Effects of dynamic ultra-high pressure homogenization on the structure and functional properties of casein

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Abstract: Dynamic ultra-high pressure homogenization (UHPH) is a novel high-pressure processing technique. In this study, the effects of dynamic UHPH on the structure and functional properties of casein were systematically investigated. It was found that the functional properties of casein changed with dynamic UHPH treatment, and the treatment at 150 MPa could significantly improve casein aqueous solubility, foaming and emulsifying properties. These functional improvements could be attributed to its structural changes, since the dynamic UHPH treatment could change the secondary structure, promote the interchange reaction between the disulfide bond and the sulfhydryl group, and increase the surface hydrophobicity. The obtained results could broaden the application of casein and provide ideas for the non-thermal processing of proteins.

Keywords: casein, dynamic ultra-high pressure homogenization, functional properties, secondary structure, hydrophobicity, non-thermal processing
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1 Introduction

Casein accounts for 80% of the total protein in milk[1]. It can be precipitated from milk at 20℃ by adjusting the pH to 4.6[2]. Casein is not only a good complete protein, but also a source of more than 100 types of active peptides[3]. Studies have shown that the bioactive peptides isolated from casein have many biological activities, such as antibacterial activity, regulation of the activities of the gastrointestinal tract, blood pressure-, blood sugar-, and blood lipid-lowering effects, free radical scavenging activity, anti-cancer activity, and immunity enhancing effect[4,5]. However, casein is insoluble in water and organic solvents, which limits the full use of its biological activities. Therefore, it is necessary to improve the utilization rate of casein. Wang et al.[6] applied the heat treatment to improve the properties of casein in yak milk; Huang et al.[7] studied the impact of ultrasound on the gelling properties of casein; Rahimi et al.[8] conducted enzymatic hydrolysis of casein in camel milk using protease K to obtain the bioactive peptides that scavenged free radicals. In recent years, researchers have used dynamic ultra-high pressure homogenization (UHPH) to improve the structural and functional properties of some proteins. Dynamic UHPH is a novel and special physical modification technique[9], by which the structures of biomacromolecules, i.e. proteins and starches, will change due to the strong shearing force, high-speed impact, high-frequency oscillation, instantaneous pressure release, and other dynamic actions applied to the materials in the reaction chamber, resulting in certain changes in the functional properties of the materials[10-12]. Dynamic UHPH has been used to improve the functional properties of peanut protein[13], soy protein[14], whey protein[15], and others. However, few studies have been reported on the application of dynamic UHPH to improve the functional properties of casein. In this study, this technique was used to treat casein, study the changes of casein structure and functional properties to broaden the application of casein and provide ideas for the pretreatment of the proteolysis.

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2 Materials and methods

2.1 Materials

Casein, glycine, Tris, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), trichloroacetic acid (TCA), 1-anilino-8-naphthalenesulfonic acid (ANS), and anhydrous ethanol were purchased from Tianjin Kemiou Chemical Reagent Co., Ltd. (Tianjin, China), and β-mercaptoethanol was purchased from Synthese (Shanghai, China).

2.2 Dynamic UHPH treatment of casein solution

The casein solution at the concentration of 10 mg/mL was prepared with the phosphate buffer solution (pH 6.25) and incubated in water bath at 30℃ for 10 min, and then homogenized with a sterile homogenizer (Xinzhi Biotech Co., Ltd., Ningbo, China) for 12 min to disperse the casein. After homogenization, the casein solution was treated by a FPG12805 ultra-high pressure homogenizer (SFP Inc., UK) for 1 cycle at the designed pressure (25 MPa, 50 MPa, 100 MPa, 150 MPa, 200 MPa, and 250 MPa) with the feeding volume of 10 mL. The temperature of the obtained solution was immediately measured by an infrared thermometer.

2.3 Determination of the particle size of casein

The effect of UHPH on the particle size of casein was evaluated by using the laser light scattering method[16]. The obtained casein solution in 2.2 was immediately injected into a BT-9300H laser particle size analyzer (Bettersize, Dandong, China) equipped with a BT-601 Circulating disperser. The particle size of casein was measured and recorded by using the software Bettersize 7.21.

2.4 Measurement of the aqueous solubility

The aqueous solubility of casein was determined based on Kjeldahl method[17,18]. The casein solution obtained in 2.2 was centrifugated at 10000 r/min for 15 min in order to remove the insoluble protein. The casein content in supernatant was evaluated by using a FOSS 8400 automatic kieldahl apparatus (Hillerød, Denmark), and the solubility of casein was calculated according to the following formula:

$$\text{Solubility (\%)} = \frac{\text{Protein content in the supernatant}}{\text{Total protein content}} \times 100\% \quad (1)$$

2.5 Determination of the foaming properties

The foaming properties of casein were measured according to previous report[19]. The treated casein solution (100 mL) was dispersed at 9500 r/min for 2 min by a high-speed dispersion homogenizer. Both the volumes of the foam at 0 min (V_0) and at 30 min (V_{30}) in the graduated cylinder were recorded, respectively. Then, the foaming capability and foaming stability were respectively obtained by using the following formulas:

$$\text{Foamability (\%)} = \frac{(V_0 - 100)}{100} \times 100\% \quad (2)$$

$$\text{Foaming stability (\%)} = \frac{(V_{30} - 100)}{(V_0 - 100)} \times 100\% \quad (3)$$

2.6 Determination of the emulsifying properties

12 mL treated casein solution was mixed with 4 mL soybean oil, and the mixture was emulsified with a high-speed homogenizer at 13500 r/min for 2 min. Then, 50 μL of the emulsion was taken from the bottom of the beaker and mixed with 5 mL of 0.1 g/100 mL sodium dodecyl sulfate (SDS) solution. The initial absorbance (A_0) was measured at 500 nm, and the corresponding absorbance (A_{30}) was also recorded using the same method after the emulsion standing for 30 min. The foamability (EA) and foaming stability (ES) were calculated according to the following formulas[20]:

$$EA = 2T \frac{A_0}{c \times \varphi \times (1-\theta) \times 10^5} \quad (4)$$

$$ES = \frac{A_0}{A_0 - A_{30}} \times 30 \quad (5)$$

where, T = 2.303; EA was emulsifying ability in m^2/g; ES was emulsifying stability in min; c was the casein concentration before the formation of emulsion in g/mL; φ was the optical path length (0.01); θ was the volume fraction of oil in the emulsion (0.25).

The particle size distribution of the casein emulsion was determined by a nano-ZS90 Malvern nanoparticle size analyzer (Malvern Instruments Ltd., Worcestershire, UK) with He/Ne laser (λ = 633 nm) and a scatter angle of 173°. The casein emulsion was added to a polystyrene cuvette (refractive index: 1.33). After held at 25℃ for 3 min, the average particle size of the emulsion was measured and recorded by Malvern Zetasizer software 7.11.

2.7 Determination of the content of the sulfhydryl group and the disulfide bond

The contents of free sulfhydryl group (SH_F) and the disulfide bond were analyzed according to previous report[21]. Briefly, 0.5 mL of the UHPH-treated casein solution was mixed with 2.5 mL of Tris-gly-8M urea solution (0.086 M Tris, 0.09 M glycine, 0.004 M EDTA and 8 M urea) and 0.02 mL of 5,5'-dithiobis-2-nitrobenzoic acid solution (DTNB, 4 mg/mL). The obtained mixture was incubated at 25℃ for 30 min, and its absorbance was measured at 412 nm (A_{412}). The mixture without casein solution was used as the blank control.

For the measurement of the total sulfhydryl group (SH_T) content, 0.2 mL of the obtained casein solution in Section 2.2 was taken to mix with 1.0 mL of Tris-glycine-10 M urea solution (0.086 M Tris, 0.09 M glycine, 0.004 M EDTA and 10 M urea) and 0.02 mL of β-mercaptoethanol, and incubated at 25℃ for 1 h. Subsequently, 10 mL of 12% TCA solution was added, and the solution was maintained for another 1 h, followed by centrifugation at 3000 r/min for 10 min. The precipitate was then rinsed with 12% of TCA solution, and followed by centrifugation at 3000 r/min for 10 min. The procedure was repeated twice, and the precipitate was dissolved by adding 3 mL of Tris-gly-8M urea and 0.03 mL of DTNB solution. Immediately, the mixture was mixed evenly to promote reaction at 25℃ for 30 min. The absorbance at 412 nm (A_{412}) was measured, and the absorbance of the blank was also determined by using the sample without casein solution. The content of the sulfhydryl group and the disulfide bond were calculated using the following formulas:

$$\text{Sulfhydryl group content (μmol/g protein)} = \frac{73.53A_{412}}{C} \quad (6)$$

$$\text{Disulfide bond content (μmol/g protein)} = \frac{SH_T - SH_F}{2} \quad (7)$$

where, A_{412} was the absorbance at λ = 412 nm; C was the casein concentration of the sample in mg/mL.

2.8 Determination of surface hydrophobicity

The surface hydrophobicity of the sample was measured using the ANS fluorescent probe method[22]. 4 mL of the treated casein solution at different concentrations (0.005%-0.2%) were mixed with 20 μL of 8.0 mM ANS solution, and the fluorescence intensity (FI') of the mixture was rapidly measured on an Agilent Cary Eclipse fluorescence spectrophotometer (Santa Clara, CA, USA). The excitation and the emission wavelengths were set at 338 nm and 496 nm, respectively. The control of fluorescence intensity, mixture without ANS, was also recorded as FI_0. The FI value was obtained by the following equation:

$$FI = FI' - FI_0 \quad (8)$$

A graph was made with the casein concentration as abscissa and FI as ordinate. The slope of the curve was the surface hydrophobicity index H_0 of casein.

2.9 Measurement of secondary structure

The secondary structure of the sample was measured using a Chirascan circular dichroic spectrometer (Applied Photophysics Ltd. Surrey, UK)[23]. The phosphate buffer solution (pH 6.25) was served as a blank control. A quartz cuvette with the path length of 1.0 mm was used. The sample was scanned from 190 nm to 250 nm at a scanning speed of 100 nm/min, resolution of 0.2 nm, slit width of 1.0 nm, sensitivity of 20 mdeg, and response time of 0.25 s. The contents of secondary structure conformational units (α-helix, β-sheet, β-turn and random coil) of casein were analyzed by the spectrometer software.

2.10 Statistical analysis

Each sample was measured in triplicate. The obtained results were expressed as mean ± standard deviation. The statistics

analysis was performed by using IBM SPSS 17.0 (Armonk, NY, USA) and Origin 8.0 (Northampton, MA, USA).

3 Results and discussion

3.1 Effects of dynamic UHPH on the temperature of casein solution

Dynamic UHPH treatment can increase the temperature of the material and the machine during the processing. It was reported that a large amount of heat could be generated when the material was forced to pass through the homogenization valve under high pressure produced by the pump[24]. As a result, the temperature of the solution was increased, and the increase magnitude of the temperature was closely related with the pressure. Figure 1 showed the effects of dynamic UHPH pressure on the temperature of casein solution. The temperature of the casein solution increased linearly with the increasing of pressure. But when the processing pressure exceeded 200 MPa, the temperature changed gently. During the process, the temperature of casein solution could be controlled to below 55 ℃. Compared with the traditional heat treatment method, it is more mild, energy-saving and environmental friendly.

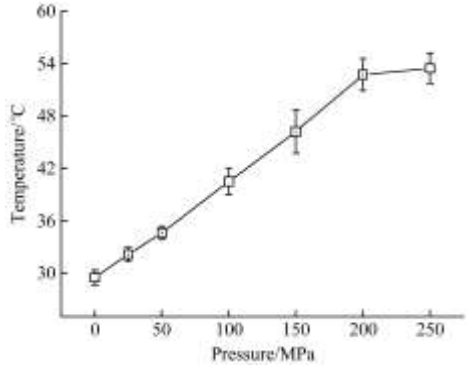

Figure 1 Effects of dynamic UHPH on the temperature of casein solution

3.2 Effects of dynamic UHPH on the particle size of casein

The effects of different dynamic UHPH treatment on the median particle size of casein were shown in Figure 2. With the increasing of pressure, the median particle size of the casein showed a gradual upward trend after the initial sharp decrease. The decrease in the median particle size of the treated casein was found because the casein was subjected to the strong shearing force, high-speed impact, high-frequency oscillation, instantaneous pressure release, and other dynamic actions in the reaction chamber, which dispersed the casein particles and destroyed the aggregates, thus reduced the median diameter of the casein particles. With the further increase of pressure, the casein particles were further broken down, the casein polymer was broken down into smaller aggregates, and the new surfaces were created. Because of the existence of thermodynamically unstable parts, the newly formed polymers were aggregated again. As shown in Figure 2, no significant differences were found in the median particle size among the casein samples treated in the pressure range of 50-250 MPa, indicating that the particle breakage and particle recombination reached a dynamic equilibrium when the pressure was between 50-250 MPa. In summary, the casein with a relatively small particle size could be obtained by dynamic UHPH treatment in the range of 50-250 MPa. It was also found that both casein samples treated at 150-300 MPa had the similar particle size distribution.

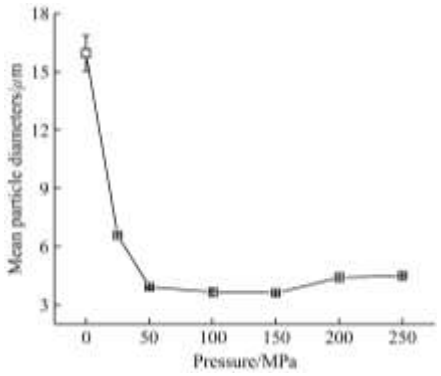

Figure 2 Effects of dynamic UHPH on the median particle size of casein

3.3 Effects of dynamic UHPH on the aqueous solubility of casein

The aqueous solubility of a protein generally refers to its water-solubility, which is the primary property of the functional properties of a protein and is the basis of other functions. A protein with a good solubility has good emulsifying ability, foamability, gelling properties, and other functional properties, and is also easier to be utilized in food processing applications[25]. The effects of dynamic UHPH on the aqueous solubility of casein were shown in Figure 3, which indicated that the solubility of casein increased firstly and then decreased with the increasing of pressure. This might because the dynamic UHPH treatment decreased the casein particle size, increased the contact area of casein with water, and resulted in an increased aqueous solubility. But according to previous report, higher pressure could expose more hydrophobic residues hidden in the interior of the protein, which could decrease the aqueous solubility of casein[26].

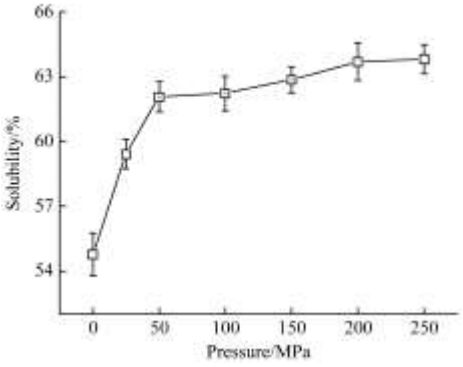

Figure 3 Effects of dynamic UHPH on the aqueous solubility of casein

3.4 Effects of dynamic UHPH on the foaming properties of casein

The foaming properties of a protein are its capacity of forming a thin film at the gas-liquid interface and stabilizing the foam, which can be characterized by foaming capacity and foaming stability. The effects of dynamic UHPH on the foaming capacity and foaming stability of casein were shown in Figure 4. With the increasing pressure level, the foam capacity of the casein was firstly increased and then decreased, while the foam stability of the casein was firstly decreased and then increased. As the block copolymers from hydrophilic and hydrophobic amino acids, casein possesses a strong surface activity, and was easy to create foams in the process of mixing. The main factors that affect the foam capacity of casein include solubility, molecular chain flexibility, hydrophobicity, protein concentration, foam generation methods, and so on. For its foaming stability, it mainly depends on the

rheological properties of the protein, such as hydration of the protein in the adsorption film, protein concentration and thickness of the film[27,28]. It was reported that the dynamic UHPH could improve the solubility and molecular flexibility of proteins, and reduce their interfacial tension. Therefore, casein molecules were more likely to be unfolded and adsorbed at the gas-water interface and to form a hard film at the gas-water interface, thus improving the foaming properties of casein[29]. However, when the pressure level further increased, the dynamic UHPH destroyed the equilibrium between hydrophobic/hydrophilic groups. Most of the hydrophobic structures of the casein were exposed, which was not favorable for the equilibrium of the protein film in the gas-water interface and the formation of foams[30]. Result from this study coincided with these previous reports.

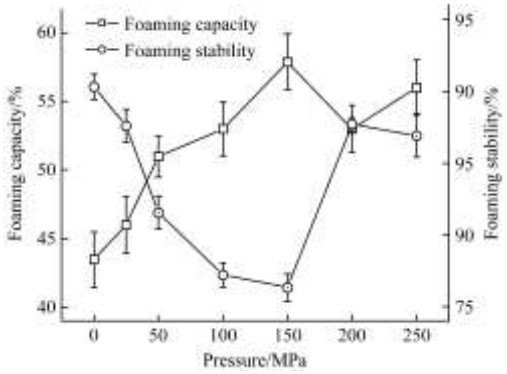

Figure 4 Effects of dynamic UHPH on the foaming properties and foaming stability of casein

3.5 Effects of dynamic UHPH on the emulsifying properties of casein

The emulsifying activity of proteins is their capacity to form the emulsions by reducing the interfacial tension of the oil-water surface. The effects of dynamic UHPH on the emulsifying capability (EA) and the emulsifying stability (ES) of casein were exhibited in Figure 5. With the increasing pressure level, the emulsifying capability of casein exhibited a downward trend after the initial increase and showed the maximum value at 100 MPa, while the emulsifying stability showed a downward trend after the initial increase. The enhancement in the emulsifying capability of casein might be due to the exposure of the hydrophobic and hydrophilic groups of the casein molecules during the homogenization process. Additionally, the dynamic UHPH improved the solubility and surface hydrophobicity of casein, thereby increased the emulsifying properties. When the pressure further increased, the unfolded casein molecules formed the polymers again by the hydrogen bonds, the disulfide bond and other forces. The molecular chain flexibility decreased so that protein and oil droplets were difficult to combine. The thermal effect of high-pressure homogenization became more significant, and the mechanical action and thermal effect were mutually affected, causing a certain degree of protein denaturation, a decrease in the surface hydrophobicity of protein and a decrease in the emulsifying capability of casein. Figure 6 exhibited the effects of dynamic UHPH on the particle size of the casein emulsion. The mean particle size of the emulsion gradually decreased with the increase of pressure, and tended to reach equilibrium. Cheng et al. also found that the dynamic ultra-high pressure treatment could decrease the particle size of the emulsion[31]. Results from this study suggested that when the pressure was higher than 50 MPa, the dispersion and aggregation of the emulsion reached a relative equilibrium, and the mean particle size of the emulsion tended to be stable.

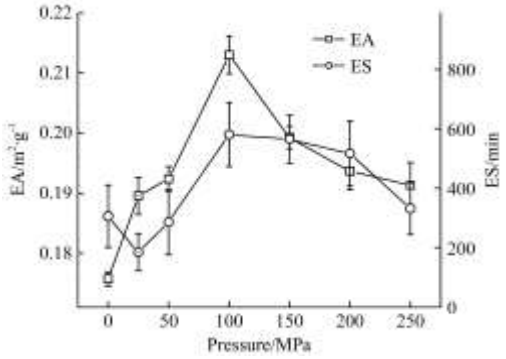

Figure 5 Effects of dynamic UHPH on the emulsifying capability (EA) and emulsifying stability (ES) of casein

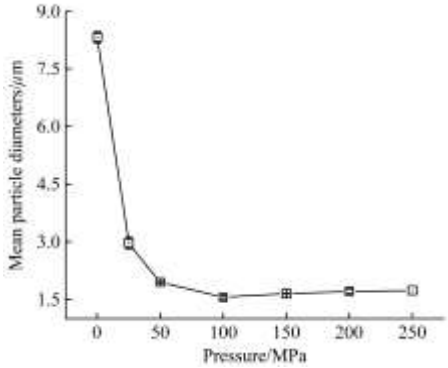

Figure 6 Effects of dynamic UHPH on the average particle size of casein emulsion

3.6 Effects of dynamic UHPH on the content of the sulfhydryl group and the disulfide bonds

The mercapto groups and the disulfide bonds are important functional groups of proteins, which belong to the weak secondary bonds that maintain the three-dimensional structure of protein. Changes in their contents can reflect the degree of protein denaturation and play an important role in the use of protein functional properties. Some processing methods (such as high pressure, heating, and others) will lead to the changes in the contents of the sulfhydryl group and the disulfide bond, causing protein denaturation. Li et al.[32] and Yang et al.[33] reported that the increased content of the sulfhydryl group in high-pressure treated soybean protein suggested a change in the protein conformation. Figure 7 shows that with the increasing pressure level, the content of the sulfhydryl group in the protein first increased to a peak value and then decreased, while the disulfide bond content decreased to a low value first and then increased. It was referred that under dynamic UHPH, the casein molecules underwent the following changes: First, the structure of casein was unfolded and became loose, allowing the internal disulfide bonds to be exposed to the molecular surface. Due to some strong local molecular activities, some disulfide bonds were broken and reduced to form sulfhydryl groups. Secondly, some sulfhydryl groups that were exposed on the surface of molecules combined with the oxygen in the air to form disulfide bonds. Furthermore, since the interface newly formed by protein particles was thermodynamically unstable, the sulfhydryl groups were folded and embedded inside the molecules. The results suggested that the breakage of the disulfide bond was dominant in the range of 0.1-150 MPa, so the content of the sulfhydryl group was gradually increased, and the content of disulfide bond was reduced

accordingly. At the pressures of 150-250 MPa, the oxidation and embedment of the sulfhydryl group were dominant, so the content of the sulfhydryl group and the disulfide bonds decreased and increased, respectively.

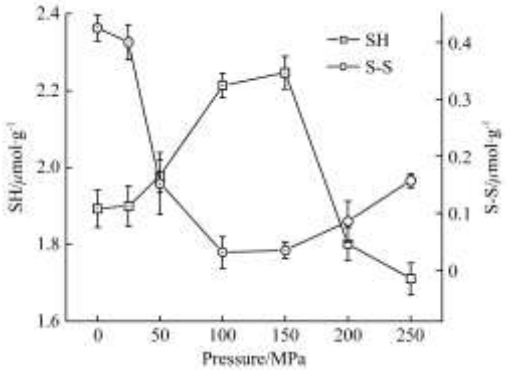

Figure 7 Effects of dynamic UHPH on the content of the sulfhydryl group and the disulfide bond in casein

3.7 Effects of dynamic UHPH on the surface hydrophobicity of casein

Hydrophobic interaction is the main force affecting the tertiary structure of proteins, has a significant effect on the functional properties of proteins such as solubility, emulsifying property and foaming property. The effects of dynamic UHPH on the surface hydrophobicity of casein were shown in Figure 8. The surface hydrophobicity of casein could be significantly increased by the treatment at 100 MPa. In the natural state, most of the non-polar amino acid residues are located in the intramolecular regions to form a stable hydrophobic core, while the polar amino acids are distributed on the protein molecular surface to maintain the interactions with water molecules, thus ensuring the stability of the hydrophilic environment[34]. Figure 8 indicated that the surface hydrophobicity could be enhanced after treated by dynamic UHPH.

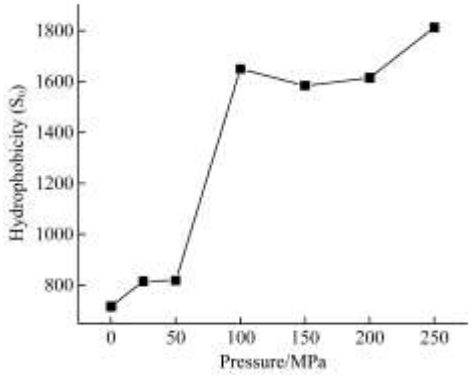

Figure 8 Effects of dynamic UHPH on surface hydrophobicity of casein

3.8 Effects of dynamic UHPH on the secondary structure of casein

The effects of dynamic UHPH on the secondary structure of casein were shown in Table 1. The conformation of casein changed in the pressure-dependent manner. The contents of α-helix and β-Sheet structures gradually decreased and increased, respectively. The hydrogen bonds between carbonyl groups and amino groups in proteins account for maintaining the α-helical structure[35], which was sensitive to the dynamic UHPH treatment. β-sheet is a typical structure of conversion from globule to fibers and probably accompanies the occurrence of protein denaturation. It could be concluded that the casein structure was stretched and rearranged, and its flexibility was enhanced, which caused the change of the functional properties of casein.

Table 1 Effects of dynamic UHPH on the secondary structure of casein

Sample	α-Helix	β-Sheet	β-Turn	Random coils
0 MPa	11.30%	26.2%	27.30%	37.80%
25 MPa	10.10%	28%	27.60%	38.30%
50 MPa	9.80%	29.1%	27.20%	38.30%
100 MPa	10.10%	28.1%	27.30%	38.10%
150 MPa	9.60%	29.3%	27.30%	38.30%
200 MPa	9.30%	30.5%	26.60%	38.20%
250 MPa	8.30%	34.7%	25.20%	38.10%

4 Conclusions

In this study, the dynamic UHPH treatment affecting the functional properties of casein was investigated. It was found that the treatment at 150 MPa improved the solubility, foaming properties and emulsifying properties of casein. These functional improvements could be attributed to casein structural changes, since the dynamic UHPH treatment changed the secondary structure, promoted the interchange reaction between disulfide bond and sulfhydryl group, and increased the surface hydrophobicity. The obtained results could broaden the application of casein and provide ideas for the non-thermal processes of proteins.

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