products. The main function of casein in bovine milk is thought to be nutritional [6], however there are many techno-functional properties in food products. α_{S1}, α_{S2}-, β-, and κ-casein are the different types and they found in the ratio 4:1:4:1.3 [7]. In the native form, κ-caseins forms a hairy layer around the micelles and these hairs becomes inactive upon acidification at or around the iso-electric point (IEP) of casein (pH 4.6) and thereby forming aggregates through formation of salt bridges [8]. On the other hand, stability of casein against aggregation upon heating is quite high compared to whey proteins. Heat coagulation of casein could happen above 120°C [9]. Therefore, gelation or aggregation of caseins is often achieved by acidification or enzymatic crosslinking [10].

Caseins are often found in micelle form in milk, however in food products salt derived forms, which are caseinates, are more commonly used. At the IEP of caseins, washing is done to obtain pure precipitate of caseins and then it is neutralized. When NaOH is used for neutralization process, sodium caseinate (NaCas) is obtained [11]. In food systems, NaCas has several techno-functional properties such as texturizer, thickening or gelling agent, or more commonly as emulsifier due to its amphiphilic property [12].

Another protein from animal source is gelatin, which is obtained through hydrolysis of collagen [13]. Molecular structure of gelatin includes single or multiple strands of polypeptides in a helix conformation [14]. The most commonly known characteristic of gelatin is being a thermo-reversible gel. Below the melting point, gelatin has a triple-helix structure; whereas above the melting point, hydrogen bonds between helical structures are broken and the molecular structure turns into a random-coil state [15].

Gelatin could be obtained from different sources such as pork, bovine or fish, each of which has different properties. Gelling and melting temperatures of gelatin changes with respect to the species. For instance, cold water fish gelatin can melt at 4°C and gel below at 17°C, whereas warm water fish gelatin can melt at 18°C and gel at 24°C. On the other hand, melting temperature of pork or bovine gelatin can be above 30°C [13]. Depending on the pretreatment during the extraction process, either alkaline or acid conditions, two main types of gelatin are obtained, which are namely type-A and type-B. The IEP of type-A gelatin is around at pH 8-9, whereas that of type-B is around at pH 4-5 [13]. Another classification of gelatin can be made based on the gel strength, which is often expressed using Bloom number. Bloom number is mainly a function of source of gelatin and its processing conditions [16]. Source of gelatin determines the amino acid composition and processing conditions could change the molecular weight distribution, both of which affect the bloom number. Bloom number affects the physical properties of gelatin, and thereby determining its use area.

Whey proteins, NaCas and gelatin are all from different sources and have different physical and chemical properties, thereby having different functional properties. Their gelation conditions are also different from each other. Their potential use areas vary from solid state food materials, such as dough, to fluids, such as emulsion and foam systems. Therefore, in this review we focus on the particle formation through controlled gelation of these proteins and their behavior against changing pH in an aqueous medium.

2. Preparation Methods of Protein Particles

Preparation of functional foods and functional components are important [17]. One possible way to give extra functionality is to create particles through gelation of proteins. Particles produced using different biomaterials were studied previously [18, 19, 20]. Presence of proteins in these studies supply many functional and structural properties, such as biocatalysis, bioactivity, thickening, drug delivery or possible applications in tissue engineering.

Protein aggregation or gelation under certain conditions can be turned into an advantage for further stability of proteins. To make protein particles with a better control over the physicochemical properties, several methods were described in literature, such as heat-set or cold-set gelation [21, 22]. In aggregation or gelation of proteins, peptide chains interact through hydrophobic interactions, hydrogen bonding, electrostatic interactions, disulfide bridges or van der Waals interactions, and therefore rearrangement of proteins occurs [23]. Once the aggregation or gelation is done in a controlled way, protein particles with different properties could be obtained.

Heat-set gelation of proteins is a commonly used method to make particles from globular proteins, especially from the whey proteins [21, 22, 24, 25]. Whey protein solutions were heated above their denaturation temperature (68°C), which allows the reactive groups of amino acids to be free, and subsequently aggregates were formed via hydrophobic interactions, hydrogen bonding, and disulfide bonding [24]. Heat-set gelation is irreversible; therefore, stable forms of aggregates can be obtained. The size of

aggregates, which depended on the heating time and temperature, has ranged between a few hundred nm to a few microns [24, 26]. Larger aggregate formation was reported at higher temperatures during the gelation process and longer heating times through nucleation and growth process. Heat application can also be used as a pre-application in particle formation, as many proteins are susceptible to heating.

Cold-set gelation is another method to prepare protein particles. In cold-set gelation, often acidification [27, 28, 29], salt addition [26, 30], or enzymatic crosslinking [31, 32, 33] methods were used. A heating step either before or after the cold process can be used to create aggregates or to stabilize the aggregates [22, 34].

To create protein particles, acidification can be done using either acid solutions [27, 28] or acidifying agents, such as glucono-δ-lactone (GDL) [35, 36]. This method uses the principle of decreasing or totally inhibiting the net charge of the proteins and as a result, aggregates are formed. For many proteins, acidification is done until IEP is reached as the net charge is zero at the point. Salt addition also has a similar effect on the net charge of proteins; however the mechanism is different from pH-induced aggregation. Salt ions screen the charges of proteins, which are responsible for the repulsive forces. Monovalent salt (e.g. NaCl) ions can only screen the charges, whereas multivalent salt (e.g. $MgCl_2$, $AlCl_3$) ions can have additional effects, such as salt bridge formation or specific ion adsorption, besides screening [25, 37].

Enzymatic cross-linking of proteins is an alternative method for the production of particles [31, 32]. In this method, protein solutions with or without a pretreatment, such as heating or pH adjustment, were mixed with an enzyme. As enzyme functions, protein cross-linking has been achieved, and therefore particles have been formed as separate stable entities.

A recent method to produce protein particles is the emulsification method [32, 38]. In this method, a protein solution has been emulsified in an oil solution to create droplets. Then, the gelation of these droplets has been induced using one of the methods explained above. After gelation of the protein in droplets, particles have been separated from the water-in-oil emulsion using a centrifuge. This method was used for several proteins using different gelation techniques and was shown to be flexible to change the size, source, concentration, and pH of the protein particles [39, 40]. Changing these properties allow changing the internal and surface structure of particles, influencing the functional properties and thus the use area of them.

3. Effect of pH on Whey Protein Particles and Their Dispersions

Whey proteins have an IEP of around pH 5.1 and native whey proteins in their neutral aqueous solution are negatively charged. At different pH values, overall charge of whey proteins varies. The net charge of proteins at pH values away from the IEP prevents the aggregation due to the presence of repulsive forces. The pH of the aqueous phase influences the stability against aggregation of proteins, which could limit the acceptability of beverages. Such stability problems could be solved to some extent by using controlled aggregation and thereby creating protein particles or changing the surface properties of protein particles [41].

Protein particles behave like colloidal particles and they can be stabilized against coagulation by changing their surface properties. For instance, increasing the electrostatic or steric repulsive forces through changing pH, adding salt, or coating the particles with different polymers prevents the particles from approaching to each other, thereby preventing the coagulation [42].

In a study, soft and hard whey protein isolate (WPI) particles were prepared using a two-step emulsification method [43]. Sağlam and co-workers [43] prepared soft WPI particles, which were at pH 6.8, and hard WPI particles, which were at pH 5.5. These particles had different physical properties from each other [44]. For instance, soft WPI particles, which were spherical, had lower internal protein concentration, more open microstructure and smoother surface structure than the hard WPI particles [44]. On the other hand, hard WPI particles had a denser network inside particle than soft ones and a cauliflower-like shape. These properties affect the stability and colloidal behavior of particles. For example, the maximum volume fraction of the particles for a stable dispersion in the case of soft ones was found to be lower than that in the case of corresponding rigid particles. The theoretical approach for this difference explained the reason as the smaller energy barrier for two interacting colloids, which includes the electrical energy and van der Waals energy, for the soft particles than for the rigid ones [45]. Practically, the porous structure and swelling ability of the soft particles, as a result of the interaction with the co-solvents, allow a lower volume fraction of particles for a stable dispersion. At the same protein concentrations (in terms of weight) and the same size of particles, soft WPI particles had a volume fraction of 0.35, whereas hard WPI particles had a volume fraction of 0.15 [46]. This finding suggests that the volume fraction of soft particles increase faster due to the flexible network inside particles, which disrupts the colloidal stability, than that of hard particles.

Other than the colloidal stability, the stability of particle integrity is also highly dependent on the electrostatic interactions. Both soft and hard WPI particles were found to be sensitive to changes in pH of the dispersion [47]. When pH of the dispersion was changed from neutral to alkaline values, the protein network inside the particle was disrupted with the increasing repulsive force. Depending on the strength of this repulsive force, particles either swelled or at the extremes they disintegrated. When the pH of the dispersion was lowered, until the IEP was reached, WPI particles were reported to shrink, and below the IEP, due to the increased repulsive forces, particles again showed swelling. Soft WPI particles had relatively an open structure compared to the hard particles, and therefore the sensitive characteristics of soft particles to any changes in the medium, like pH or salt concentration, is expected. In the case of hard WPI particles, due to their dense and compact structure, the sensitivity to pH or salt may be less expected. However, they showed the similar vulnerability to changes in electrostatic forces [47]. At highly alkaline pH, the protein leakage from the hard particles was even more than the soft ones. The reason of the situation could be that during preparation of the hard particles, dominant contribution of electrostatic forces to the aggregation of proteins besides covalent interactions, such as disulfide bonds, which can stabilize the network as in the case of heat-set gelation [23, 34].

Disintegration of soft WPI particles could have been observed from the appearance of dispersions [47]. Particularly at pH 2 and pH 9.5, dispersions became translucent, most probably due to the disintegration of the particles. On the other hand, at other pH values in between pH 2 and pH 9.5, dispersions seemed more homogeneous and opaque, which means particle integrity was not largely disrupted. At or around the IEP of proteins, at pH 5, dispersions showed phase separation with a clear upper phase. In the same study, a flow-cell set-up for CLSM indicated that increasing pH of the continuous phase up to 10.5 resulted in swelling of particles and decreasing pH till 5.5 resulted in shrinking of particles. These results indicate that soft WPI particles were reversible pH-responsive micro-gels, as they kept their integrity upon consecutive increase and decrease of pH of the continuous phase. Such pH-responsive micro-gels could be promising in controlled delivery systems, particularly in pharmaceutical applications.

4. Effect of pH on Sodium Caseinate (NaCas) Particles and Their Dispersions

NaCas particles were produced using two-step emulsification method and using acidification in the gelation step [40]. In this case, emulsification was followed by acidification using GDL, which hydrolyses slowly in the aqueous phase, and thereby lowering the pH of NaCas droplets in the emulsion till its IEP. At this point, electrostatic repulsion between proteins decreased and therefore aggregation occurred. NaCas particles were a few hundred microns, highly porous and had irregular shapes. In that study, there was whey protein as co-solvent in the aqueous phase, and therefore pH-dependent phase separation was observed at pH 5 due to reduced electrostatic repulsion. NaCas dispersions were stable and opaque at pH values between 3 and 4. At lower and higher pH values than these, appearances of dispersions were less opaque and translucent, indicating disintegration of protein particles. SEM images of NaCas particles at pH 2, pH 3.5, and pH 6 also proved the pH-dependent disintegration away from the IEP of proteins. Above pH 6, translucent appearance was more pronounced and few particles could have been observed under CLSM, indicating that the particle integrity was not kept due to strong electrostatic repulsion [40]. In the same study, rheological behavior of dispersions at different pH values was also investigated to determine the effect of swelling of particles. Before disintegration of particles due to strong repulsion, there were pH values at which particles still kept their integrity, however depending on the strength of repulsion size of particles could have increased and microstructure of particles have changed. Viscosity measurements in rheological tests showed that at pH 2, the viscosity of dispersions increased, indicating the swelling of particles; whereas at pH 6, viscosity of dispersions decreased, indicating the disintegration of particles.

In another study, NaCas was used as a stabilizer for zein colloidal particles [48]. Zein particles were prepared using a controlled precipitation method, and NaCas was then added to cover the zein particles. Zein particles with negative surface charge density attracted the NaCas electrostatically, and the IEP of the new system became in between those of native proteins. As the pH of the system shifted from the IEP of zein, where zein aggregated, particles became stable against aggregation. In the same study, zein dispersion system was found to be stable in the presence of different NaCl concentrations, which means screening of charges did not affect the stability of colloidal particles and there was still enough repulsion. Stability of dispersion in the presence of NaCas was attributed to both electrostatic and steric interactions. Repulsive forces kept particles from approaching to each other and full surface coverage of particles by NaCas supplied steric stabilization. Thus, the concentration of NaCas could determine the strength of steric and electrostatic interactions; at higher concentrations complete surface coverage could have been reached and therefore dispersions became more stable against aggregation or precipitation [48].

A more recent and similar study was done with zein particles including NaCas for encapsulation and

controlled release systems [49]. An aromatic organic chemical, coumarin, was encapsulated using zein/NaCas-mix particles and also using NaCas coated zein particles. They have investigated the effect of several parameters during fabrication of particles, such as mixing ratio, concentration of protein, and temperature; and thus they obtained either nano- or micro-particles with optimized concentration and temperature for high stability against aggregation. In addition, the mechanisms of particle formation in both cases (either mix particles or NaCas coated particles) were explained and resulting particles were found to be similar. In the case of zein/NaCas mix particle formation, they proposed adsorption driven particle formation and coacervate formation during mixing. Both adsorption and coacervation could have occurred in short time during the course of mixing, therefore particles probably included both proteins inside their structure, changing the microstructure of particles. On the other hand, the size distribution and zeta potential values of particles showed that NaCas most probably sat on the surface of zein particles. Similar to this structure, already-formed zein particles with a NaCas coating on their surface had a core and shell structure. However, for the coated particles, FTIR results showed a shift to lower peak values, indicating the bonding interactions were different from zein/NaCas mix particles [49].

In another study, effect of pH on the accumulation of NaCas on alginate micro-gels was studied [50]. In that study, interaction between NaCas and alginate micro-gels was determined at different pH values. Alginate gels were always negatively charged at pH values between 3 and 7, whereas as NaCas has an IEP around pH 4.1, at pH 3, it is positively charged and at and above pH 5 it is negatively charged. Therefore, at these pH values, interaction was shaped by the electrostatic forces. Alginate particles could have been coated with NaCas at pH 3 as a result of attractive forces, whereas at and above pH 5 they repel each other. Interestingly, authors showed an accumulation of NaCas around alginate particles at pH 4, where no electrostatic attraction was expected. They reported the reason of this behavior as the presence of positively charged patches on the surface of NaCas protein at the IEP, as the point was actually indicated a narrow range of pH values. The IEP being a range rather than a single value is due to the presence of NaCas from different sources. Different sources of caseins provided a different distribution of carboxyl and amine groups, and this distribution resulted in a range of IEP of protein instead of specifying an exact single pH value [51].

5. Effect of pH on Gelatin Particles and Their Dispersions

As gelatin is a thermo-reversible protein, its particles are highly sensitive to temperature changes [40]. Gelatin micro-particles were prepared via two-step emulsification method with cold-set gelation. Physical properties of these particles were investigated in a broad pH range, from pH 2 to pH 12. SEM pictures showed homogeneous and spherical structures for all tested pH values between 2 and 12, indicating that gelatin micro-gels were stable against disintegration upon pH changes. Alternatively, dispersions of gelatin particles in whey protein solution showed stability against sedimentation for all pH values except pH 6. At pH 6, a phase separation was observed probably due to the electrostatic attraction between gelatin and whey proteins, which occurred as a result of charge neutralization [40].

Gelatin gels have triple-helix structure and flexible, therefore changes in the strength of repulsive forces affect the strength of gels [52]. Gelatin micro-gels were able to swell; they had an average diameter of 4 μm at neural pH of the medium and at other pH values their sizes were found to increase [40]. Viscosity measurements of dispersions also indicated the swelling of particles at pH values away from pH 6, at which the overall charge of particles was close to zero. Strength of repulsive forces increased at pH values away from pH 6 and thereby increasing the effective size of particles, which increased the volume fraction of particles in the dispersion. Dispersions showed higher viscosities at acidic pH values than the alkaline pH values, indicating that acidic pH values had stronger repulsive effect on gelatin micro-gels.

Other than emulsification method, gelatin nano-particles were produced using desolvation method [53, 54]. With this method under controlled conditions, homogeneous and monodisperse gelatin particles could have been obtained. Such particles, due to their flexible network structure and thermo-sensitive properties, were used in controlled release systems. In controlled release systems, size of particles and their internal network density were found to be important [54]. Microstructure of nano-particles was shown to depend on the changing pH of gelatin. Adjusted pH values of gelatin solutions altered the hydration characteristics, and thereby changing the electrostatic interactions [29]. As a result of changing electrostatic forces; different shapes of gelatin particles such as spherical, needle-like or irregular-shaped were obtained. Such morphological differences were found to be dependent also on the concentration of gelatin through hydrophobic interactions, hydrogen bonding, or van der Waals forces. Particularly, at the IEP of gelatin, as there was no net charge on the protein, these weak non-covalent interactions were more favored [53]. On the other hand, Ahsan and Rao [54] explained that at lower pH values than the IEP of proteins could have increased the number of hydrogen bonds via increasing available hydrophilic sections of gelatin with water. As a result of

increasing water retention, gelatin network became denser. Particle size increase at pH values away from the IEP was mainly a result of increased electrostatic repulsion. When gelatin concentration was high, the reason of particle size increase at pH values lower than the IEP was further explained with the macromolecular crowding, which could induce swelling of particles or deposition of biomaterials on the surface of particles. It was also reported that even under strong electrostatic repulsion, it was still possible to produce nano-particles using desolvation method and the particles could keep their integrity well at different pH values.

6. Conclusion

In this review, we considered three different types of protein in micro- or nano-sized particles and their stability against disintegration or swelling at different pH values. We have explained the chemical and physical properties of whey proteins, caseins and gelatin. As a globular protein, whey proteins aggregate above denaturation temperature and therefore most of the time heat-set gelation was used with different techniques. Whey proteins have a net charge except their IEP, so pH of the medium affected the integrity and stability of the particles. Whey protein particles could have swollen depending on the strength of the electrostatic forces. On the other hand, sodium caseinate particles were heat stable but highly sensitive to pH changes. As they were often formed via acidification to the IEP of casein, they kept their integrity only around that pH. Alternatively, gelatin particles were highly stable against pH changes; they could keep their integrity well at different pH values. As gelatin also has a net charge in aqueous environment, flexible micro-particles can swell or shrink depending on the strength of the electrostatic repulsion, which was similar to the case of whey protein particles. These particles have a high potential use in food and pharmaceutical industries, as texturizer and/or in controlled delivery systems. Therefore, it is important to know the physicochemical properties of these particles in aqueous media. To gain more insight on the behaviour of the protein particles, further research should focus on the interaction of these particles with other biopolymers in the matrix.

Author's Contributions

Alev Emine İnce Coşkun: Structured and wrote the manuscript.

Özgül Özdestan Ocak: Helped in preparing the manuscript and explaining the chemistry of the materials.

Buğra Ocak: Helped and designed the 5th part, which is about gelatin particles.

Semih Ötleş: Decided the main topic and narrowed down the subtitles.

Ethics

There are no ethical issues after the publication of this manuscript.

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