**Effect of Papain on the Demulsification of Peanut Oil Body Emulsion and the Corresponding Mechanism**

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**Abstract:** This study investigated the effect of papain on the demulsification of peanut oil body emulsion extracted using an aqueous enzymatic method and the associated mechanism. The highest free oil yield using papain (92.39%) was obtained under the following conditions: an enzymatic hydrolysis temperature of 55°C, sample-to-water ratio of 1:3, enzyme concentration of 1400 U/g, and an enzymatic hydrolysis time of 3 h. Papain degraded the peanut oil body protein to small-molecular-weight peptides (≤ 14.4 kDa). Compared to the emulsion before enzymatic hydrolysis, the amino acid content in the aqueous phase was higher after enzymatic hydrolysis, the viscosity of the oil body emulsion was lower, and the particle diameter of the emulsion was significantly larger. The following demulsification mechanism was derived. Papain degrades the protein on the peanut oil body and dissolves it in water. The outer side of the oil body loses the protection of electrostatic repulsion and steric hindrance provided by the membrane protein. This causes the viscosity of the emulsion system and the molecular steric hindrance to decrease. As a result, the oil droplets gather and eventually demulsify. The results of this study provide the theoretical basis for the instability in oil body emulsions and are expected to promote the application of enzymatic demulsification in industry.

**Key words:** peanut oil body, demulsification, papain, rheological properties, particle diameter

**1 Introduction**

Peanut is one of the most important oilseeds in the world. China’s peanut production exceeds 17 million tons per year. Peanut oil, which has a delicate fragrance and high nutritional value, is a glyceride mixture containing approximately 80% unsaturated fatty acids and 20% saturated fatty acids. Aqueous enzymatic extraction is considered to be a safe and environmentally friendly method for extracting oil and protein from peanut. This method does not require the use of organic solvents, which create health and safety concerns, and has relatively low cost and energy requirements. In one aqueous enzymatic method, peanut oil body emulsion is obtained using an enzyme that degrades the cell wall, and the oil body emulsion is then demulsified to obtain free oil. This method can be used to obtain peanut oil along with unhydrolyzed peanut protein, improving the utilization of peanut protein. During the aqueous enzymatic extraction of oil, emulsification is easily caused by the presence of oils, phospholipids, proteins, and other substances in the aqueous system; this emulsification acts as a bottleneck that restricts further aqueous enzymatic extraction.

In the emulsion obtained during the aqueous enzymatic extraction of peanut oil, the hydrophobic groups of the protein can be adsorbed on the surface of the oil in the emulsion, resulting in the formation of a stable interfacial protein film. This film wraps up the oil, resulting in steric hindrance and preventing the oil droplets from coming close to each other and polymerizing. Meanwhile, the protein film exhibits electrification, which increases the electrostatic repulsion between oil droplets and improves the stability of the emulsion. At present, the main methods for emulsion demulsification include the isoelectric point method, freeze-thawing method, phospholipase method, and protease method. Among them, the enzymatic and freeze-thawing method provide the highest oil extraction rates. While the freeze-thawing method provides a better demulsification effect, it has several disadvantages including long cycle time and difficulty scaling for continuous mass production. In contrast, enzymatic demulsification has better application prospects. Some researchers have applied protease preparation to break down the emulsion produced during aqueous extraction. The degree of protein hydrolysis was positively correlated with
the yield of free oil\(^{10}\). Furthermore, the demulsification efficiency was significantly affected by proteases. Li et al.\(^{11}\) found papain and Protex 50FP were effective in destabilizing the cream emulsion during aqueous extraction, with free oil yield of 90.70\% and 93.50\%, respectively. However, there are few studies on enzymatic demulsification of peanut oil body emulsion in the process of aqueous enzymatic extraction, and the emulsion breaking mechanism has not been reported.

In our previous study, the peanut oil body emulsion extracted by aqueous enzymatic extraction was demulsified with papain, Protex 50FP, Alcalase 2.4 L, and Protex 7L, with the highest free oil yield of 90.30\% obtained by papain. Therefore, papain was used for the demulsification of peanut oil body emulsion in this study. First, the enzymatic demulsification process using papain was optimized. Subsequently, to determine the mechanism of demulsification, the oil body proteins, particle size, rheological properties, and microstructure of the oil body emulsion were investigated.

2 Materials and Methods

2.1 Materials

Peanuts (KaiNong KN-9326) were purchased from Kaifeng Agricultural and Forestry Research Institute (China). Viscozyme L (plant cell wall hydrolytic enzyme, optimal pH 7.0, optimal temperature 50°C) was procured from Novozymes (China). Papain (EC 3.4.22.2, molecular weight 23 kDa, optimal pH 7.5, optimal temperature 60°C) was procured from Yuanye (China). Other chemicals and reagents of analytical reagent grade or higher were procured from Sigma-Aldrich Trading Co., Ltd.

2.2 Extraction of peanut oil bodies

Peeled peanuts were crushed for 10 s using a high-speed universal grinder, and the crushed peanuts (20 g) were dispersed in distilled water (1:5 w/v). Subsequently, Viscozyme L was added to the solution followed by incubation for 90 min at 52°C in a shaker bath (PuTian, SHY-2A, Changzhou, China). After sufficient enzymatic hydrolysis, the mixture was transferred to a boiling water bath for 2 min. The cooled solution was centrifuged at 5000 rpm for 20 min (Anting, DZ267-32C6 centrifuge, Shanghai, China). The upper oil body emulsion was used in the following demulsification, and the oil body yield was calculated using Eq. (1):

\[
\text{Oil body yield \%} = \frac{\text{oil body weight (g)}}{\text{peanut weight (g)}} \times 100\%. \tag{1}
\]

2.3 Demulsification of the oil body emulsion

Enzymatic treatment was carried out following the method reported by Wu et al.\(^{12}\) with some modifications. The appropriate amount of oil body emulsion was dispersed in distilled water (1:5 w/v). A certain amount of enzyme was added, and the sample was placed in a constant-temperature oscillating water bath to adjust the sample to the appropriate temperature. After reaction for 3 h, the sample was removed to inactivate the enzyme. After cooling, the sample was centrifuged at 5000 rpm for 20 min, resulting in an upper layer of free oil, a stubborn emulsion, and a lower aqueous phase. The upper layer of free oil was removed and weighed. The free oil yield was calculated using Eq. (2):

\[
\text{Free oil yield \%} = \frac{\text{free oil from emulsion (g)}}{\text{emulsion \times oil content (g)}} \times 100\%. \tag{2}
\]

The effect of temperature (40°C, 45°C, 50°C, 55°C, 60°C, 65°C, and 70°C) on demulsification was determined under the following conditions: enzyme concentration, 1400 U/g; hydrolysis temperature, 55°C; hydrolysis time, 3 h; and sample-to-water ratio, 1.5 (w/v). The optimal demulsification conditions were determined by evaluating different sample-to-water ratios (1:1, 1:2, 1:3, 1:4, 1:5, 1:6 w/v), enzyme concentrations (400, 600, 800, 1000, 1200, 1400, and 1600 U/g), and enzymatic hydrolysis times (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 h).

2.4 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

The original oil body emulsions and the emulsions enzymatically hydrolyzed for different times were frozen and thawed at −20°C and centrifuged at 5000 rpm for 20 min. The lower aqueous phase was then collected and used after lyophilization. SDS-PAGE was performed on a gel slab comprising 5% stacking gel and 12% separating gel in a SDS–Tris–glycine discontinuous buffer system. Protein was diluted to 2 mg/mL with sample buffer, then 10 μL was loaded into sample well. After electrophoresis, the gels were stained with 0.1% Coomassie Brilliant Blue to reveal the protein bands\(^{13}\).

2.5 Amino acid composition and content analysis

The amino acid composition and contents were analyzed according to the method reported by Liu\(^{14}\). The lower aqueous phase was collected in a clean aluminum box and placed in a freeze-dryer for lyophilization. Samples (50 mg) were subjected to acid hydrolysis with 6 mol/L HCl at 110°C for 24 h. The hydrolysate was vacuum-dried until waterless and then mixed with sodium citrate buffer. The contents of 17 amino acids were determined: aspartate (Asp), threonine (Thr), serine (Ser), glutamate (Glu), glycine (Gly), alanine (Ala), cysteine (Cys), valine (Val), methionine (Met), isoleucine (Ile), leucine (Leu), tyrosine (Tyr), phenylalanine (Phe), histidine (His), lysine (Lys), arginine (Arg), and proline (Pro).
2.6 Rheological properties

The temperature-dependent viscosity of the emulsion was measured at a shear rate of \(5 \text{ S}^{-1}\) and a heating rate of \(5^\circ\text{C/min}\) using a Huck rheometer with a parallel plate geometry (PP40; 40-mm diameter and 1-mm gap setting). The viscosity was also measured at 25°C at different shear rates. In the linear viscoelastic region, the elastic storage modulus \(G'\) and loss modulus \(G''\) of the emulsion were measured under changing oscillation frequency.

2.7 Particle size measurement

The oil body emulsion and stubborn emulsion were added to deionized water in a ratio of 1:3 (w/v) and leached in an ultrasonic bath to ensure homogeneity. The particle sizes of the prepared emulsions were analyzed using a laser particle size analyzer (Microtrac S3500, Montgomeryville, PA, USA). Each sample was measured in triplicate, and the average value was calculated.

2.8 Confocal laser scanning microscopy (CLSM)

CLSM (Leica DM6000B, Heidelberg, Germany) was used to observe the distributions of lipids and proteins during the enzymatic hydrolysis of the oil body emulsion. Fluorescent stain selection and staining were performed according to the method of Li et al.\(^{11}\). Nile red was selected for oil staining, and fluorescein isothiocyanate (FITC) was used for protein staining. The sample (5 mL) was mixed with 25 \(\mu\text{L}\) of 0.1% nile red and 0.01% FITC and then observed using CLSM.

2.9 Statistical analysis

Data are presented as means ± standard deviation (SD). The level of significance was set at \(p < 0.05\) and was determined using Duncan’s multiple range test with SPSS software (version 20, SPSS Inc., Chicago, IL, USA). The same letters indicate no significant difference, while different letters indicate significant differences.

3 Results and Discussion

3.1 Optimization of the demulsification process using papain

According to Eq. (1), the yield of peanut oil body extracted by Viscozymel L was 51.54\%. The protein content in the oil body was only 2.25\%, and the oil content in the peanut oil body was 76.69\%. A significant increase in oil yield was observed when the enzymatic hydrolysis temperature increased from 40°C to 55°C, and the maximum oil yield was obtained at 55°C (Fig. 1a). However, when the

\(\text{Fig. 1} \quad \text{Effects of enzymatic hydrolysis conditions on free oil yield: (a) temperature, (b) sample-to-water ratio, (c) enzyme concentration, and (d) time.}\)
enzymatic hydrolysis temperature increased further, the demulsification rate of the peanut oil body emulsion showed decreased gradually. This may be because the higher temperature inhibited the enzymatic activity of papain, leading to a decline in demulsification rate. Thus, the optimal enzymatic hydrolysis temperature was considered to be 55°C.

Oil yield increased with increasing sample-to-water ratio up to 1:3 (Fig. 1b), which produced the highest oil yield of 92.39%. Oil yield then decreased when the ratio increased beyond 1:3. At ratios above 1:3, the enzyme becomes diluted, which reduces the probability of collision between papain molecules and substrate molecules. The resulting decrease in the enzymatic hydrolysis rate leads to a decrease in the demulsification rate. Therefore, a sample-to-water ratio of 1:3 was selected for subsequent tests.

With increasing enzyme concentration, the demulsification rate of peanut oil body emulsion first increased and then gradually leveled off (Fig. 1c). The demulsification rate was maximized at the enzyme concentration of 1400 U/g. Therefore, this concentration was selected for subsequent optimization tests.

Oil yield increased with increasing extraction time up to 3 h (Fig. 1d) and then decreased slightly. This may be because at a certain enzyme concentration, proteins are continuously degraded with increasing enzymatic hydrolysis time. After approximately 3 h of enzymatic hydrolysis, the oil body proteins were almost completely degraded, and the demulsification rate no longer increased. Over time, new emulsification of the free oil has formed, reducing the extraction rate of the free oil. Thus, an extraction time of 3 h was selected for further testing.

3.2 SDS-PAGE and amino acid profile analysis

The oil body contains three natural proteins: oleosin (molecular weight = 15–26 kDa); caleosin (molecular weight = 27 kDa); and steroleosin (molecular weight = 39–41 kDa). Proteins with molecular weights exceeding 41 kDa are commonly referred to as storage proteins. High-molecular-weight storage proteins can adsorb on the surfaces of oil bodies to improve the strength and viscoelasticity of the surface film of the oil body, enhance the charge stability and space stability of the oil body, and prevent the mutual fusion of oil bodies.

The gel electrophoresis results are shown in Fig. 2 for different enzymatic hydrolysis times. The peanut oil contained the three natural proteins (oleosin, caleosin, and steroleosin), and contains foreign proteins with a molecular weight higher than 41 kDa. During the extraction of oil bodies, a part of peanut protein is adsorbed on the surface of the oil body, consistent with the findings of Huang. However, after enzymatic hydrolysis for 1–3 h, the protein bands with large molecular weights gradually disappeared, leaving only a shallow protein band at 14.4 kDa. This indicates that after the enzymatic hydrolysis using papain, oleosin, caleosin, steroleosin, and macromolecular storage protein, which play an important role in the stability of the oil body, were completely degraded. After degradation, the functional properties of the proteins were destroyed, and no emulsification occurred; thus, steric hindrance and electrostatic repulsion were no longer present, leading to the aggregation and fusion of the oil body emulsion and finally to demulsification.

The contents of amino acids in the aqueous phase after different enzymatic hydrolysis times are shown in Table 1. The oil body proteins contained much greater amounts of polar amino acids than non-polar amino acids, which is conducive to the stability of the oil body emulsion and makes demulsification difficult. In addition, as the enzymatic hydrolysis time increased, the amount of amino acids in the aqueous phase also increased. This indicates that the protein hydrolyzed by papain on the oil body surface were degraded into amino acids or small-molecule peptides and dissolved in the aqueous phase. The amino acid distribution in the aqueous phase after enzymatic hydrolysis was similar to that of the protein in the original oil body. This indicates that enzymatic hydrolysis only degraded the protein into amino acids or peptides of small molecules. The small-molecule peptides are more soluble in the aqueous phase than proteins. Although the small molecule peptide does not maintain the stability of the oil body emulsion, the amino acid distribution in the whole system does not change.
Demulsification of Oil Body Emulsion

3.3 Rheological properties of the emulsion during enzymatic hydrolysis

The protein attached to the oil body is an important factor in determining the stability of the oil body emulsion. As a surfactant, protein can be adsorbed at the oil–water interface, leading to changes in the rheological properties of the emulsification system, including elasticity and viscosity. These changes may affect the accumulation of oil droplets in the oil body emulsion and thus have important effects on demulsification.

As shown in Fig. 3a, the viscosity of the oil body emulsion after enzymatic hydrolysis was much lower than that before enzymatic hydrolysis at all tested temperatures. This is because enzymatic hydrolysis degrades proteins into small-molecule peptides that are dissolved in the aqueous phase, reducing the viscosity of the system and facilitating the accumulation of oil droplets in the emulsion. The viscosity of the oil body emulsion before enzymatic hydrolysis decreased with increasing temperature until 80°C and then increased. After enzymatic hydrolysis, the viscosity of the oil body emulsion decreased with increasing temperature until 50°C and then fluctuated irregularly. This indicates that enzymatic hydrolysis increased the sensitivity of the oil body emulsion to temperature. As temperature increased, the viscosity of the oil body emulsion decreased rapidly. Accordingly, the resistance of the oil drops decreased, and the oil droplets in the emulsion collided, accumulated, and demulsified. As shown in Fig. 3b, the viscosity of the oil body emulsion both before and after enzymatic hydrolysis decreased with increasing shear velocity. This behavior is typical of pseudoplastic fluids, indicating that the fluid type of the emulsion did not change during enzymatic hydrolysis.

The viscoelasticity of an oil body emulsion can be characterized by $G'$ and $G''$. The dynamic rheological properties of the oil body emulsion before and after enzymatic hydrolysis using papain are shown in Figs. 3c and 3d. Within the studied frequency range, $G''$ was consistently lower than $G'$, indicating that the oil body emulsion before enzymatic hydrolysis was a stable system dominated by elasticity. After enzymatic hydrolysis, $G''$ was greater than $G'$. The elastic deformation of protein, starch, and other macromolecular

| Amino Acid | 0 h  | 1 h  | 2 h  | 3 h  |
|------------|------|------|------|------|
| Asp        | 1.94 ± 0.05a | 1.62 ± 0.04b | 1.7 ± 0.08b | 1.90 ± 0.06a |
| Thr        | 0.70 ± 0.03a | 0.56 ± 0.02b | 0.54 ± 0.03b | 0.66 ± 0.02a |
| Ser        | 0.90 ± 0.02a | 0.72 ± 0.05b | 0.74 ± 0.04b | 0.86 ± 0.02a |
| Glu        | 3.00 ± 0.16a | 2.54 ± 0.08b | 2.70 ± 0.16ab | 3.00 ± 0.12a |
| Gly        | 0.98 ± 0.02a | 0.78 ± 0.07b | 0.78 ± 0.01b | 0.92 ± 0.04a |
| Ala        | 0.86 ± 0.03a | 0.68 ± 0.02b | 0.64 ± 0.02b | 0.80 ± 0.03a |
| Cys        | 0.08 ± 0.00a | 0.06 ± 0.00a | 0.10 ± 0.01a | 0.10 ± 0.04a |
| Val        | 0.94 ± 0.02a | 0.74 ± 0.03b | 0.74 ± 0.03b | 0.88 ± 0.04a |
| Met        | 0.16 ± 0.00b | 0.18 ± 0.02b | 0.16 ± 0.02b | 0.24 ± 0.01a |
| Lle        | 0.78 ± 0.03a | 0.62 ± 0.04b | 0.58 ± 0.02b | 0.72 ± 0.01a |
| Leu        | 1.38 ± 0.07a | 1.04 ± 0.03bc | 1.00 ± 0.04c | 1.20 ± 0.09b |
| Tyr        | 0.82 ± 0.02a | 0.70 ± 0.08b | 0.68 ± 0.03b | 0.82 ± 0.01a |
| Phe        | 0.92 ± 0.02a | 0.72 ± 0.03c | 0.72 ± 0.02c | 0.84 ± 0.02b |
| His        | 0.68 ± 0.03a | 0.58 ± 0.02b | 0.54 ± 0.02b | 0.66 ± 0.02a |
| Lys        | 0.74 ± 0.02a | 0.64 ± 0.05b | 0.64 ± 0.05b | 0.78 ± 0.01a |
| Arg        | 1.82 ± 0.10a | 1.50 ± 0.15b | 1.6 ± 0.08ab | 1.8 ± 0.06a |
| Pro        | 0.88 ± 0.02a | 0.64 ± 0.03c | 0.66 ± 0.01c | 0.74 ± 0.02b |
| NAC        | 6.90 ± 0.10a | 5.40 ± 0.08b | 5.28 ± 0.13b | 6.34 ± 0.11c |
| PAC        | 10.66 ± 0.17a | 8.94 ± 0.13b | 9.28 ± 0.15c | 10.56 ± 0.16a |
| TAA        | 17.56 ± 0.26a | 14.34 ± 0.16b | 14.56 ± 0.21b | 16.90 ± 0.30a |

Means for the same amino acid followed by the same letter are not significantly different ($p > 0.05$).

NAC: nonpolar amino acid. PAC: polar amino acid. TAA: total amino acids.
substances in the emulsion is important in determining its elastic properties. The $G'$ and $G''$ values in this study indicate that the protein molecules of the oil body emulsion were degraded by enzymatic hydrolysis, causing the viscoelasticity to decrease and resulting in decreased stability and demulsification.

3.4 Change in particle diameter of the oil body emulsion during enzymatic hydrolysis

Particle diameter is an important parameter to evaluate the stability of peanut oil body emulsion. To study the demulsification process of peanut oil body emulsion, a laser particle size analyzer was used to detect the mean particle diameter of peanut oil body emulsion after enzymatic hydrolysis for different time periods. The mean particle diameter of peanut oil body emulsion increased with increasing enzymatic hydrolysis time (Fig. 4a), indicating that protease degraded the proteins on the oil body during enzymatic hydrolysis. This degradation destroyed the interfacial membrane structure of the oil body emulsion, causing the oil droplets to polymerize and form larger oil droplets. In addition, with increasing enzymatic hydrolysis time, the...
number of oil droplets with sizes of 10–1000 μm gradually increased, while the number of droplets with sizes of 10 μm or less gradually decreased. The particle diameter distribution also gradually changed from a clear bimodal distribution to a unimodal one with increasing hydrolysis time, and the peak in the particle diameter distribution gradually moved toward a larger diameter. This indicates that with increasing enzymatic hydrolysis time, the particle diameter distribution gradually became more uneven, with an increase in peak particle diameter and the accumulation of oil droplets into larger droplets. These effects are likely to lead to the final demulsification of the oil body emulsion.

After demulsification, some oil body emulsions remained; these are referred to as stubborn emulsions. As shown in Fig. 4b, the particle diameter distribution of the stubborn emulsion was similar to that of the oil body emulsion. As the enzymatic hydrolysis time increased, the mean particle diameter of the stubborn emulsion gradually increased. However, the increase in particle diameter in the stubborn emulsion was smaller than that in the regular emulsion, indicating less accumulation and fusion between oil droplets in the stubborn emulsion, which explains why it was not demulsified. This phenomenon may be attributed to a higher protein concentration on the surface of the stubborn emulsion, lower oil droplet aggregation rate, and higher stability compared to the regular emulsion27. According to the particle diameter distribution, as enzymatic hydrolysis time increased, the number of oil droplets with diameters below 10 μm gradually decreased, while the number of droplets with diameters in the range of 10–1000 μm gradually increased. The particle diameter of the stubborn emulsion gradually increased with increasing enzymatic hydrolysis time; however, because of the low concentration of oil droplets, demulsification did not occur.

3.5 Lipid and protein distributions during enzymatic hydrolysis

Nile red exhibits intense fluorescence in lipid environments because of its solubility in neutral lipids28. FITC is a dye that is electrostatically attracted to the charged groups of proteins29. The oil and protein distributions in the emulsion before and after enzymatic hydrolysis were observed by CLSM. The original oil body emulsion contained more proteins than the hydrolyzed emulsion, and the outer layer of the oil body was tightly surrounded by proteins; as a result, few oil bodies could be observed (Fig. 5). After enzymatic hydrolysis, the protein content was drastically reduced, and the oil body content was significantly increased. The size of the oil body was significantly increased compared to the original emulsion, consistent with the particle size distributions discussed above. These results indicate that the protein in the oil body emulsion was degraded under the action of papain. Thus, the oil body emulsion was destabilized and polymerized, eventually leading to demulsification30.

The microscopic structure of the oil body emulsion is shown in Fig. 6. After papain was added to the oil body emulsion, the proteins on the surface of the oil body were enzymatically decomposed into amino acids or small-molecule peptides, so that they were solubled in the water phase and had no emulsifying effect. The oil droplets lost their protein films and gradually aggregated into larger oil droplets. Finally, when the centrifuge was rotating at high speed, different centrifugal forces were generated due to the density difference between oil and water, and the different phases were separated.
4 Conclusions

Papain was used to demulsify peanut oil body emulsion obtained by aqueous enzymatic extraction, and the demulsification mechanism was studied. The optimal demulsification rate was 92.39%. Due to the combined action of phospholipids and oleosin in oil body emulsion and the resulting electrostatic repulsion and steric hindrance, the oil droplets exist independently and stably in the emulsification system. Thus, it is difficult to demulsify the emulsion and extract clean oil. In this study, the addition of papain into peanut oil body emulsion caused the exogenous proteins and proteins on the oil surface to be degraded into amino acids and small-molecule peptides, which dissolved in the aqueous phase without emulsification. As a result, the outer part of the oil body lost the protection provided by the electrostatic repulsion and steric resistance afforded by the membrane proteins and exogenous adsorbed proteins. Thus, the oil droplets came into close proximity with each other and fused in the emulsification system, and the small oil droplets gradually formed larger oil bodies. Meanwhile, the degradation of proteins reduced the viscosity of the system, facilitating the movement of oil bodies and their aggregation, which led to demulsification.

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Author Contributions

R. Niu and Z. Zhao designed and conducted the experiments, performed data analysis, and wrote the manuscript. F. Chen supervised the study and helped to initiate the project. Y. Xin, X. Duan revised the manuscript. B. Zhao helped complete the experiment.

Conflict of Interest

We declare that we have no conflict of interest.

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