

Solvent-free pH-driven encapsulation of hesperidin by ovalbumin: binding, antioxidant activity, and gel network properties

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Abstract: Hesperidin (HES) is a citrus flavanone glycoside with promising bioactivities but limited aqueous solubility, which restricts its utilization in food systems. Here, a solvent-free pH-driven strategy was developed to encapsulate HES using ovalbumin (OVA) as a protein carrier, and the structural responses and antioxidant performance of the resulting OVA-HES systems were systematically evaluated at the alkaline driving stage (pH 12) and after neutralization (pH 7). Encapsulation efficiency (EE) increased monotonically at pH 12 and reached 74.75% at 0.8 mg/mL HES, whereas EE exhibited a bell-shaped dependence after neutralization and peaked at 51.64% at 0.6 mg/mL. Fluorescence quenching, Stern-Volmer analysis and docking collectively supported complex formation dominated by static quenching and multiple hydrogen-bond interactions, with stronger binding at pH 12 ($K = 49.3 \times 10^{10}$ L/mol) than at pH 7 ($K = 3.45 \times 10^{10}$ L/mol). Moderate HES loading enhanced free sulfhydryl exposure (maximum at 0.6 mg/mL) and improved 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) cation radical scavenging ability, with a peak value of 64.51% at pH 7. Fourier transform infrared spectroscopy, water-holding capacity, low-field nuclear magnetic resonance, and scanning electron microscope analyse further indicated that intermediate HES levels (0.4–0.6 mg/mL) promoted a denser and more continuous gel network, whereas excessive HES induced heterogeneous aggregation and reduced water retention. Overall, this work demonstrates a pH- and dosage-dependent route for incorporating HES into OVA matrices and provides mechanistic insights for designing antioxidant protein gels via solvent-free encapsulation.

Keywords: ovalbumin; hesperidin; pH-driven encapsulation; antioxidant activity; gel microstructure

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1 Introduction

Hesperidin (HES), a flavanone glycoside consisting of hesperetin and the disaccharide rutinose, is a predominant citrus bioflavonoid and a key bioactive constituent of Citri Reticulatae Pericarpium (dried tangerine peel) and Aurantii Fructus immaturus (immature bitter orange fruit)^[1]. HES has been associated with antioxidant, anti-inflammatory, neuroprotective, and hepatoprotective effects^[2]. Nevertheless, its poor water solubility remains a major barrier to broader utilization across food, cosmetic, and pharmaceutical applications. To address this limitation, a range of stabilization and delivery strategies has been explored, including nanoemulsions (NEs), microcapsules, and biopolymer-based complexes. For instance, incorporation of citrus flavonoids into NEs improved the stability of hydrophobic bioactives^[3], while soy protein isolate-polysaccharide complexes enabled aqueous-phase stabilization and markedly increased the bioaccessibility of HES^[4]. In addition, enzymatic conversion of HES using *Aspergillus sojae* naringinase generated hesperetin-7-O-glucoside with enhanced solubility and bioavailability^[4]. Collectively, these approaches can be broadly classified as i) chemical modification, which converts HES into more soluble derivatives via enzymatic or chemical catalysis, and ii) physical modification, which integrates HES into carrier matrices to form stable delivery systems such as nanoparticles, emulsions, solid dispersions, and micelles^[5].

Despite their effectiveness, many physical approaches rely on cosolvent-rich formulations, where HES is pre-dissolved in organic cosolvents (e.g., ethanol, isopropanol, or *n*-propanol) prior to carrier association^[6]. This dependence on organic solvents raises concerns regarding safety and potential toxicity. In parallel, chemical modification often involves operational complexity, higher costs, uncertain intermediates, and increased energy demand. Accordingly, developing a low-energy, safe, and solvent-free strategy that enables efficient encapsulation while improving the apparent water solubility and bioavailability of hydrophobic bioactives is of considerable interest.

The pH-driven method has emerged as a solvent-free encapsulation strategy that exploits proteins as primary carriers^[7]. Under alkaline conditions, disruption of intermolecular hydrogen bonding and strengthened electrostatic repulsion promote protein unfolding and expose additional hydrophobic residues, thereby facilitating hydrophobic association with polyphenolic compounds, including flavonoids such as HES^[8]. Subsequent neutralization induces protein refolding, and non-covalent interactions stabilize the guest molecules, often accompanied by reduced crystallinity and partial amorphization that favor retention within protein hydrophobic domains^[9]. These features make pH-driven assembly particularly attractive for food-grade encapsulation without organic solvents.

Ovalbumin (OVA), the predominant egg-white protein (~45 kDa; 385 amino acids), is highly digestible and nutritionally favorable^[10–11]. OVA is rich in hydrophobic amino acids (> 50%) and contains hydrophobic pocket-like domains, supporting efficient loading of hydrophobic small molecules^[12]. Previous work further demonstrated that OVA forms stable complexes with HES and related derivatives, improving their functionality and chemical stability, including methyl hesperidin (MH) and hesperidin methyl chalcone (HMC)^[13]. Given these attributes, OVA represents a promising carrier for pH-driven encapsulation and delivery of HES^[3].

The gel properties of OVA hold significant application value in the food industry. However, research on the effects of pH-driven encapsulation on subsequent processing performance of proteins remains limited. Given that OVA-based products typically undergo thermal processing and are consumed in gel or foam forms, it is essential to systematically evaluate the structural integrity and biological activity of HES-loaded OVA following heat-induced gelation. Therefore, this study investigated the interaction mechanisms and encapsulation efficiency (EE) of HES in the OVA system and characterized the associated changes in thermal gelation behavior. By integrating molecular-level analysis with textural and functional evaluations, this work aims to advance the understanding of solvent-free encapsulation of hydrophobic bioactives and its practical implementation in food systems.

2 Materials and methods

2.1 Materials

OVA (purity 95%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). HES (analytical grade) was obtained from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; analytical grade) was purchased from Sigma-Aldrich. Hydrochloric acid and anhydrous ethanol (analytical grade) were supplied by Beijing Lanjiek Technology Co., Ltd. (Beijing, China). All chemicals were of analytical grade, and deionized water was used in all experiments.

2.2 Sample preparation

OVA stock solution (7 g/100 mL) was prepared in deionized water, magnetically stirred at 25 °C for 30 min, and hydrated at 4 °C overnight to ensure complete dissolution. The pH of the system was adjusted to 12.0 using 1.0 mol/L NaOH solution. HES was then added to achieve final concentrations of 0.2, 0.4, 0.6, and 0.8 mg/mL, and the samples were denoted as OVA/HES-0.2, OVA/HES-0.4, OVA/HES-0.6, and OVA/HES-0.8, respectively. After mixing for 5 min in the dark with minimal oxygen exposure, the pH was readjusted to 7.0 using 1.0 mol/L HCl and stirred for a further 10 min. The resulting suspensions were centrifuged at 3 000 r/min for 10 min, and the collected supernatants were used as the OVA-HES complex solutions. Transfer the prepared OVA-HES complex supernatant and pure OVA solution (blank control) into clean glass vials. Add 20 mL of sample solution to each vial to ensure a level liquid surface. Subsequently, place the vials in a 90 °C constant-temperature water bath and heat for 30 min to obtain the corresponding gel samples (invert the sample vial 90° for 1 min; the gel remains intact and does not flow).

2.3 Determination of EE

EE was determined by dimethyl sulfoxide (DMSO) extraction followed by ultraviolet (UV) quantification. Specifically, 1.0 mL of the OVA-HES complex solution was diluted with 9.0 mL DMSO, vortexed for 2 min, and equilibrated for 3 min to ensure complete dissociation of HES. The absorbance was recorded at 364 nm (UV-2600; Shimadzu Corp., Kyoto, Japan), and HES concentration (ρ_1 , mg/mL) was obtained from the calibration curve ($y = 1.129 4x + 0.022 3$, $R^2 = 0.994 6$). EE was calculated as equation (1):

$$EE (\%) = \frac{\rho_1}{\rho_0} \times 100 \quad (1)$$

Where ρ_0 denotes the initial HES concentration (mg/mL) added to the system. All measurements were conducted in triplicate.

2.4 Determination of free sulphhydryl (–SH) content

Free –SH groups were quantified using the dithionitrobenzoic acid (DTNB) assay (Shanghai Aladdin Biochemical Technology Co., Ltd., Shanghai, China). Tris-glycine buffer was prepared by dissolving Tris (2.423 g), glycine (1.501 g), and ethylenediaminetetraacetic acid (EDTA) (0.234 g) in 200 mL deionized water, followed by dividing the solution into two equal aliquots. Urea (48.048 g) was added to one aliquot to obtain solution A, whereas DTNB (0.4 g) was added to the other to obtain solution B. For analysis, 0.5 mL of the OVA-HES complex supernatant was mixed with 2.5 mL solution A and 20 μ L solution B, vortexed for 1 min, and incubated in the dark for 30 min. Absorbance was measured at 412 nm, and –SH content was calculated according to Ellman's method using the molar extinction coefficient of trinitrobenzene (TNB) at 412 nm ($\epsilon = 13\,600$ L/(mol·cm), path length = 1 cm). Each sample was analyzed in triplicate, and the experiment was independently repeated three times.

2.5 Determination of intrinsic fluorescence spectroscopy

Intrinsic fluorescence spectra were collected on a spectrofluorometer (RF-6000; Shimadzu Corp., Kyoto, Japan). Samples were diluted to 0.2 mg/mL OVA, with HES concentrations adjusted to 0–0.025 mg/mL. The excitation wavelength was 280 nm, and the excitation/emission slit widths were both set to 10 nm. Emission spectra were recorded over 300–400 nm at a scan speed of 200 nm/min. Fluorescence intensity changes and shifts in the maximum emission wavelength were used to probe OVA-HES interactions. All measurements were conducted in triplicate at room temperature.

2.6 Determination of molecular docking simulation

Molecular docking was conducted in Discovery Studio v2019 (Dassault Systèmes, Paris, France) to elucidate the binding mode between OVA and HES. The OVA crystal structure (PDB ID: 1UHG; 1.9 Å) was obtained from the RCSB Protein Data Bank and prepared by removing crystallographic waters/ligands and assigning appropriate protonation states. The HES structure was generated in ChemDraw (Revvity Signals Software, Inc., Waltham, MA, USA) and subjected to energy minimization prior to docking. Docking was performed within the hydrophobic cavity of OVA using the CDOCKER protocol (pose cluster radius, 0.5 Å; remaining parameters at default). The resulting poses were evaluated based on binding energy, hydrogen-bonding patterns, and hydrophobic contacts to determine the most probable binding conformation.

2.7 Determination of antioxidant capacity

ABTS cation radical and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities were measured to assess antioxidant capacity. ABTS cation radical solution was prepared by reacting ABTS (7 mmol/L) with potassium persulfate (2.45 mmol/L) in the dark at room temperature for 12–16 h. The working solution was diluted with deionized water to an absorbance of 0.70 ± 0.02 at 734 nm. Subsequently, 0.1 mL of sample was mixed with 0.9 mL ABTS cation radical solution, vortexed, and incubated in the dark for 6 min before measuring absorbance at 734 nm (A_{Sample}). Deionized water served as the blank (A_{Blank}). ABTS cation radical scavenging rate was calculated as equation (2):

$$\text{ABTS cation radical scavenging rate (\%)} = \left(1 - \frac{A_{\text{Sample}}}{A_{\text{Blank}}}\right) \times 100 \quad (2)$$

Additionally, prepare a DPPH solution by dissolving DPPH in ethanol. Mix the OVA-HES complex supernatant with an equal volume of DPPH solution (517 nm) having an absorbance of 0.7. Allow the mixture to stand at room temperature in the dark for 30 min, then measure the absorbance of the solution at 517 nm (A_{Sample}). Deionized water served as the blank (A_{Blank}). DPPH radical scavenging rate was calculated as equation (3):

$$\text{DPPH radical scavenging rate (\%)} = \left(1 - \frac{A_{\text{Sample}}}{A_{\text{Blank}}}\right) \times 100 \quad (3)$$

All measurements were performed in triplicate at pH 12 and pH 7 to compare HES-associated antioxidant responses under alkaline and neutral conditions.

2.8 Determination of Fourier transform infrared (FTIR) spectroscopy

Freeze-dried gel samples were finely ground and thoroughly blended with dried KBr at 1:100 (*m/m*). The mixture was further milled (particle size < 2 μm) and compressed into transparent pellets. FTIR spectroscopy were acquired using a FTIR spectrometer (Nicolet iS50; Thermo Fisher Scientific, Waltham, MA, USA) over 400–4 000 cm^{-1} at a resolution of 4 cm^{-1} with 64 scans per sample. Background correction was performed using KBr pellets^[14].

2.9 Determination of water-holding capacity (WHC)

WHC of gels was evaluated using a centrifugation-based method^[15]. Briefly, 10 g of gel was placed in a 50 mL centrifuge tube and centrifuged at $8\,000 \times g$ for 30 min at 4 °C. After centrifugation, tubes were inverted to drain released water, which was removed using filter paper prior to weighing. WHC was calculated as equation (4):

$$\text{WHC (\%)} = \frac{m_3 - m_1}{m_2 - m_1} \times 100 \quad (4)$$

Where m_1 is the mass of the empty tube (mg), m_2 is the mass of the tube containing gel before centrifugation (mg), and m_3 is the mass after centrifugation (mg)^[16].

2.10 Low-field nuclear magnetic resonance (LF-NMR) analysis

LF-NMR measurements were performed to determine transverse relaxation time (T_2 , ms). Gel samples (5 g) were placed into dried glass tubes (inner diameter 20 mm) and analyzed using a LF-NMR analyzer (MesoMR23-060H-I; Shanghai Niumag Corp., Shanghai,

China). A Carr-Purcell-Meiboom-Gill (CPMG) sequence was applied with waiting time 5 000 ms, repetition time 400 ms, and echo time 20 ms^[17].

2.11 Scanning electron microscopy (SEM) observation

SEM was used to examine gel microstructure. Samples were sectioned into small pieces (~0.4 cm $\times$ 0.2 cm), fixed in 2.5% glutaraldehyde, and dehydrated using a graded ethanol series. After freeze-drying, specimens were sputter-coated with gold for 60 s and observed using a SEM (S3400; Hitachi, Tokyo, Japan) at 5 kV with a magnification of $5\,000\times$ ^[18].

2.12 Statistical analysis

All experiments were conducted at least in triplicate, and data are reported as mean $\pm$ standard deviation. Statistical analyses were performed using SPSS 28.0 (IBM, Chicago, IL, USA). Differences among treatments were evaluated by one-way analysis of variance followed by Duncan's multiple range test, with significance set at $P < 0.05$. Graphs were generated using Origin 9.0 (OriginLab Corp., Northampton, MA, USA).

3 Results and discussion

3.1 EE and free -SH content

As shown in Figure 1A, EE depended strongly on HES concentration and on the stage of the pH-driven process. At pH 12, EE increased monotonically with HES concentration, reaching 74.75% at 0.8 mg/mL. After neutralization (final stage), EE exhibited a bell-shaped profile, with a maximum of 51.64% at 0.6 mg/mL. The reduced EE at higher HES levels after neutralization may stem from partial refolding of OVA, which re-buries hydrophobic domains and masks binding sites, thereby weakening HES retention and promoting partial desorption. In addition, excessive HES loading can introduce steric constraints that lower the effective encounter frequency between HES and OVA^[19], ultimately limiting encapsulation. The higher EE observed at pH 12 is also consistent with enhanced apparent solubility of HES in alkaline media, allowing a larger fraction of HES to be present in a form accessible for association. A comparable concentration optimum has been reported for β -cyclodextrin-carvacrol complexes, where EE peaked at an intermediate carvacrol concentration and decreased at higher loadings due to inhibited host-guest binding^[20]. Together, these results indicate a nonlinear dependence of EE on HES concentration, highlighting the need to optimize HES dosage to maximize encapsulation.

Free -SH provide molecular-level insight into conformational rearrangements of OVA. As shown in Figure 1B, pH-driven treatment alone increased the free -SH content of OVA by ~50% relative to native OVA, suggesting alkalization-induced partial unfolding and exposure of thiol groups previously buried in hydrophobic regions^[21]. Upon HES addition, the free -SH content displayed a concentration-dependent response, increasing to a maximum at 0.6 mg/mL and then decreasing at higher HES concentrations at both pH 12 and pH 7. The initial increase may reflect the reducing capacity of HES, which can promote conversion of disulfide bonds (S-S) to thiols under suitable loading, consistent with the positive association reported for glutenin systems^[22]. Additionally, Jiang et al.^[23] observed a similar pattern: the content of free sulfhydryl groups showed a significant positive correlation with increasing HES concentration. This change is

closely related to HES's reducing properties, which promote the conversion of disulfide bonds into free sulfhydryl groups. By contrast, the decline at higher HES concentrations is plausibly related to solubility- and mass-transfer constraints that reduce effective OVA-HES association, thereby diminishing its influence on thiol exposure.

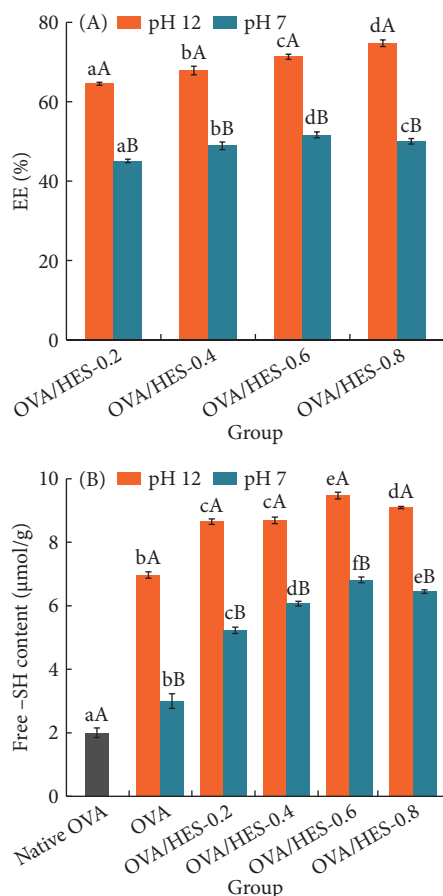


Figure 1 Encapsulation performance and free -SH content of OVA-HES systems during the pH-driven process. (A) EE of HES-loaded OVA at the alkaline driving stage (pH 12) and after neutralization (pH 7). (B) Free -SH contents of native OVA (no processing) and OVA-HES samples collected at pH 12 and 7. Different lowercase letters indicate significant differences among different groups under the same pH conditions ($P < 0.05$), while different uppercase letters indicate significant differences within the same group under the different pH conditions ($P < 0.05$).

3.2 Intrinsic fluorescence spectroscopy

Intrinsic fluorescence measurements indicated pronounced quenching of OVA upon addition of HES. As shown in Figure 2, increasing HES concentration progressively decreased the fluorescence intensity at both pH 12 (intermediate stage) and pH 7 (final stage), supporting the formation of OVA-HES complexes. A similar quenching response has been reported for HES interacting with hydrophobic domains of zein⁷. In addition to intensity changes, the maximum emission wavelength exhibited a stage-dependent shift. At pH 12, a slight blue shift was observed (337 nm→336 nm), whereas at pH 7, a red shift occurred (329 nm→331 nm). Because intrinsic fluorescence is highly sensitive to the polarity of the microenvironment surrounding aromatic residues, these shifts suggest distinct interaction microenvironments at different stages. Under alkaline conditions (pH 12), HES association

with hydrophobic regions likely reduces the local polarity around fluorophores, resulting in a small blue shift²⁴. After neutralization (pH 7), partial refolding of OVA re-buries hydrophobic domains, and the bound HES is associated with a more polar microenvironment, leading to a red shift. Consistently, pH-driven binding of curcumin to OVA has also been reported to induce emission shifts²⁵.

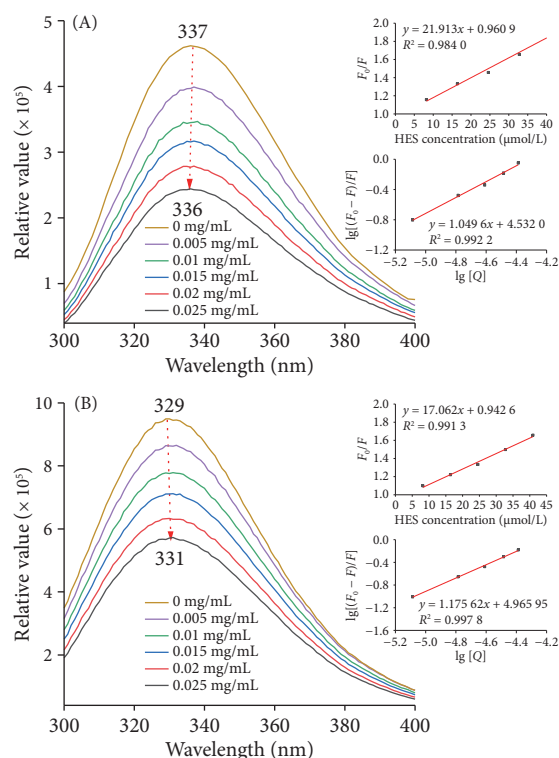


Figure 2 Intrinsic fluorescence analysis of OVA-HES interactions at different stages of the pH-driven process. Fluorescence emission spectra of OVA in the presence of increasing HES concentrations at (A) pH 12 (intermediate stage) and (B) 7 (final stage).

To clarify the quenching mechanism, the Stern-Volmer relationship was applied, as shown in equation (5):

$$F_0/F = 1 + K_q \tau_0 [Q] \quad (5)$$

Where F_0 and F represent the maximum fluorescence intensities in the absence and presence of HES, respectively; τ_0 is approximately 10^{-8} s; K_q denotes the biomolecular quenching rate constant; and $[Q]$ is the concentration of HES (g/mL). K_q was calculated from the slope of the linear regression equation between F_0/F and $[Q]$.

As shown in Figure 2 and Table 1, all K_q values were greater than the maximum dynamic quenching rate constant of biomolecules (2×10^{10} L/(mol·s)), indicating that the quenching of OVA by HES is a static quenching process²⁶.

Table 1 K_q , K , fitted number of binding sites (n) of HES binding to OVA at the intermediate (pH 12) and final (pH 7) stages during the pH-driven process.

Stage	pH	K_q (10^{12} L/(mol·s))	K (10^{10} L/mol)	n
Intermediate	12	2.09	49.3	1.85
Final	7	1.71	3.45	1.69

Accordingly, binding parameters were further analyzed using the double-logarithmic model:

$$\lg[(F_0 - F)/F] = \lg K + n \lg [Q] \quad (6)$$

After neutralization (pH 7), the binding constant (K) obtained from the double-logarithmic model decreased markedly, indicating reduced affinity between HES and OVA^[27]. In contrast, the n values remained close to unity (1.0–1.2), suggesting that the number of effective accessible binding sites did not appreciably decrease and that binding is dominated by a single-site contribution under the tested conditions^[28]. These results imply that partial refolding and conformational rearrangement of OVA during neutralization primarily weakens the interaction strength at the binding site. Such weakening likely arises from changes in the local microenvironment and shifts in the relative contributions of hydrophobic interactions and hydrogen-bonding networks, rather than from substantial masking of binding sites. Together with the EE results in section 3.1, this supports that the partial desorption of HES during the pH-driven process is mainly attributable to decreased affinity and reduced complex stability after neutralization, rather than a loss of accessible binding sites.

3.3 Molecular docking

To elucidate the interaction mode between HES and OVA, molecular docking was employed to construct an OVA-HES complex model. At pH 7.0, the three-dimensional (3D) docked conformation is shown in Figure 3A, and the corresponding two-dimensional (2D) interaction diagram in Figure 3B, which depicts the key contact sites, highlighting the hydrogen-bond network and other non-covalent interactions between HES and

OVA. HES was predicted to be stabilized by hydrogen bonding with multiple OVA residues, including Lys375, Thr276, Asn380, Ala378, Ile377, and Arg228. Conventional hydrogen bonds (green dashed lines) constituted the predominant interaction type, while π -donor hydrogen bonds (light green dashed lines) were also observed, further strengthening ligand-protein association. These results indicate that HES can bind to OVA through multiple hydrogen-bonding contacts, which may contribute to the conformational and functional changes observed experimentally^[34].

At pH 12, the 3D docking conformation of the OVA-HES complex is shown in Figure 3C. Its binding energy (BE; -17.1242 kcal/mol) is lower than that at pH 7 (-16.4170 kcal/mol), suggesting that the binding between HES and OVA is more stable under alkaline conditions. The corresponding 2D interaction diagram (Figure 3D) reveals a significant shift in the interaction pattern between HES and OVA. Conventional hydrogen bonds (green dashed lines) remain the dominant interaction force, while carbon-hydrogen bonds (light green dashed lines) also contribute to complex stabilization. Key amino acid residues involved in hydrogen bond formation include Ala378, Asn380, Ile377. Compared to pH 7 condition, a greater number of amino acid residues participate in interactions at pH 12, and the BE is lower. This indicates that in an alkaline environment, the conformation of OVA changes, exposing more sites that can bind to HES, thereby forming a more stable complex.

Consistent with the 3D model, HES is accommodated within a relatively open binding pocket of OVA. The glycoside moiety predominantly forms hydrogen bonds with residues located near the pocket surface, whereas the phenolic moiety participates in hydrophobic interactions or π - π stacking, collectively reinforcing

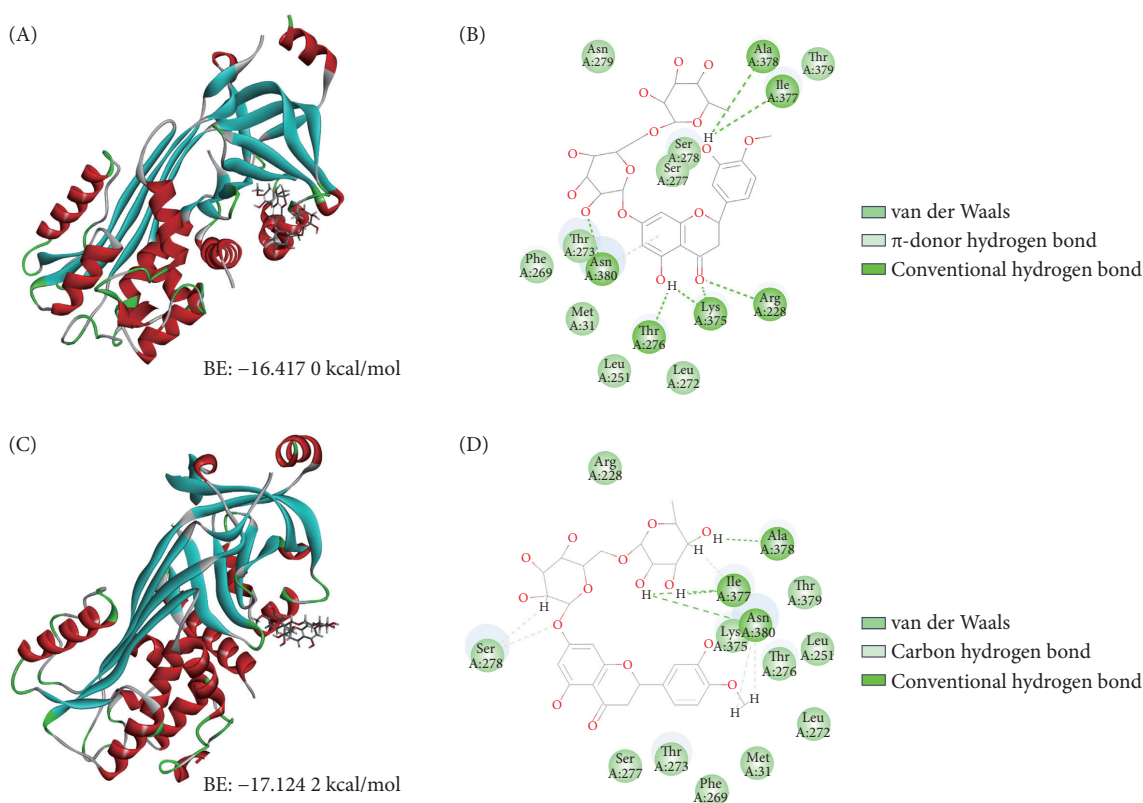


Figure 3 Molecular docking of the OVA-HES complex. (A) 3D docking pose and (B) 2D interaction map under pH 7 condition. (C) 3D docking pose and (D) 2D interaction map under pH 12 condition.

the stability of the complex structure. Changes in pH significantly regulate the binding affinity and interaction patterns between OVA and HES by affecting OVA's conformation and charge distribution.

3.4 Radical scavenging activity

3.4.1 Intermediate stage (pH 12)

As shown in Figures 4A and C, incorporation of HES significantly increased ABTS cation radical and DPPH radical scavenging activities of the OVA-HES system at pH 12 compared with untreated OVA ($P < 0.05$). Notably, the scavenging rate reached a plateau when HES concentration exceeded 0.4 mg/mL, indicating a saturation behavior. This trend suggests that, under alkaline conditions, complex formation at lower HES levels enhances antioxidant performance, whereas additional HES provides limited benefit once the available binding/association capacity is approached^[29].

3.4.2 Final stage (pH 7)

After neutralization (pH 7), the OVA-HES system retained significantly higher ABTS cation radical/DPPH scavenging activity than untreated OVA ($P < 0.05$) (Figures 4B and D), indicating that the antioxidant enhancement persisted after pH adjustment. However, the overall activity was lower than that at pH 12 and showed a non-monotonic dependence on HES concentration. The ABTS cation scavenging rate increased to a maximum of 64.51% at 0.6 mg/mL HES and then declined at higher HES levels. This

bell-shaped response implies that an appropriate HES loading favors an antioxidant-active microenvironment, whereas excessive HES compromises efficiency, potentially due to self-association that masks reactive phenolic sites and/or reduced effective association with OVA under neutral conditions. Collectively, these results demonstrate that maximizing antioxidant performance requires optimization of HES addition in concert with pH, rather than a simple increase in HES concentration^[30].

3.5 Gel network

The FTIR spectra of OVA gels are shown in Figure 5A. Compared with the OVA gel, HES incorporation induced discernible band shifts and intensity variations in three characteristic regions, including 3 400 (O–H/N–H stretching), 1 600 (amide-related region), and 2 800–2 850 cm^{-1} (C–H stretching), indicating that gel formation was accompanied by a reorganization of the hydrogen-bonding and hydrophobic microenvironments. The band at 3 439 cm^{-1} , which is associated with –OH/–NH groups involved in hydrogen bonding^[31], displayed a pronounced blue shift to 3 456 cm^{-1} for OVA/HES-0.4, suggesting weakened hydrogen bonding and/or increased exposure of these groups to a less polar microenvironment^[31]. At higher HES levels (OVA/HES-0.6 and OVA/HES-0.8), the band shifted back toward lower wavenumbers, implying partial re-stabilization of the hydrogen-bond network, potentially due to progressive association/aggregation that shields polar groups.

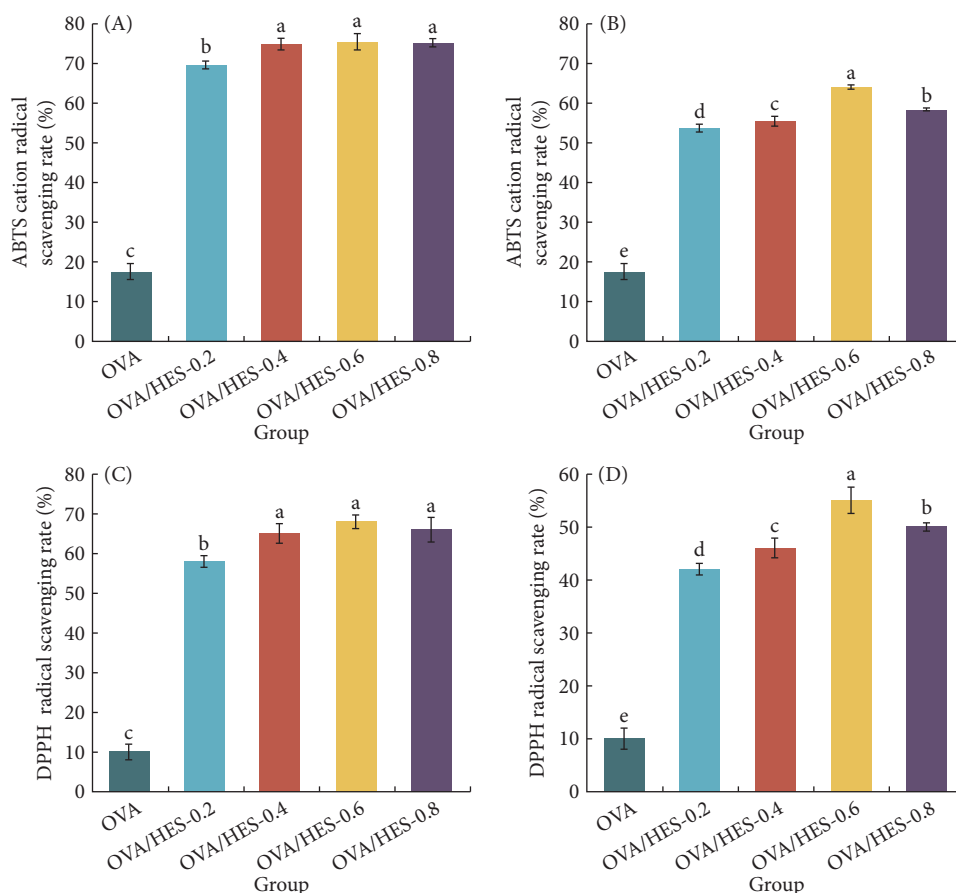


Figure 4 ABTS cation and DPPH radical scavenging activities of OVA and OVA-HES systems at different stages of the pH-driven process. (A, C) Intermediate stage (pH 12) and (B, D) final stage (pH 7).

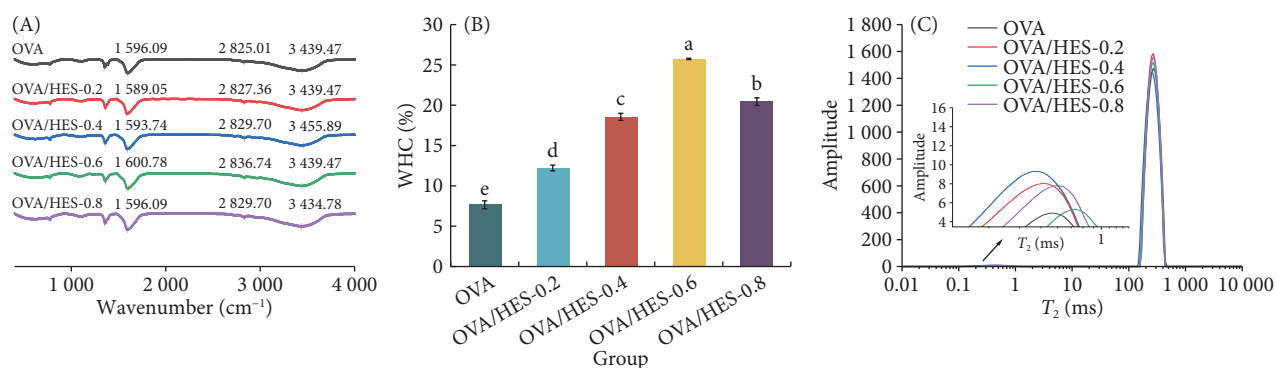


Figure 5 Structural and hydration characteristics of OVA gels containing HES. (A) FTIR spectra, (B) WHC, and (C) LF-NMR transverse relaxation time (T_2) profiles of OVA gel and OVA/HES gels.

In the amide-related region (1596 cm^{-1}), modest shifts were also observed, reflecting changes in local bonding environments and dipole coupling within the protein network. Specifically, OVA/HES-0.2 and OVA/HES-0.4 exhibited slight red shifts, consistent with strengthened local hydrogen bonding and/or specific interactions between HES and polar side-chain groups, whereas OVA/HES-0.6 shifted toward higher wavenumbers, indicating further rearrangement of the hydrogen-bonding pattern and microenvironmental polarity^[27]. The spectrum of OVA/HES-0.8 returned toward 1596 cm^{-1} , suggesting that additional association at excessive HES loading counterbalanced the earlier spectral displacement. In the C–H stretching region (2825 cm^{-1}), OVA/HES-0.6 showed an increased wavenumber, indicative of a more hydrophobic and/or more densely packed environment of aliphatic groups, consistent with relatively strong hydrophobic association between OVA and HES^[19]. By contrast, the reduced wavenumber observed for OVA/HES-0.8 suggests re-shielding of hydrophobic groups, plausibly associated with excessive aggregation or micro-phase separation.

Consistent with these spectroscopic signatures, WHC increased markedly for gels containing 0.2–0.6 mg/mL HES, indicating improved water retention within the gel network, whereas WHC declined at 0.8 mg/mL (Figure 5B), likely due to formation of dense, heterogeneous aggregates that facilitate water expulsion under centrifugation^[16]. LF-NMR (Figure 5C) further showed broadly similar water-population profiles among samples, with non-flowing water as the dominant component, suggesting that all formulations retained a 3D hydrogel network. To further analyze

the state of water distribution, we qualitatively distinguished water components corresponding to different T_2 : short T_2 components correspond to bound water, medium T_2 components correspond to immobile water, and long T_2 components correspond to free water. Taken together, these results indicate that HES modulates the water retention of OVA gels primarily by tuning hydrogen-bonding and hydrophobic associations within the network, while the gel framework remains intact across the tested formulations.

3.6 SEM micrographs

We first captured macroscopic morphology images of the gels (Figure S1), revealing no significant differences in gel state among the various samples. To further elucidate their microstructural characteristics, we subjected the samples to SEM characterization. As shown in Figure 6, SEM micrographs reveal that all gels formed porous 3D networks, whereas pore uniformity and network compactness were strongly dependent on HES loading. The OVA gel exhibited a relatively homogeneous fine-pore architecture with continuous pore walls, characteristic of a self-assembled protein network. At low HES loading (OVA/HES-0.2), the network became looser with enlarged pores, suggesting that initial OVA-HES association perturbed intermolecular hydrogen bonding before the network fully reorganized^[31]. At 0.4 mg/mL HES, pore walls thickened and pore size became more uniform, consistent with a more stabilized composite network arising from reorganized hydrogen bonding and strengthened hydrophobic associations. Increasing HES to 0.6 mg/mL led to locally compact pore walls accompanied by slight shrinkage and micro-aggregation, indicative of excessive local association. At the highest loading (0.8 mg/mL),

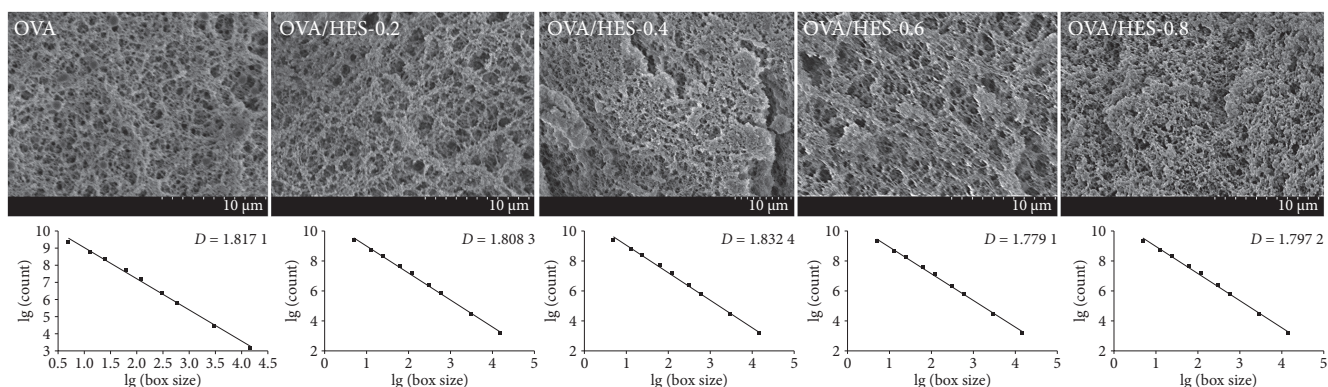


Figure 6 Microstructure and fractal characterization of OVA gels with different HES loadings. D denotes the fractal dimension calculated via the box-counting algorithm.

heterogeneous pores and pronounced aggregates emerged, and the network continuity deteriorated, implying that excessive HES promoted heterogeneous aggregation and/or micro-phase separation of protein-polyphenol assemblies^[32]. This microstructural evolution aligns with the FTIR observations, where the most pronounced network reorganization occurred at intermediate HES levels (0.4–0.6 mg/mL), whereas excessive HES was associated with non-uniform aggregation and disrupted continuity.

Fractal dimension analysis provided complementary quantitative evidence of microstructural complexity. The OVA gel showed a fractal dimension of 1.817. Upon HES addition, the value slightly decreased at 0.2 mg/mL (1.808) and increased to a maximum at 0.4 mg/mL (1.832), indicating the highest pore-wall complexity and branching at this intermediate loading^[33]. Further increases in HES reduced the fractal dimension (1.779 at 0.6 mg/mL and 1.797 at 0.8 mg/mL), consistent with microstructural coarsening and partial collapse. In summary, intermediate HES loadings (0.4–0.6 mg/mL) promoted a denser and more continuous porous network, whereas excessive HES compromised network uniformity.

4 Conclusion

This study demonstrates a solvent-free, pH-driven strategy for incorporating HES into OVA matrices and clarifies the pH- and dosage-dependent relationships among encapsulation behavior, binding interactions, antioxidant activity, and gel network properties. EE increased with HES concentration during the alkaline driving stage (pH 12) but exhibited a non-monotonic profile after neutralization (pH 7), indicating that partial refolding and microenvironmental reorganization reduced effective retention at high loadings. Intrinsic fluorescence quenching, Stern-Volmer analysis, and molecular docking consistently supported complex formation dominated by static quenching and extensive hydrogen-bond interactions, with higher binding affinity at pH 12 than at pH 7. Functionally, HES addition significantly enhanced ABTS cation radical scavenging ability, while an optimal loading window (0.4–0.6 mg/mL) maximized antioxidant performance and maintained favorable network organization; excessive HES led to diminished gains, consistent with reduced effective association and increased heterogeneous aggregation. Complementary FTIR spectroscopy, WHC, LF-NMR, and SEM/fractal analyses further indicated that intermediate HES levels promoted a denser and more continuous porous gel network with improved water retention, whereas excessive HES compromised uniformity without disrupting the overall hydrogel framework. Collectively, these findings provide mechanistic guidance for designing antioxidant protein gels via pH-programmed, solvent-free encapsulation of poorly water-soluble flavonoids. However, in this study, static gelation under laboratory conditions struggles to meet the demands of large-scale industrial production. Additionally, the resulting gel exhibits sensitivity to thermal processing and long-term storage, readily triggering protein denaturation and premature release of HES. Furthermore, achieving uniform HES loading proves challenging during scaled-up preparation. Future research will systematically optimize these critical industrial application challenges to further enhance system stability and scalability, thereby advancing the practical industrialization of this technology in functional food development.

Declaration of competing interest

All co-authors have no conflicts of interest to declare.

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Author contributions

Sibing Li: Writing-original draft, validation, data curation. Minghui Zhao: Data curation. Jinghong Li: Methodology. Hanyu Li: Writing-review & editing, funding acquisition, supervision. Huajiang Zhang: Writing-review & editing.

Appendix A. supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.26599/FSAP.2026.9240172>.

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