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Investigation Of Influencing Factors Of The Particle Size And Distribution Of Zein

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Abstract:

Background*: Zein is amphiphilic and ethanol-soluble, which can be loaded with drugs and functional ingredients to prepare microcapsules which could release at specific sites within body. However, the natural zein grain size is general large (150-550 nm) and the distribution is not concentrated, which is not conducive to the loading and release of the core material, therefore, it is urgent to explore the influencing factors of its particle size and distribution.*

Materials and Methods*: In this study, we used anti-solvent precipitation method to obtain different particle sizes of zein by adjusting pH value, ethanol concentration, heating temperature, protein concentration, the ratio of ethanol to water. Then, the particle size, chroma, absorption peak were characterized to illustrate the influence of each factors on zein.*

Results*: The average diameter of zein were ranged of 0.1~1 μm in neutral and alkaline environments, with small particle size, large colorimetric value, and high absorption peak at 280 nm. Among the zein prepared at different heating temperatures, zein showed the best dispersion and small particle size at 60 ℃. The change of ethanol concentration also had an obvious effect on the particle size distribution and the particle size. When the ethanol concentration reached 85% or above, the particle size of zein increased significantly and the dispersibility became worse. Zein particles with the ratio of ethanol to water1:6 and 1:7 were of small size and uniform distribution.*

Conclusion: *Results from this work indicated that the change of pH value had the most significant effect on the particle size and distribution of zein. Besides, different heating temperature, ethanol concentration and the ratio of ethanol to water had certain effects, but protein concentration has no obvious effect on it.*

Key Word*: Zein; Antisolvent method; Granularity; Chroma; Absorption peak*

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I.Introduction

Zein is one of the main components of corn protein, accounting for about 50% to 60% of corn protein, but it lacks tryptophan and lysine and other essential amino acids, so the biological value is low leading to low nutritional value. Zein is constituted of non-polar amino acids (e.g., leucine, proline, alanine and glutamine), while lacks basic and acidic amino acids, especially tryptophan and lysine [1], which determines the properties of zein: insoluble in water and anhydrous alcohols, but soluble in 60% ~95% ethanol solution and organic

solvents such as acetone and acetic acid [2]. In addition, its special structure and shape also affect its physical and chemical properties. Zein molecules are rod-like with a long axis ratio of 28:1 or 15:1. Zein molecules show strong optical rotation in solution, indicating that it contains a large amount of α-helix, because the conformation of α-helix fully conforms to the principle of stereochemistry, which makes it the most stable. Therefore, the molecular shape and side chain composition of zein may be the main reason for its unique properties of forming thin films.

Zein is widely used in the field of food and biomedicine. In the field of food, the type and sequence of amino acids determine that zein has good film forming properties and the film can be degraded [3]. Therefore, plasticizers and other substances can be added according to the characteristics of the required materials in practical applications. Torun et al. [4] found that the zein-based coating showed good water vapor permeability, preventing the entry of oxygen and thus preserving the color properties of the cloves. In the biomedical field, zein meets the criteria for ideal wound dressings, including non-toxicity and non-inflammatory stimulation [5], and the protein has hemostatic and adhesive properties, which can be used to accelerate wound healing [6]. Moreover, it can be used to build controlled delivery systems to protect encapsulated bioactive ingredients from degradation [7]. Zein can also be used as an adhesive, edible packaging and slow-release wall material. However, the large size of natural zein particles (150-550 nm) and their distribution are not concentrated, affecting the shape and release rate of the carrying material, and then affect the final absorption effect of the embedded material. Therefore, it is of great significance to explore the influencing factors of its particle size and distribution for expanding the application range of zein.

In this study, zein particles with different particle sizes were obtained by antisolvent precipitation method under different pH value, ethanol concentration, protein concentration, heating temperature, and the ratio of alcohol to water, respectively. Then we explored the effects of different conditions on the particle size and distribution, apparent appearance, chroma and ultraviolet absorption spectrum of zein particles.

II.Material And Methods

Materials

Zein (purity of 92.8%) was purchased from Gaoyou Rixing Pharmaceutical Co., Ltd. Sodium hydroxide was purchased from Shandong Dezhou Zhongqin Chemical Co., Ltd. Hydrochloric acid was purchased from Zhengzhou Longda Chemical Products Co., Ltd. Anhydrous ethanol was purchased from Shandong Yukang Chemical Co., Ltd.

Preparation of zein particles

A certain amount of zein powder was weighed, dissolving in ethanol solution with precision electronic balance (ME204/02, Mettler Toledo, China), and five groups of zein solution were prepared. The solution was stirred with a magnetic stirrer (ML-902, Shaoxing Satellite medicine, China) for 30 min, and then heated with an electric thermostatic water bath (DZKW-S-B, Beijing Everbright Medical, China) at 60℃ for 15 min. Then remove the beaker from the water bath and cool to room temperature. The pH of the five groups was adjusted using a pH meter (PHS-3C, Shanghai Jingke, China). Then it was equilibrated at room temperature for 12 h, placed overnight in a 4℃ refrigerator (BCD-206TS, Haier, China). Zein particles were obtained by antisolvent method. According to the different control factors (pH value, ethanol concentration, protein concentration, heating temperature, the ratio of ethanol to water), the control group was set into five groups, and except for a single control factor, other conditions should be within the optimal range. The following Table 1 represented the different levels of each single control factor.

Table 1 Different levels of each single control factor

Group number	*pH*	*Ethanol concentration/%*	*Heating temperature/℃*	*Protein concentration/%*	*the ratio of ethanol to water*
1	3	70	40	0.1	1:4
2	5	75	50	0.5	1:5
3	7	80	60	1.0	1:6
4	8	85	70	2.0	1:7
5	9	90	80	3.0	1:8

Direct observation method

The turbidity and light transmittivity of zein particles under natural light were judged by visual inspection, and the dispersion degree of zein in water was initially determined.

Particle size and distribution

The particle size and distribution of each sample could be directly determined by using the Malvern laser particle size analyzer (Mastersizer 2000, Malvern, UK). In order to ensure the rigor of the experiment and reduce the error, 5 groups of parallel tests were conducted for each group of experiments, and the average value was taken.

Chroma determination

Colorimetry could be measured directly using a colorimeter (JC-XZ-S, Qingdao Juchuang Environmental, China). Did this twice for each sample and took an average.

Ultraviolet spectrum scanning

In this experiment, the UV-visible spectrophotometer (756PC, Prius, China) was used to determine the full wavelength of 5 groups of samples between 190 and 800 nm, and the characteristic absorption peak of each group of samples was determined.

Statistic analysis

The particle size determination was repeated 5 times in each group, and the other groups were tested 3 times in parallel. Microsoft Excel 2010 and Origin 9 software were used for mathematical and statistical analysis.

III.Result

Size and distribution of zein particles

Particle size and distribution of zein at different pH

Fig.1 showed the particle size distribution and size of pH group measured by dynamic light scattering technology (DLS). When the suspension was in an acidic environment, the particle size was concentrated in the range of 10~100 μm. With the increase of pH, the particle size gradually decreased, and the peak shape became higher and wider at 0.1~1 μm. This suggested that zein in acidic conditions tended to form larger and more dispersed particles than in near-neutral and alkaline environments. Studies had shown that alkaline conditions facilitated the transition of aromatic amino acid residues in zein to a more hydrophilic microenvironment with better dispersion [8].

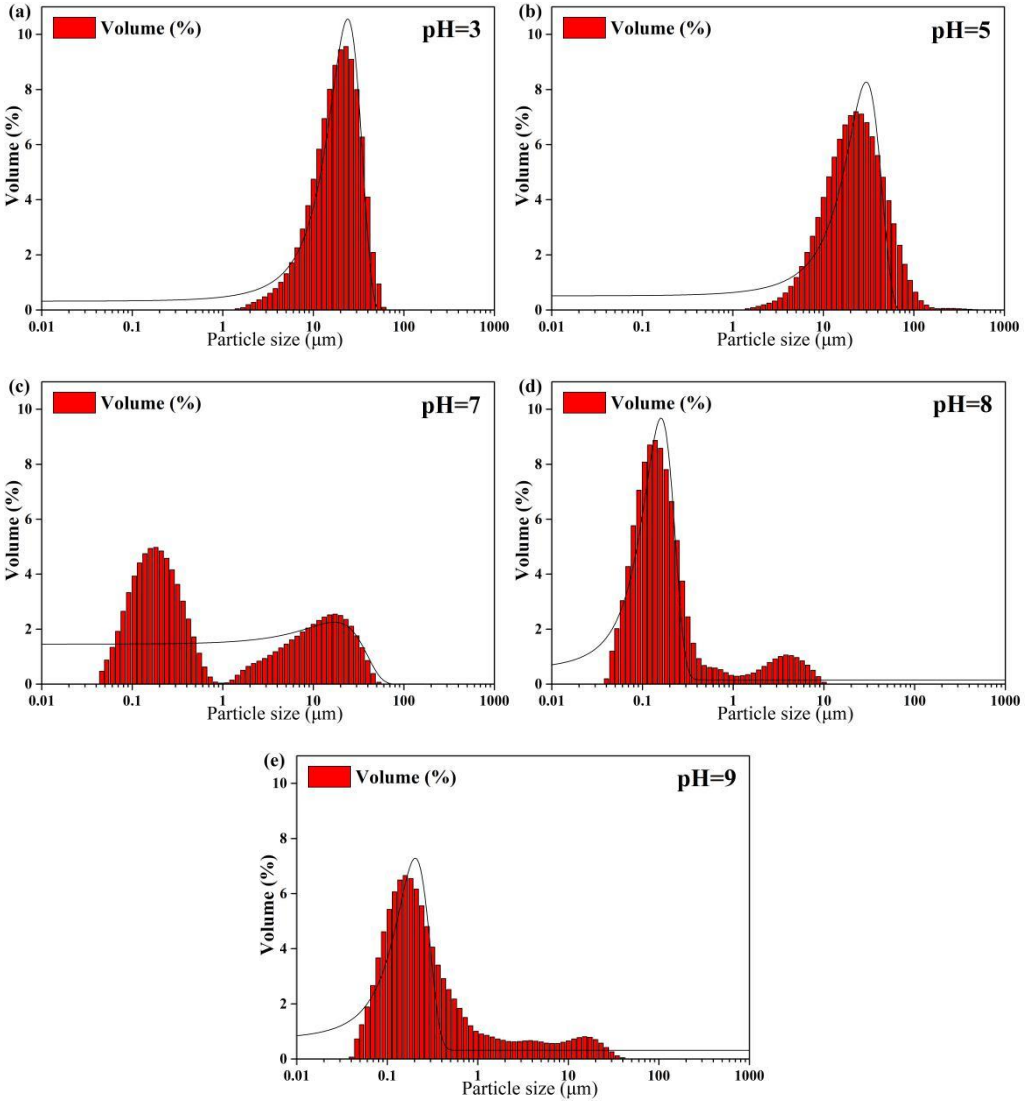


Fig.1 Particle size and distribution of zein at different pH

As could be seen from Table 2, the concentration of zein in suspension was higher than that in alkaline solution under acidic and neutral conditions, because zein particles would dissolve in a strongly alkaline environment, and the required data would not be measured if the pH continued to increase. According to Zhang et al. [9], protonation of acidic amino acids at low pH could increase the hydrophobicity of zein, while alkaline amino acids could reverse affect the surface hydrophobicity, making the protein dissolve in a strong alkali environment.

Table 2 Correlation parameters of zein particle size under different pH conditions

pH	c%	Span	Uniformity	SSA	D[3,2]/μm	D[4,3]/μm
3	0.0223	1.479	0.459	0.338	12.617	18.697
5	0.1211	2.174	0.718	0.335	14.625	26.835
7	0.0082	8.992	3.17	39.6	0.124	7.535
8	0.008	12.877	5.45	28.71	0.148	0.946
9	0.003	16.806	6.97	29.9	0.164	1.501

Particle size and distribution of zein at different ethanol concentrations

Fig.2 showed that zein particles in 70% and 75% ethanol solutions were concentrated in the range of 0.1~ 1 μm. When the concentration of ethanol reaches 80%, an obvious bimodal distribution appeared and were concentrated in ranges of 0.1~ 1 μm and 1~ 100 μm, respectively. Ethanol concentrations of 85% and 90% increased the size of zein, reaching its maximum in 90% ethanol solutions. The reason was that the polarity of different solvents was different, and the amino acid residues of zein were reshaped, which made the hydrophobic domain be exposed to the surface. The size of zein microspheres increased due to the hydrophobic interaction between molecules. It was known that the particle size was not the size of protein molecules, but the oligomerization formed by the aggregation between zein molecules and protein molecules, and the average oligomerization size was determined by the instrument [10].

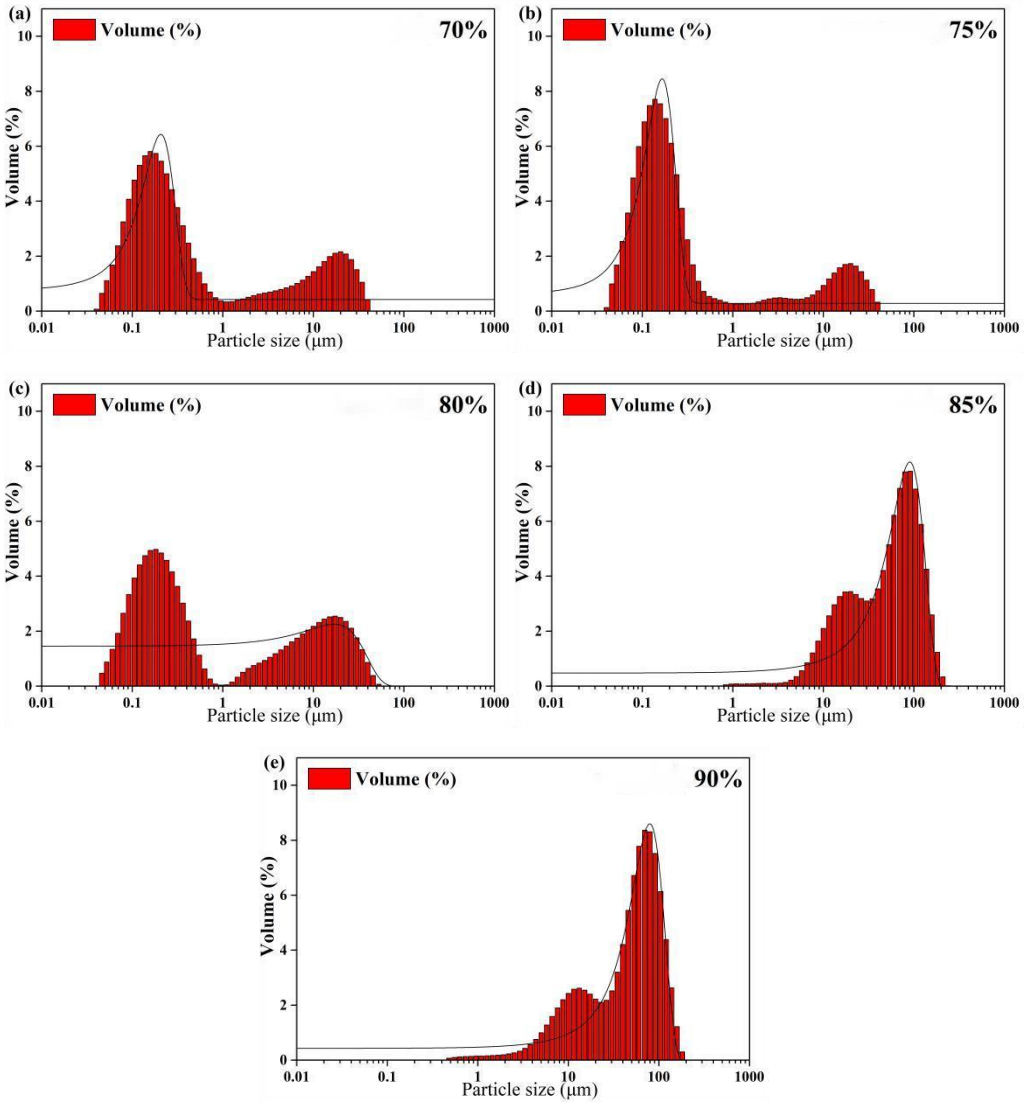


Fig.2 Particle size and distribution of zein at different ethanol concentrations

Table 3 showed the values of zein treated with different ethanol concentrations measured by particle size analyzer. D[3,2] and D[4,3] represented the volume average diameter and area average diameter of the particle size, respectively. The closer the two are, the more concentrated the particle size range was, and the smaller the particle diameter was, the larger the specific surface area was. It could be seen from the comprehensive data that

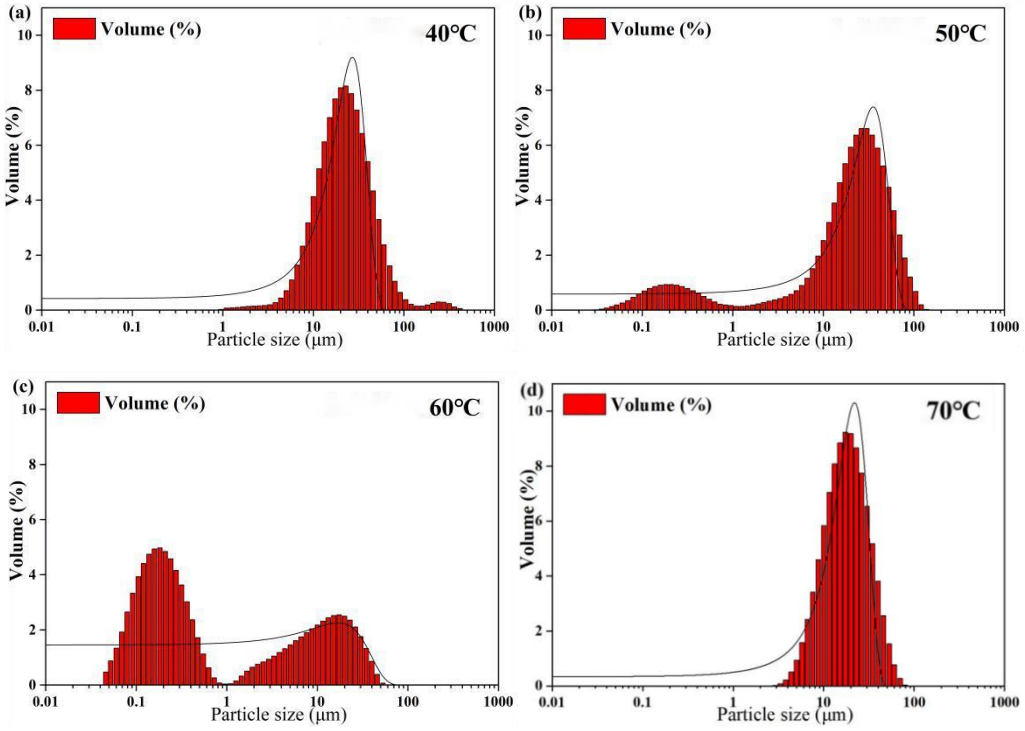
75% ethanol concentration had the most complete dissolution of protein, which was consistent with the particle size distribution of the reaction in Fig.2.

Table 3 Correlation parameters of zein particle size under different ethanol concentrations

Ethanol concentration/%	c%	Span	Uniformity	SSA	D[3,2]/μm	D[4,3]/μm
70	0.0078	69.952	16.7	26.2	0.187	4.105
75	0.0096	84.475	18.2	36.2	0.143	3.106
80	0.0082	8.992	3.17	39.6	0.124	7.535
85	0.0633	1.963	0.637	0.205	23.818	58.204
90	0.0292	1.899	0.604	0.311	15.754	51.4998

Particle size and distribution of zein at different heating temperatures

The particle size distribution results in Fig.3 showed that the particle size of zein treated at lower temperature was concentrated in the range of 10~100 μm, and the peak type was high and relatively narrow. When the temperature increased to 50℃, the peak was low at 0.1~ 1μm, and the temperature continued to increase to 60℃, where the peak value became higher. But this phenomenon did not continue, while 70℃ and 80℃ treatment of zein showed large particle aggregation state. The results showed that zein partially folded and showed a narrower particle size distribution during heat treatment at relatively low temperatures. When the heat treatment temperature was increased to 60℃, zein fully unfolded, and the fully unfolded zein molecules eventually clustered together to form protein aggregates. However, with increasing temperature, the secondary structure of zein was destroyed, forming aggregates of large particles.



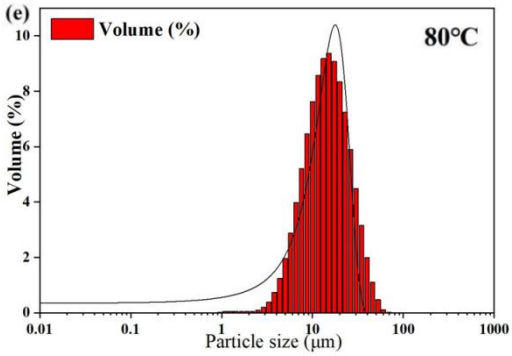


Fig.3 Particle size and distribution of zein at different heating temperatures

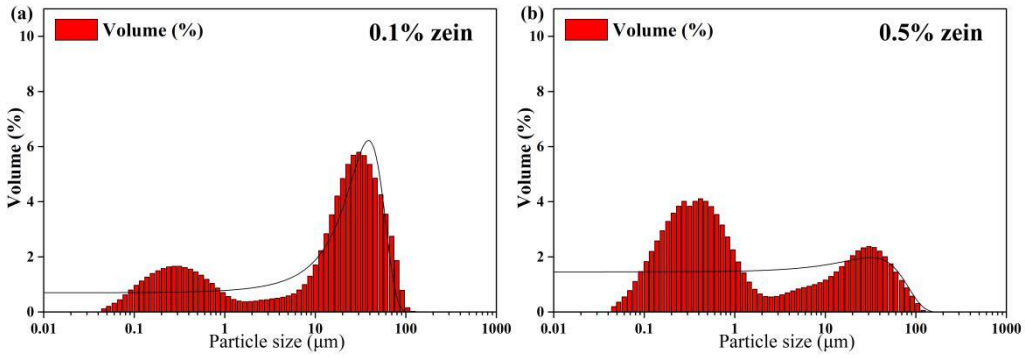
The temperature had an obvious effect on the solubility of the protein. Table 4 showed the values of zein at different temperatures. At 60℃, the secondary structure of the protein was fully developed, so the solubility was good and the particle diameter was small. As temperatures continued to rise, the proteins denatured, leading to the formation of large aggregates. Guo et al. [11] found a similar phenomenon. It was reported that the emulsion prepared by heating β-lactoglobulin at 85℃ for 7 min had a narrow distribution of droplet size, and a significant increase in droplet size was observed after heat treatment at 85℃ for 15 min.

Table 4 Correlation parameters of zein particle size at different heating temperature

Heating temperature/℃	c%	Span	Uniformity	SSA	D[3,2]/μm	D[4,3]/μm
40	0.0475	1.965	0.75	0.325	15.081	27.774
50	0.0777	2.536	0.753	4.75	1.03	24.926
60	0.0082	8.992	3.17	39.6	0.124	7.535
70	0.0511	1.608	1.23	0.347	14.084	31.52
80	0.0489	1.565	0.489	0.442	11.062	15.377

Particle size and distribution of zein at different protein concentrations

It could be seen from Fig.4 that the change of zein concentration had less effect on particle size distribution, and the peak pattern presented a two-segment peak pattern all the time. The concentration only changed the height and width of the peak, and the change was relatively small compared with the other four groups.



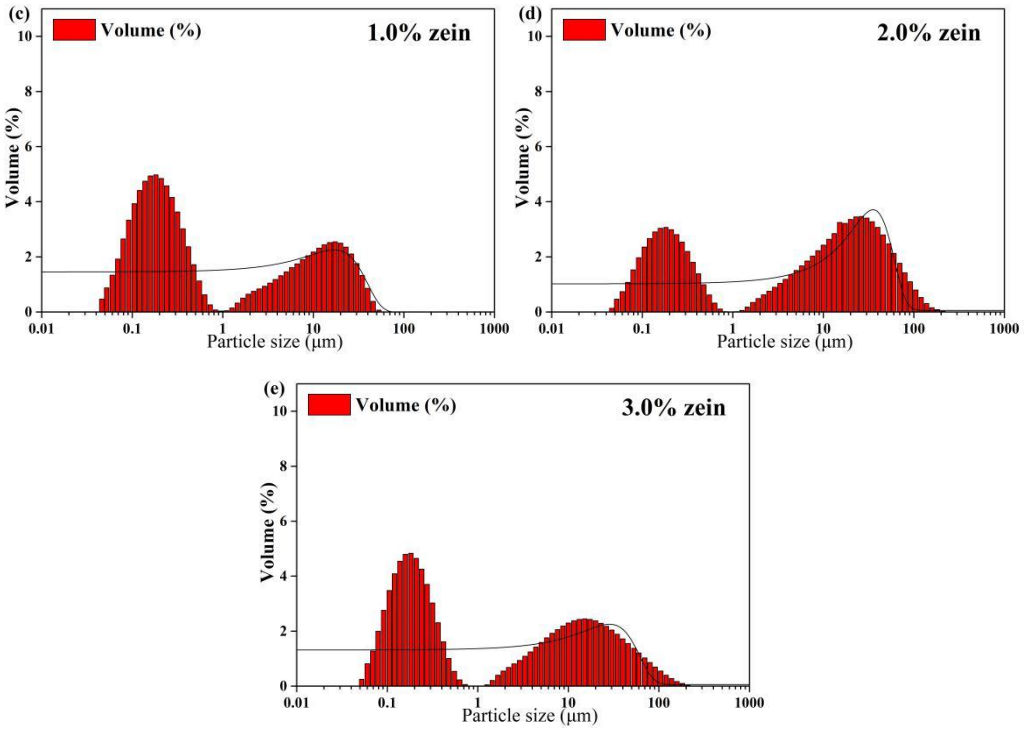


Fig.4 Particle size and distribution of zein at different protein concentrations

Table 5 showed that the particle size and distribution of zein in 80% ethanol solution had little change with the increase of protein concentration. When the concentration of D[3,2] and D[4,3] was 0.5% and 1.0%, the difference was 8.463 and 7.411, respectively, which was relatively close.

Table 5 Correlation parameters of zein particle size at different protein concentrations

Protein concentrations/%	c%	Span	Uniformity	SSA	D[3,2]/μm	D[4,3]/μm
0.1	0.0024	2.833	0.911	6.96	0.703	21.504
0.5	0.0044	52.321	14.5	14.3	0.342	8.805
1.0	0.0082	8.992	3.17	39.6	0.124	7.535
2.0	0.0129	62.238	2.05	12.9	0.378	17.091
3.0	0.0135	63.035	20.6	17.4	0.281	11.511

Particle size and distribution of zein in different ratios of ethanol to water

Fig.5 showed that the most suitable ratio was alcohol to water 1:7. The reason might be that with more water in the solution, zein could be well distributed in the solution under the action of magnetic stirring, and no microspheres would be formed. However, when the ratio of ethanol to water was 1:8, too much water was added, resulting in a decrease in ethanol concentration, and the grains of zein precipitate were concentrated in the range of 10~100 μm.

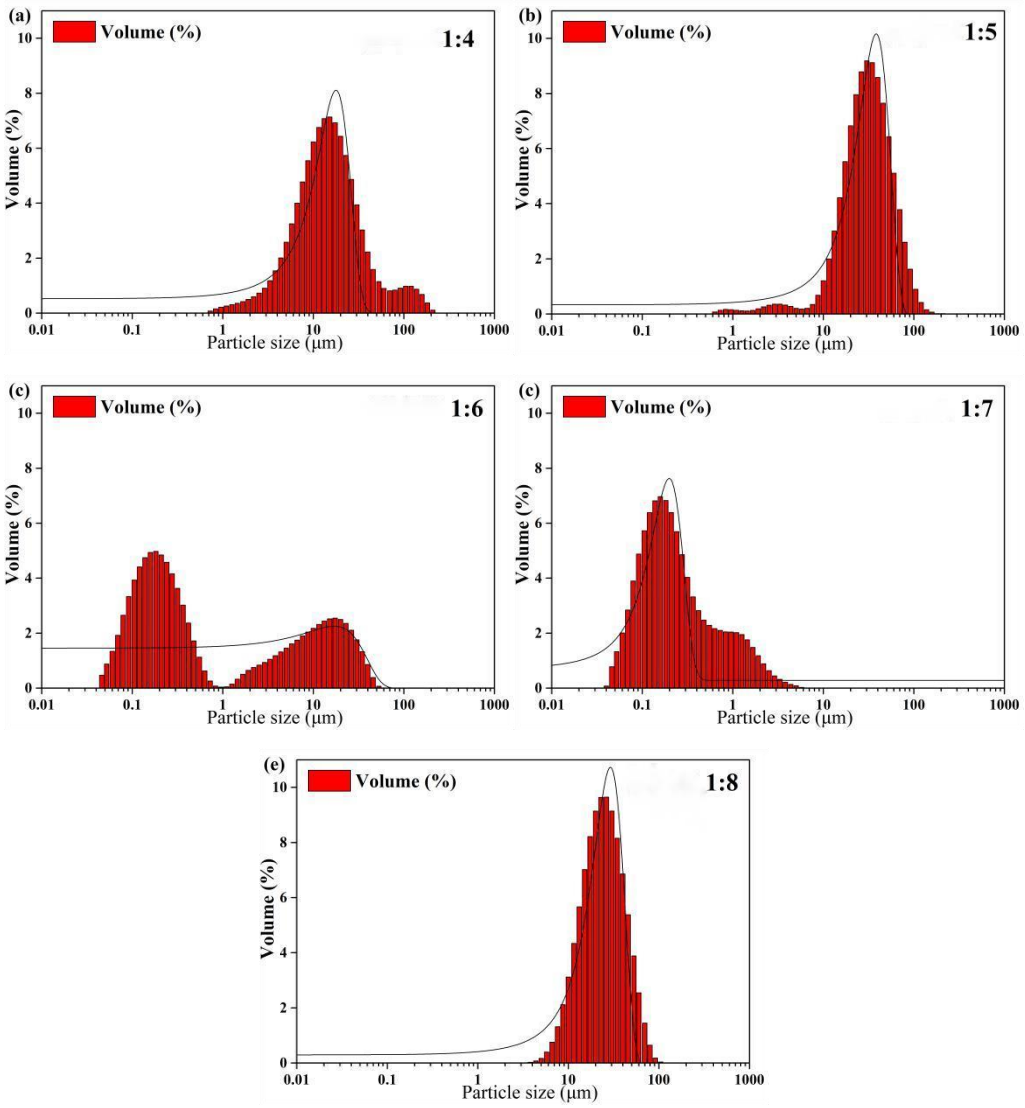


Fig.5 Particle size and distribution of zein in different ratios of ethanol to water

It could be seen from D[3,2] and D[4,3] in Table 6 that the most appropriate ratio of ethanol to water was 1:7. In this suspension, the specific surface area of zein reached the maximum in all data, which was consistent with the content in Fig.5.

Table 6 Correlation parameters of zein in different ratios of ethanol to water

The ratio of ethanol to water	c%	Span	Uniformity	SSA	D[3,2]/μm	D[4,3]/μm
1:4	0.0122	2.688	1.01	0.556	8.795	21.121
1:5	0.0271	1.636	0.52	0.285	17.201	33.043
1:6	0.0082	8.992	3.17	39.6	0.124	7.535
1:7	0.0532	1.501	4.52	41.22	0.102	0.325
1:8	0.0770	1.632	0.509	0.267	18.317	26.314

Appearance of zein particles

The solution in Fig.6 (a) showed that the zein particles in the acidic environment had large particle aggregation, forming large molecular aggregates. The solution in the alkaline environment was clearer than the other three groups, which could be speculated that zein dissolved in the strong alkaline environment and could not form particles in the water. As shown in Fig.6 (b), the turbidity of the solution increased with the increase of protein concentration, and the zein suspension with 3.0% protein concentration began to show a slight yellow color. It could be seen from Fig.6 (c) that zein formed obvious particle aggregation in water at low temperature and could not be dispersed in aqueous solution. As the temperature increases, the transmittance of the solution decreased and the solution became cloudy. In Fig.6 (d), zein also showed aggregation when the ratio of ethanol to water was 1:4. With the increase of water proportion, the dispersibility of zein in water increased. The solution became turbid and the transmittance decreased. Fig.6 (e) showed the turbidity of zein in different ethanol concentrations, and zein turbidity in 70% ethanol concentration showed a macromolecular aggregation state. The turbidity increased with the increase of ethanol concentration, and the dispersion of protein particles in water was improved.

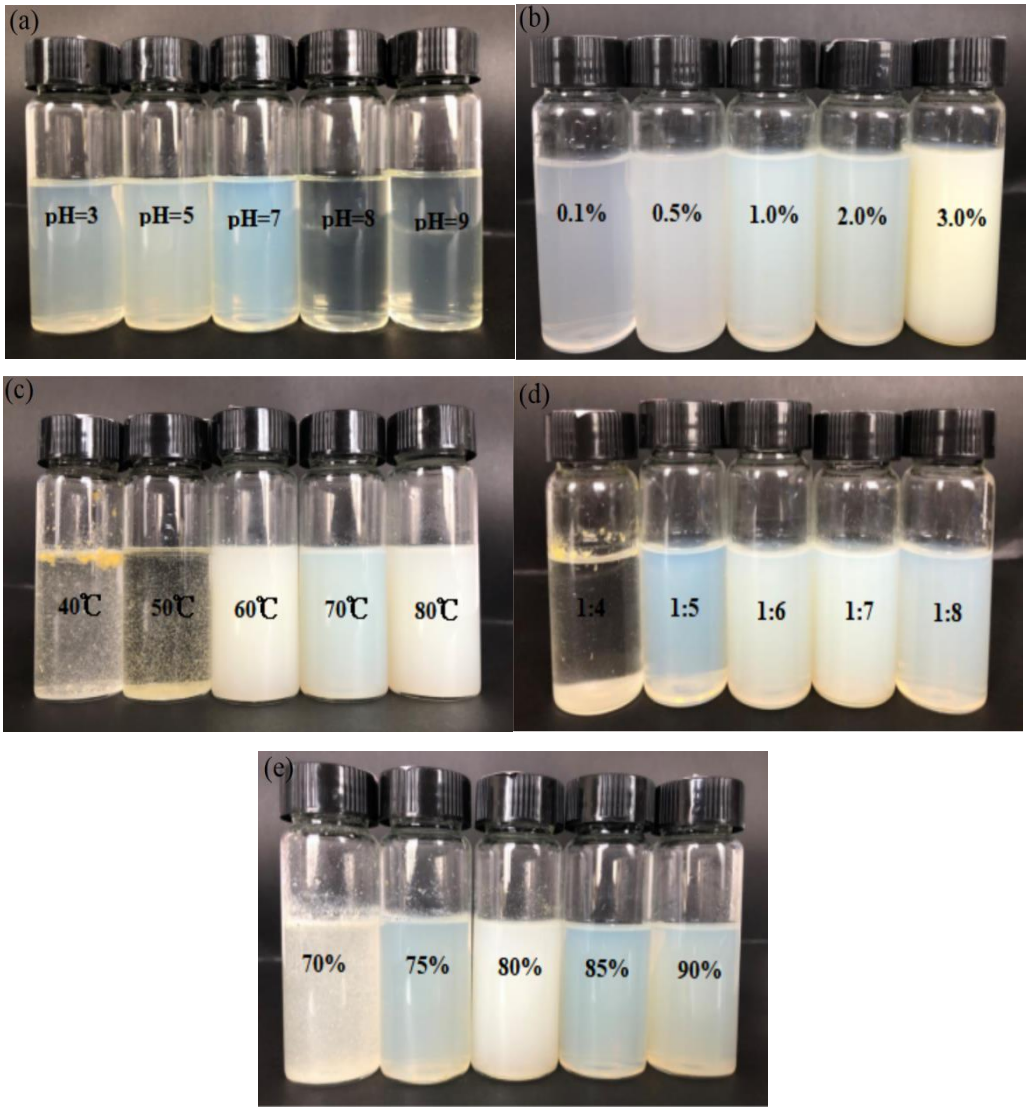


Fig.6 Photographic observation of zein particles (Note, (a) Visual images of ZEIN at different pH; (b) zein at different protein concentrations; (c) zein at different temperatures (d) zein at different ratios of

alcohol to water; (e) zein at different ethanol concentrations)

Chroma of zein particles

The L values in the colorimeter represented black and white. If the L values were positive number, the white was displayed. Otherwise, it was black, and the larger the values were, the darker they were. a* values indicated red and green, and a* values were red when the number was positive or green when it was negative. b* values referred to yellow and blue, and b* values were yellow when the number was positive or blue when it is negative. Fig.7 (a) data showed that L values in acidic and alkaline environments were smaller than these in neutral environments. These were consistent with the particle size distribution of pH-treated zein and the results of visual inspection. Fig.7 (b) showed the chroma data of zein treated with different ethanol concentrations, which was similar to the results of temperature treatment, and L values also showed a trend of first increasing and then decreasing, which was consistent with the results of visual inspection and particle size determination. Fig.7 (c) showed that with the increase of heating temperature, L values first increased and then decreased, indicating that the turbidity of the solution showed the same trend..The dispersion of zein in water reached the maximum at 60℃, which was consistent with the particle size measurement results and visual inspection. The chroma of zein samples with different protein concentrations was shown in Fig.7 (d). The L values of the first four groups of samples increased more evenly, but the L values of zein samples with 3.0% concentration increased sharply, which was consistent with the yellowish color of the samples shown in the visual examination. Fig.7 (e) showed the chroma values of zein particles with different ratios of ethanol to water. It could be seen from the particle size measurement that the particle diameter of protein particles was the smallest when the ratio of ethanol to water was 1:7, and the distribution was concentrated between 0.1 and 1 μm.

In the visual examination, the transmittance of the solution with was the lowest and the solution was the turbidity. The chroma detection results were consistent with the above two items. The results showed that zein granules had the best dispersibility and stability in the solution that the ratio of ethanol to water was 1:7.

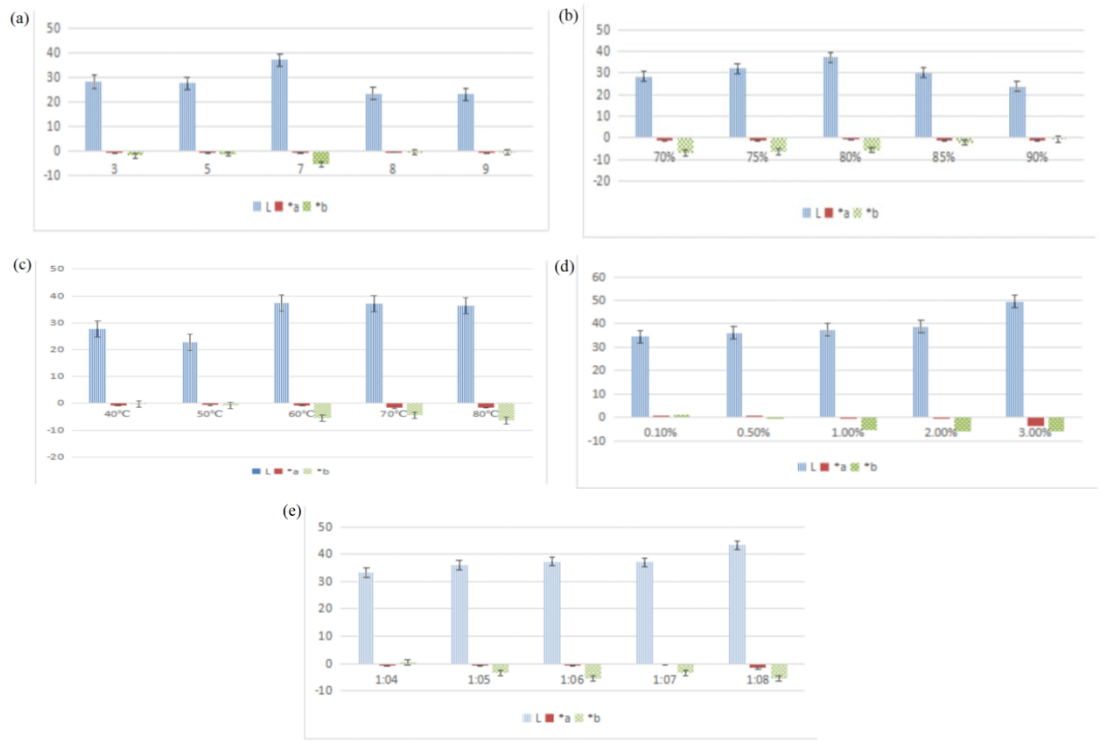


Fig.7 Colorimetric values of zein (Note, (a) colorimetric values at different pH;(b) colorimetric values

at different ethanol concentrations; (c) colorimetric values at different heating temperatures; (d) colorimetric values at different protein concentrations; (e) colorimetric values at different ratios of ethanol to water)

Ultraviolet absorption spectrum of zein

Fig.8 represented the UV absorption peak of zein particles at 198-800 nm. It could be seen from Fig.8 (a) that each group of samples had an absorption peak at 280 nm, which was close to the UV absorption peak of 80% zein reported by Sessa et al.[12].Studies had shown that zein exhibited an absorption peak at 280 nm, which might be attributed to the vibration of tryptophan and tyrosine residues, with peaks near wavelengths 285 and 277 nm. The peak value increased first and then decreased with the increase of pH, which might due to the tends that zein in acidic solution form aggregates with large particles, while in alkaline solution the particles were more dispersed, resulting in more transparency and low absorbance. Fig.8 (b) represented the full-wavelength absorption peaks of zein treated with different ethanol concentrations in the range of 195 to 800 nm. Similarly, the first absorption peak appeared at 280 nm, which was consistent with the actual protein absorption peak. With the increase of the concentration of ethanol, the peak shape of the absorption peak widened gradually, indicating that the particle range in the solution increased gradually. At the same time, the height of the peak was also increasing, indicating that the solubility of the protein was gradually increasing. UV absorption spectra were studied to elucidate the effect of heat treatment on the conformational change of zein. Fig.8 (c) showed that increasing temperature significantly increased the intensity and peak width of the UV absorption peak, and the maximum absorption peak was shown at 80℃. The increase in temperature also led to the appearance of a new peak at 345 nm, indicating that a large number of particles were concentrated in this size range. Wada et al. [13] found that with the increase of temperature, there was a difference in the UV absorption of β-lactoglobulin, and the change was significant at high temperature. Sun et al. [14] also reported that the UV absorption groups of egg transferrin were increased by heat treatment. The increase in absorption intensity indicated a conformational change in zein due to the outward turn of part of the chromophore and exposure of more tryptophan and tyrosine residues after heat treatment. As shown in Fig.8 (d), the first absorption peak appeared in each group of samples at 209 nm. For the ultraviolet spectrum of proteins, the absorption peak in the wavelength range of 200~220 nm corresponded to the backbone structure of the peptide chain, while the absorption peak near 280 nm was the characteristic peak of proteins containing aromatic amino acid residues [15]. As the protein concentration increased, a second absorption peak also appeared at 345 nm. Fig.8(e) showed that when the ratio of ethanol to water was 1:6, the peak value was the highest and the peak type was wider.

The results showed that the solubility of protein in this solution was the best, and the particle dispersion was more uniform than the other four groups. The absorption peak showed a trend of first increasing and then decreasing. The possible reason for the decline in the later stage was that the ethanol was diluted, and the concentration decreased, so the protein could not be dissolved.

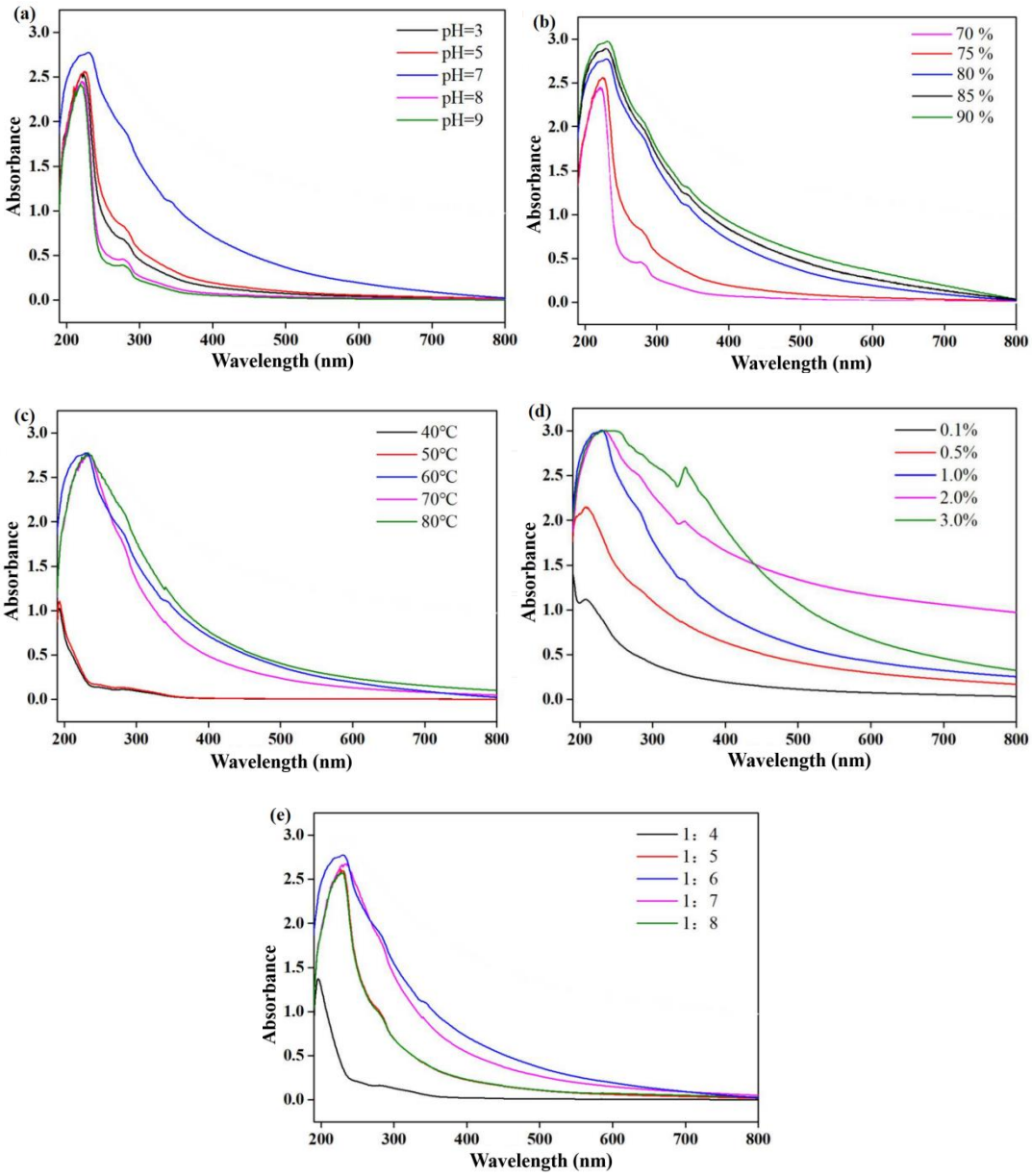


Fig.8 Zein absorption peaks under different conditions (Note, (a) different pH; (b) different ethanol concentrations; (c) Different temperatures; (d) different protein concentrations; (e) Different ratios of ethanol to water)

IV.Conclusion

The size and distribution of zein particles were greatly affected by pH. In the acidic environment, the size of zein particles was larger and the distribution was uneven, and the opposite was true in the alkaline environment. The change of ethanol concentration also showed obvious effects on the distribution and size of zein particles. When the ethanol concentration reached 85% or more, the size of zein particles increased significantly. Different heating temperatures on zein led to the different particle size and distribution. At 40℃and 80℃, the particle size concentrated in the range of 10~100 nm. At 60℃, bimodal distribution was presented. The change of protein concentration had little significant effect on zein particle size. The ratio of ethanol to water would affect the dispersion of the particles, and the ratio of ethanol to water of 1:6 and 1:7 were the best.

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