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Soy protein nanoparticles prepared by enzymatic cross-linking with enhanced emulsion stability

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1. Purification of SPI

In this experiment, SPI was purified by isoelectric precipitation method and dialysis [1]. 60 g SPI powder was taken and dispersed in 900 mL phosphate buffer solution (10 mM, pH 8.0) at a ratio of 1:15, magnetically stirred overnight to fully dissolve it, and centrifuged the next day (10000 rpm, 15 min, 4 ℃), the supernatant was taken and adjusted to pH 4.5 with 2 M HCl, and centrifuged again (10000 rpm, 15 min, 4 ℃), and the precipitate was taken and dispersed in phosphate buffer solution (10 mM, pH 8.0) again. After being fully dissolved, it was loaded into a dialysis belt for dialysis for 48 hours, freeze-dried to obtain SPI. The protein concentration was determined by BCA protein assay kit (Beyotime Biotechnology, Shanghai, China).

2. Determination of mTG enzyme activity

2.1. The effect of temperature on mTG enzyme activity

In this study, mTG enzyme was used to cross-link Gln and Lys residues between DTT-treated molten globule protein particles to form SPNPs. MTG enzyme activity was significantly affected by the change of ambient temperature, in order to explore the optimal reaction temperature of mTG enzyme, the influence of temperature on enzyme activity was investigated at 25 ℃, 37 ℃, 50 ℃ and 55 ℃, respectively. As shown in Fig. 1A, enzyme activity increased with the temperature from 25 ℃ to 50 ℃, and then decreased. 50 ℃ was the optimal activity temperature of mTG enzyme, which is basically consistent with the 45-55 ℃

optimal activity temperature of mTG enzyme reported by [2]. This conclusion provides experimental basis for the preparation of SPNPs in this work. 50 ℃ was chosen as the temperature condition of crosslinking reaction.

2.2. The influence of salt ionic strength on mTG enzyme activity

In order to explore the optimal salt ion strength of mTG enzyme activity, mTG enzyme activity was measured at different salt ion strengths (0 mM, 100 mM, 300 mM). As shown in Fig. 1B, mTG enzyme activity decreased slightly with salt ion strength increased from 0 mM to 300 mM. This conclusion is consistent with that reported by [3], salt ion strength increased from 0 g/L (0 mM) to 300 g/L (5.13 M), mTG enzyme activity decreased by more than 30 %. However, salt ion strength increased from 0 g/L (0 mM) to 25 g/L (430 mM), the enzyme activity hardly decreased [3]. The influence of salt ion strength on mTG enzyme cross-linked SPNPs was explored in this work, salt ion concentrations of 100 mM and 300 mM were selected as reaction conditions for subsequent experiments.

2.3. The effect of pH on mTG enzyme activity

Mingfei Jin, et al. [2-4] showed that mTG enzyme had a good activity from pH 5.0 to pH 8.0. In this study, pH 6.0, pH 7.0 and pH 8.0 were selected to explore the influence of different pH value on mTG enzyme activity. As shown in Fig. 1C. pH 6.0 – 8.0 showed no significant difference in mTG enzyme activity (P > 0.05), which is consistent with previous studies. pH 8.0 was selected as the reaction condition of mTG enzyme cross-linked SPNPs in this study.
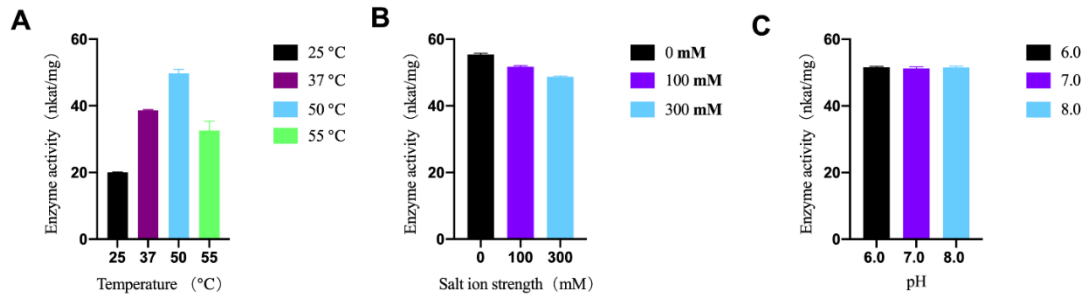
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Figure S1 - (A) the influence of temperature on mTG enzyme activity; (B) the influence of salt ion concentration on mTG enzyme activity; (C) the effect of pH on mTG enzyme activity

3. Particle size of nanoparticles prepared under different combination conditions

The influencing factors involved in the preparation of nanoparticles are protein concentration, pH, salt ion strength, mTG enzyme to protein ratio and reaction temperature. In this study, three factors were selected to explore: protein concentration, salt ion strength, and the ratio of mTG enzyme to protein. The alkaline condition selected the weak alkaline environment of pH 8.0 with better protein solubility, and the temperature selected the optimal activity temperature of mTG enzyme at 50 ℃. In order to minimize the number of tests, and

find out the best combination of levels, we use the unique balanced dispersion of orthogonal test design to design tests with three factors and three levels, and use orthogonal tables to reasonably arrange tests. We only need to make 9 highly representative teats, which can fully reflect the comprehensive test results. Table 1 is a three-factor and three-level orthogonal table designed by us, in which A represents the protein concentration, B represents the salt ion strength, and C represents the ratio of enzyme to protein, and through the orthogonal test result analysis table (Table 2), different combinations of the size of the prepared nanoparticles were obtained, and the significance order of the factors affecting the size of the nanoparticles was obtained.

Table S1 Factors and levels affecting the preparation size of nanoparticles

Levels	Factors		
	A (Protein concentration) (mg/mL)	B (Salt ion strength) (mM)	C (mTG enzyme: protein w: w)
1	A_1 (30)	B_1 (0)	C_1 (1:20)
2	A_2 (20)	B_2 (100)	C_2 (1:50)
3	A_3 (10)	B_3 (300)	C_3 (1:100)

Table S2 Table of test results

Test groups	Factors			Particle size (nm)
	A (Protein concentration) (mg/mL)	B (Salt ion strength) (mM)	C (mTG enzyme: protein w: w)	
1	A_1	B_1	C_1	16.22
2	A_1	B_2	C_2	22.34
3	A_1	B_3	C_3	36.61
4	A_2	B_1	C_2	12.71
5	A_2	B_2	C_3	16.40
6	A_2	B_3	C_1	21.03
7	A_3	B_1	C_3	14.75
8	A_3	B_2	C_1	11.76
9	A_3	B_3	C_2	13.46
K1	75.17	43.68	50.71	
K2	50.14	50.50	46.81	

K3	39.97	71.10	67.76	
k1	25.05	14.56	16.90	
k2	16.71	16.83	15.60	
k3	13.32	23.70	22.59	
R	11.74	9.14	6.99	

4. The distribution of particle size

In the table of test results (Table 1 and Table 2), Ki represents the sum of the test results corresponding to the level number i on any column, ki represents the mean value of the sum of the test results corresponding to the level number i on any column, R represents the range, the maximum k in any column minus the minimum k. According to the size of the R value, the order of the significance of the factors can be sorted out, and the optimal level combination can be selected by comparing the ki value. According to the Orthogonal Design Table, the influencing factors for the size of nanoparticles were A>B>C, and the optimal combination (the largest particle size) was $A_1B_3C_3$, namely test group 3, the combination with the middle particle size was $A_2B_2C_3$, namely test group 5, and the combination with the smallest particle size was $A_3B_2C_1$, namely test group 8, the size changes of the three particles are shown in Figure 3.
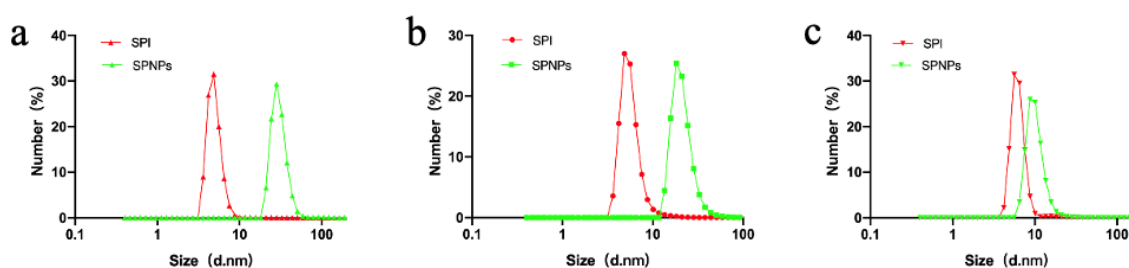

Figure S2 - The distribution of particle size

(a) No. 3 test group (SPNP-L); (b) No. 5 test group (SPNP-M); (c) No. 8 test group (SPNP-S)

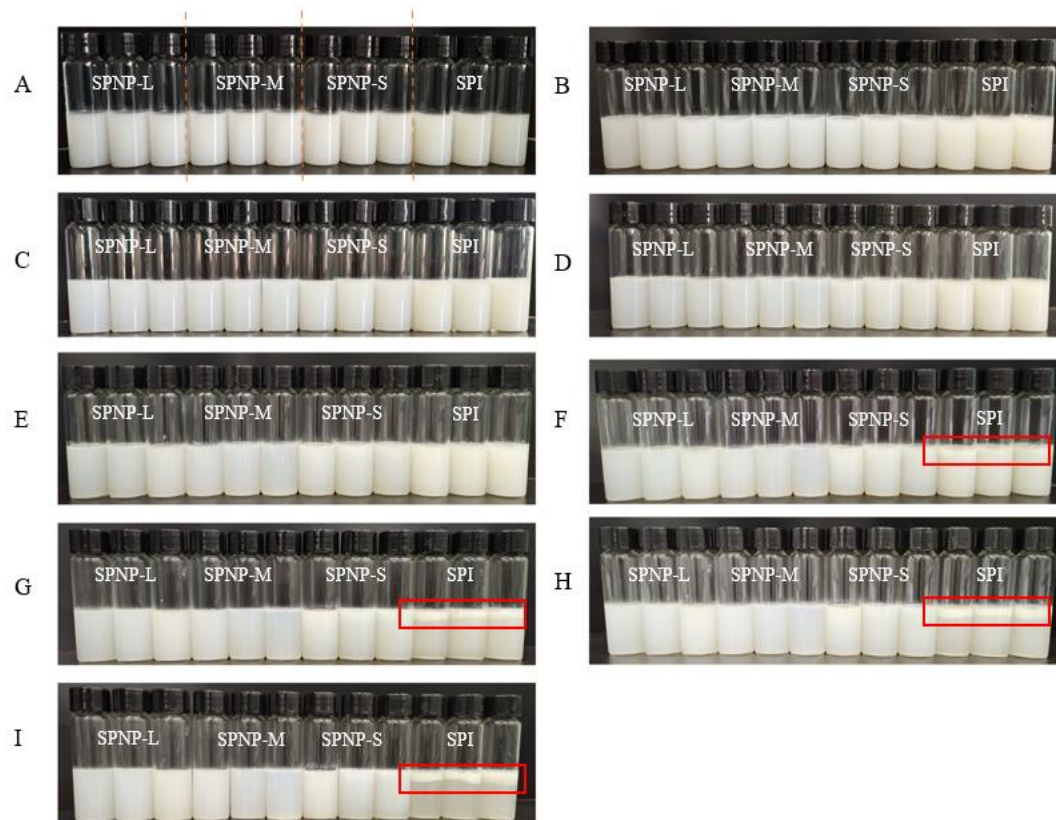## 5. Emulsions were stored at room temperature for 20 days

Figure S3 - (A) Picture of fresh emulsion; (B) Picture of emulsion after 1 day; (C) Picture of emulsion after 5 days; (D) Picture of emulsion after 10 days; (E) Picture of emulsion after 15 days; (F) Picture of emulsion after 16 days; (G) Picture of emulsion after 17 days; (H) Picture of emulsion after 18 days; (I) Picture of emulsion after 20 days

6. Analysis of the significant difference of EAI and ESI

Table S3 Significant difference analysis table		
Emulsion sample	EAI（m2/g）	ESI（min）
SPI	0.556±0.033a	188.067±28b
SPNP-L	0.512±0.033a	313.367±20a
SPNP-M	0.560±0.006a	68.643±0.65d
SPNP-S	0.523±0.024a	110.5±7.102c

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