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Protective Effect of Eggshell Powder on the In Vitro Titer Stability of Immunoglobulin Y

Yi Li, Huan Xiong, Zhi Zhang *, Xiaohong Jian *, Zaixin Li *

School of Food and Liquor Engineering, Sichuan University of Science and Engineering, Yibin, 644000, China

*Corresponding Author

ABSTRACT

Objective Aiming at the problem that immunoglobulin Y (IgY) is susceptible to acid-base, temperature and enzymatic hydrolysis in vitro, resulting in reduced titer, this study was designed to systematically evaluate the protective effect of eggshell powder on the titer stability of IgY under different pH, temperature, time and packaging conditions, and to verify its anti-digestive ability in simulated gastric fluid. Methods Using IgY as the research object and eggshell powder as the protective agent, multi-gradient experiments were set up: the titer changes were investigated at different pH (1, 3, 7.8), temperatures (-20, 4, 24, 37℃) and storage time (0-360 d); the effects of conventional packaging and vacuum packaging were compared; and incubation in simulated gastric fluid was performed for 0–60 min. The IgY titers of each group were determined by indirect enzyme-linked immunosorbent assay (ELISA), and the retention rates were calculated. Results Compared with the control group, eggshell powder significantly increased the titer retention rate of IgY under strong acid condition (pH = 1.0) ($P<0.05$). During the 360-day storage period, the titer of the eggshell powder group showed a downward trend. The antibody retention rate of vacuum packaging was higher than that of non-vacuum packaging, but the difference was not significant ($P>0.05$). After treatment in simulated gastric fluid for 60 min, the antibody retention rate reached 95.6% at 60 min, showing excellent acid resistance and anti-enzymolysis properties. Conclusion Eggshell powder can effectively maintain the titer stability of IgY in complex in vitro environments and simulated digestive juice, indicating its application potential as a natural oral delivery carrier.

KEYWORDS

Immunoglobulin Y (IgY); Eggshell powder; Titer

1. INTRODUCTION

Immunoglobulin Y (IgY) has a molecular weight of approximately 180 kDa, consisting of two heavy chains (about 65–70 kDa) and two light chains (about 22–30 kDa). As the avian homologue and evolutionary precursor of human immunoglobulin G (IgG), IgY exhibits remarkable sequence conservation. Owing to species differences, IgY does not activate the classical complement pathway in mammals nor bind to mammalian IgG Fc receptors, thus avoiding nonspecific inflammatory reactions and demonstrating excellent biosafety. These physicochemical properties render IgY particularly suitable for oral administration, enabling targeted neutralization locally in the gastrointestinal tract. IgY remains stable over a wide pH range (4.0–11.0) and retains most antigen-binding activity after heating at temperatures below 60℃ for 30 min. It also shows certain tolerance to pepsin; however, it is prone to irreversible denaturation under strong acid ($pH<3.0$) or upon exposure to pepsin [1]. Kyong Ae Lee, Yuan Ang, and co-workers reported that the activity of IgY under acidic conditions could be preserved by adding stabilizers such as sorbitol, trehalose, and

maltose [2, 3]. At present, the main strategies for protecting IgY include microencapsulation, liposome entrapment, and chemical modification. Nevertheless, these approaches suffer from limitations including complex preparation processes, high costs, potential solvent residues, and difficulties in large-scale production, which restrict their widespread application in the prevention and eradication of H. pylori infection. Therefore, exploring natural, low-cost, and readily available protective carriers has become an important direction for expanding the application scenarios of IgY.

As a byproduct of the poultry industry, eggshell is produced in huge annual quantities. Its main component is calcium carbonate, accounting for 93%–97%, with small amounts of trace elements including magnesium, phosphorus, sodium, potassium, zinc, and strontium. Due to its porous structure and high specific surface area, eggshell powder has been used as a functional carrier for numerous drugs. For instance, Qicang Wang et al. applied biogenic calcium carbonate derived from eggshells in the targeted delivery of antitumor drugs; calcium carbonate decomposes under acidic conditions, accelerating drug release while synergistically inducing apoptosis in tumor cells [4]. Meihan Tao et al. found that eggshell powder could significantly enhance the antibacterial efficacy of silver nanoparticles [5]. This dual function of acid resistance for symptom relief and performance as a functional carrier provides a reasonable theoretical basis for its use in combination with IgY to construct novel oral delivery systems.

Accordingly, the present study aimed to use eggshell powder as a natural protective matrix for IgY, systematically investigate the effects of different pH values, temperature gradients, and storage durations on the titer stability of IgY, and further explore the protective effect of vacuum packaging on long-term storage stability. Through in vitro digestion experiments in simulated gastric fluid, the protective efficacy of eggshell powder on IgY titer was comprehensively evaluated. The results of this study provide a theoretical basis and data support for the development of low-cost and environmentally friendly oral IgY preparations.

2. MATERIALS AND METHODS

2.1. Main reagents and Materials

Potassium chloride (KCl), concentrated hydrochloric acid (HCl), sodium carbonate (Na_2CO_3), and sodium hydroxide (NaOH) were purchased from Chengdu Kelong Chemical Co., Ltd. (Chengdu, China). Pepsin was obtained from Feijing Biology (Shanghai, China). The single-component TMB substrate kit was supplied by Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Goat anti-chicken IgG-HRP conjugate was acquired from Sigma-Aldrich (St. Louis, MO, USA). Unstained protein markers were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Acrylamide (30% solution) was obtained from Beijing Solarbio Science & Technology Co., Ltd.

IgY, eggshell powder, and the specific antigen protein used in this study were prepared in-house at our laboratory. Deionized water was used for all solution preparations.

2.2. Preparation of Solutions

Coating Buffer (0.5 mol/L Carbonate-Bicarbonate Buffer, pH 9.6):

The coating buffer was prepared by dissolving 1.59 g of anhydrous sodium carbonate (Na_2CO_3) and 2.94 g of sodium bicarbonate ($NaHCO_3$) in approximately 800 mL of deionized water. The pH was adjusted to 9.6 using 4% (w/v) NaOH solution, and the final volume was brought to 1000 mL with deionized water.

Simulated Gastric Fluid (SGF, pH 2.0):

SGF was prepared according to a modified protocol. Briefly, 2.0 g of sodium chloride (NaCl) and 3.2 g of pepsin were dissolved in approximately 800 mL of deionized water. The pH was carefully

adjusted to 2.0 using 1 mol/L HCl. The solution was then diluted with deionized water to a final volume of 1000 mL. The SGF was freshly prepared before each digestion experiment.

2.3. Instruments and Equipment

The pH measurements were performed using a pH meter (TCPI, INESA Scientific Instrument Co., Ltd., Shanghai, China). Enzyme-linked immunosorbent assay (ELISA) absorbance was read on a microplate reader (Model 800TS, BioTek Instruments, Inc., Winooski, VT, USA). Protein concentrations were determined using a micro-volume spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific, Waltham, MA, USA). Electrophoresis gels were visualized and analyzed with a UV gel imaging system (Model 2500B, Shanghai Tanon Science & Technology Co., Ltd., Shanghai, China). Electrophoresis was conducted using a constant voltage electrophoresis power supply (DYY-6C, Nanda Biotechnology Co., Ltd., Beijing, China).

2.4. Methods

2.4.1. Determination of Protein Concentration by Bradford Assay

WhenProtein concentrations were determined using the Bradford method with Bovine Serum Albumin (BSA) as the standard. A standard curve was constructed using six BSA solutions with concentrations ranging from 0 to 0.1 mg/mL (prepared by adding 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mL of 0.1 mg/mL BSA stock solution into separate tubes and diluting to 1.0 mL with distilled water). To each tube, 5.0 mL of Coomassie Brilliant Blue G-250 reagent was added. After mixing and incubating for 2 min at room temperature, the absorbance was measured at 595 nm (OD_{595}). Each standard concentration was analyzed in triplicate. A linear regression equation was generated by plotting absorbance (OD_{595}) against BSA concentration. The concentration of IgY samples was calculated based on this regression equation.

2.4.2. Determination of IgY Purity and Initial Titer

Lyophilized IgY powder was dissolved in deionized water, and the concentration was adjusted to 1–2 mg/mL. For SDS-PAGE analysis, an aliquot of the protein sample was mixed with 5× SDS loading buffer at a ratio of 5:1 (v/v). The mixture was boiled for 15 min to ensure complete denaturation, followed by centrifugation at 10,000 rpm for 2 min at 4°C. The supernatant was collected and stored at 4°C until use. Protein purity was assessed using reducing Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) on a discontinuous vertical slab gel system. The gel consisted of a 5% stacking gel (pH 6.8) and a 12% separating gel (pH 8.8). Approximately 20 µg of total protein per well was loaded, alongside a pre-stained protein marker (Thermo Fisher Scientific). Electrophoresis was performed in 1× running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). The initial voltage was set at 80 V during the stacking phase; once the tracking dye entered the separating gel, the voltage was increased to 120 V and maintained constant until the bromophenol blue front reached the bottom of the gel. Following electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 solution for 1 h at room temperature with gentle shaking. Destaining was performed using a solution containing 10% acetic acid and 10% methanol until the background was clear. Gel images were captured and analyzed using a UV gel imaging system (Tanon 2500B, Shanghai, China).

Three Helicobacter pylori antigens—Blood group antigen-binding adhesin (BabA2), Urease B subunit (UreB), and Flagellin A subunit (FlaA)—were diluted to a final concentration of 10 µg/mL in carbonate-bicarbonate coating buffer (0.5 mol/L, pH 9.6). The antigen solutions (100 µL/well) were added to 96-well microtiter plates and incubated overnight at 4°C. The following day, the coating solution was discarded, and the plates were washed three times with Phosphate-Buffered Saline containing 0.05% Tween-20 (PBST), 5 min per wash. To block non-specific binding sites, 100 µL of PBST containing 5% (w/v) skim milk powder was added to each well, followed by incubation

at 37°C for 2 h. After blocking, the plates were washed three times with PBST (5 min/wash). Eggshell powder (ESP)-treated IgY antibodies were serially diluted in antibody dilution buffer at ratios of 1:5,000, 1:10,000, 1:20,000, 1:40,000, 1:80,000, and 1:160,000. Untreated IgY antibodies at corresponding concentrations served as the positive control, while the antibody dilution buffer alone served as the negative control. Aliquots of 50 μL of each sample were added to the wells in triplicate. The plates were incubated at 37°C for 2.5 h, followed by three washes with PBST (5 min/wash). Horseradish peroxidase (HRP)-conjugated goat anti-chicken IgG (Sigma-Aldrich, USA) was diluted 1:15,000 in PBST. A volume of 50 μL was added to each well, and the plates were incubated at 37°C for 30 min. Subsequently, the plates were washed five times with PBST (5 min/wash) to remove unbound conjugate. For color development, 50 μL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was added to each well. The plates were incubated at room temperature in the dark until optimal color development was observed. The reaction was terminated by adding 50 μL of stop solution (2 M H_2SO_4). The absorbance was measured at 450 nm (OD_{450}) using a microplate reader.

2.4.3. Effect of pH on the Stability of Eggshell Powder-IgY Mixtures

Lyophilized IgY powder and eggshell powder (ESP) were mixed thoroughly at a mass ratio of 1:9 (w/w). The mixture was aliquoted into individual units of 1.0 g each, ensuring that each unit contained 100 mg of IgY. To evaluate pH stability, each 1.0 g sample was dissolved in phosphate buffers with adjusted pH values of 1.0, 3.0, and 7.8, respectively, to achieve a final IgY concentration of 10 mg/mL. The solutions were incubated at 37°C for 2 h to simulate stress conditions. Immediately after incubation, the pH of each solution was neutralized to 7.0 by adding an appropriate volume of 1.68 mol/L carbonate-bicarbonate buffer. The residual antibody activity (titer) was assessed using the indirect ELISA method described in Section 2.4.2. Prior to the assay, the treated samples were diluted to a working concentration of 10 μg/mL. An untreated IgY-ESP sample (dissolved directly in neutral buffer without pH stress) served as the control. The optical density at 450 nm (OD_{450}) was measured to evaluate the stability of the IgY antibodies under different pH conditions. All experiments were performed in triplicate.

2.4.4. Stability of IgY under Different Temperatures and Packaging Conditions

Lyophilized IgY powder and eggshell powder (ESP) were thoroughly mixed at a mass ratio of 1:9 (w/w). The mixture was divided into two groups based on packaging methods: one half was vacuum-sealed, and the other half was stored in non-vacuumed packages (ambient air). Each package contained 1.0 g of the mixture. Samples from both packaging groups were randomly assigned to four storage temperatures: -20°C, 4°C, 24°C (room temperature), and 37°C. To evaluate long-term stability, the antibody titer of the samples was measured every 30 days. For comparative analysis, lyophilized IgY powder without eggshell powder stored at -20°C served as the reference control to assess the protective effect of ESP. In the ELISA assay, wells without primary antibody served as the negative blank. Prior to measurement, all test samples were diluted to a working concentration of 10 μg/mL. The residual antibody activity was evaluated by measuring the optical density at 450 nm (OD_{450}) using the indirect ELISA method described previously.

2.4.5. In Vitro Digestion Stability in Simulated Gastric Fluid (SGF)

To evaluate the anti-digestive properties of the Eggshell Powder (ESP)-IgY mixture, the sample was dissolved in Simulated Gastric Fluid (SGF) containing pepsin to achieve a final IgY concentration of 10 mg/mL. The enzyme-to-substrate mass ratio (pepsin:IgY) was maintained at 1:20. The mixture was incubated at 37°C with continuous shaking (150 rpm) to simulate gastric motility. Aliquots of 1.0 mL were withdrawn at specific time intervals: 0, 20, 40, and 60 min. Immediately upon collection, each aliquot was neutralized to pH 7.0 by adding an appropriate volume of 1.68 mol/L carbonate-bicarbonate buffer. This step was critical to instantly inactivate pepsin and terminate the digestion reaction. A control group was prepared identically but using pepsin-free SGF to account for any potential effects of low pH alone on antibody stability. The residual antibody titer in each sample was determined using the indirect ELISA method described in Section 2.4.3. Prior to the assay, all

digested samples were diluted to a working concentration of 10 μg/mL. The optical density at 450 nm (OD_{450}) was measured to calculate the percentage of remaining antibody activity relative to the 0-min sample. All experiments were performed in triplicate.

2.5. Statistical Analysis

Data were analyzed using R (v4.5.1) and expressed as mean ± SD (n=3). Graphs were generated with GraphPad Prism 6.0. Normally distributed data were compared using one-way ANOVA followed by Tukey's post-hoc test. Differences were considered significant at $P<0.05$ and extremely significant at $P<0.01$.

3. RESULTS AND ANALYSIS

3.1. Determination of Protein Content by the Bradford Method

A standard curve was constructed by plotting the absorbance at 595 nm (OD_{595}) against various concentrations of Bovine Serum Albumin (BSA). As illustrated in Fig. 1, a strong linear correlation was observed between absorbance and BSA concentration, yielding the following linear regression equation:$y=0.6637x+0.06753$($R^2=0.9968$) where Y represents the OD_{595} value and X denotes the protein concentration (mg/mL). Given the high coefficient of determination ($R^2>0.99$), this regression model was deemed reliable and subsequently applied to quantify the protein concentration of the lyophilized IgY powder samples.

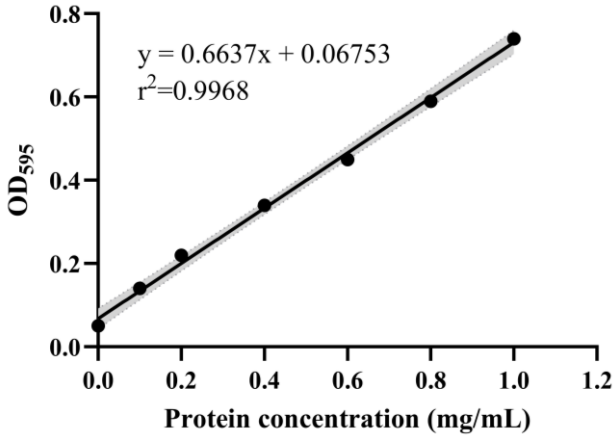

Figure 1. Standard curve of BSA for protein quantification using the Bradford assay

3.2. Characterization of IgY Purity and Titer Determination

SDS-PAGE Analysis: Under denaturing conditions, 12% SDS-PAGE analysis revealed that IgY underwent disulfide bond cleavage, resolving into two distinct bands corresponding to the heavy chain (~70 kDa) and light chain (~25 kDa) (Fig. 2A). These molecular weights are consistent with previously reported values for avian IgY.

Titer Determination: In the indirect ELISA assay, the absorbance at 450 nm (OD_{450}) exhibited a dose-dependent decrease with increasing dilution factors. Notably, even at a high dilution of 1:160,000 (corresponding to an antibody concentration of 73 pg/mL), a significant signal was detected (Fig. 2B). The positive-to-negative ratio (P/N value) at this dilution exceeded 2.1, meeting the criterion for positivity. Consequently, the titer of the lyophilized IgY powder was determined to be 1:160,000.

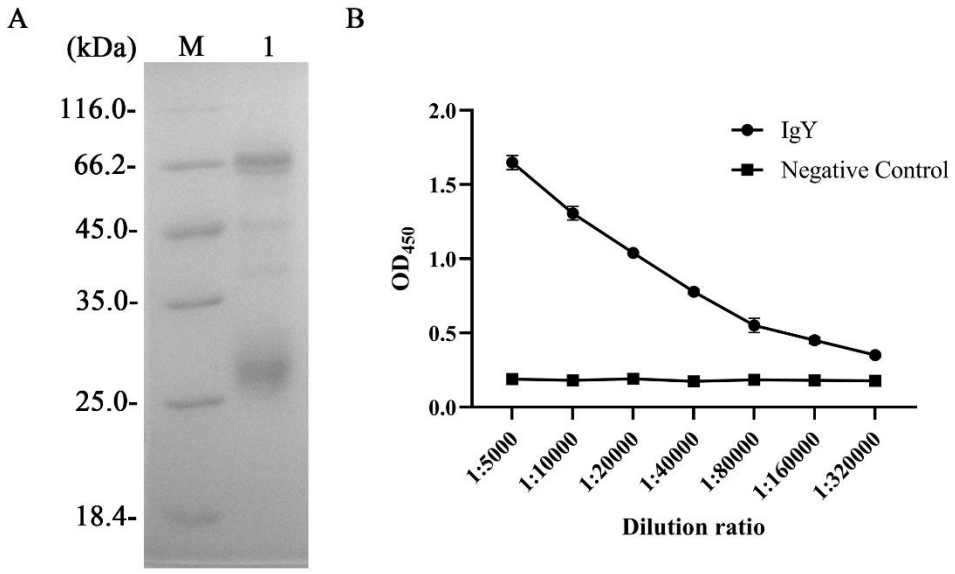

Figure 2. Characterization of IgY purity and titer determination

3.3. Effect of pH on the Stability of Eggshell Powder-Protected IgY

The immunological stability of IgY lyophilized powder, with or without eggshell powder (ESP) protection, was evaluated after exposure to varying pH conditions for 2 h by measuring the OD_{450} values (Fig. 3). The blank control group (untreated IgY) exhibited an OD_{450} of 1.328±0.024, which was defined as 100% antibody retention. Under highly acidic conditions (pH 1.0), the unprotected IgY group showed a drastic reduction in activity, with the OD_{450} dropping to 0.624±0.019 (47.0% retention). In contrast, the ESP-protected group maintained significantly higher activity, with an OD_{450} of 1.139±0.024 (85.8% retention). Similarly, at pH 3.0, the unprotected IgY retained only 67.4% of its activity OD_{450}=0.895±0.010), whereas the ESP-protected group retained 93.7% OD_{450}=1.244±0.038). Under neutral conditions (pH 7.8), both groups exhibited high stability, with retention rates of 97.8% and 99.6%, respectively. Statistical analysis revealed significant differences between the treated groups (both unprotected and protected) and the control at pH 1.0 and 3.0 (P<0.05). However, no significant difference was observed at pH 7.8 (P>0.05). Notably, the ESP-protected group showed significantly higher retention than the unprotected group under acidic conditions (P<0.05). These results demonstrate that while strong acid exposure (pH 1.0) severely compromises the titer of free IgY, encapsulation with eggshell powder provides substantial protection, preserving the immunological activity of IgY in harsh acidic environments.

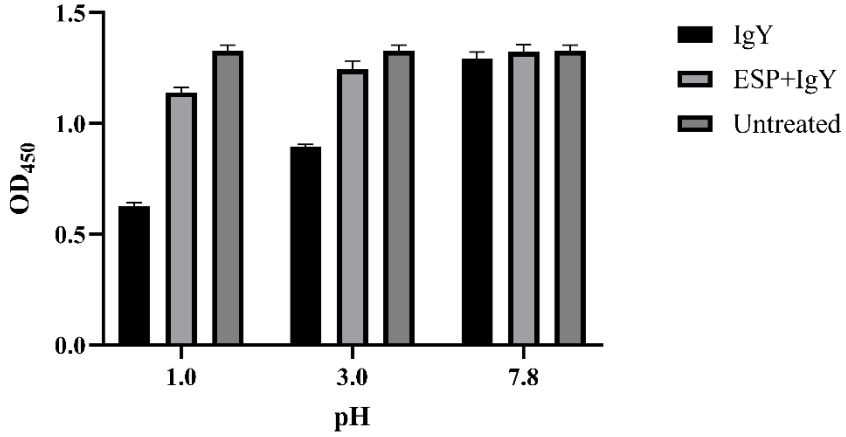

Figure 3. pH stability of IgY protected by ESP

3.4. Long-term Stability of IgY under Different Temperatures and Packaging Conditions

The stability of ESP-protected IgY was monitored over 360 days (Fig. 4). Using day 0 OD_{450} values as 100% baseline (1.868±0.006) for non-vacuum, antibody retention rates were calculated. Temperature emerged as the critical factor affecting stability. In non-vacuum packaging, retention rates at day 360 decreased significantly with rising temperature: 52.4% (-20°C), 49.4% (4°C), 38.3% (24°C), and 32.8% (37°C). The loss at 37°C was statistically significant compared to day 0 ($P<0.05$). Under vacuum packaging, retention rates were slightly higher across all temperatures (55.5%, 55.3%, 51.2%, and 33.9% for -20°C, 4°C, 24°C, and 37°C, respectively). Although vacuum packaging consistently yielded higher retention than non-vacuum controls at equivalent temperatures, the differences were not statistically significant ($P>0.05$). These results indicate that low-temperature storage (≤4°C) is essential for maintaining IgY potency, while vacuum packaging offers a marginal, albeit non-significant, protective benefit.

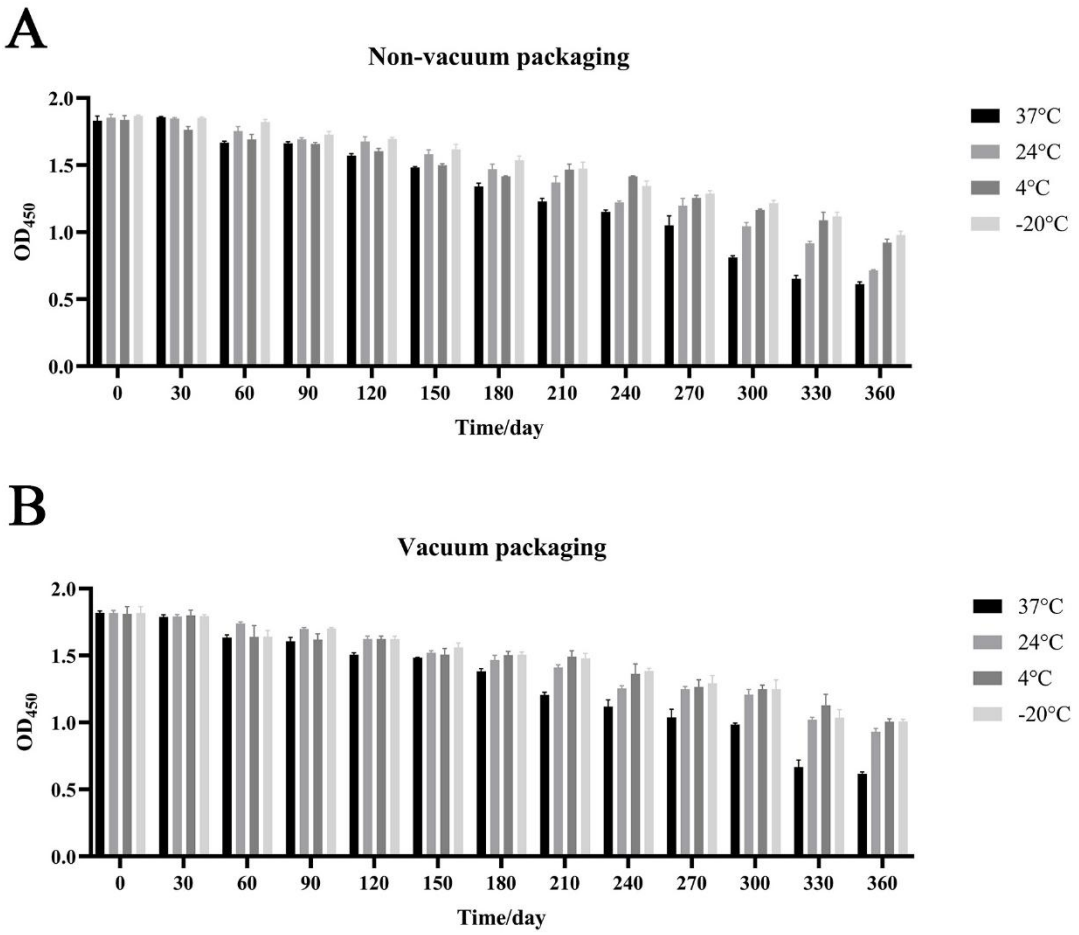

Figure 4. Long-term stability of eggshell powder-protected IgY under varying temperatures and packaging conditions

3.5. Anti-digestive Properties of ESP-IgY Complexes in Simulated Gastric Fluid

As shown in Fig. 5, after 60 min in simulated gastric fluid, free IgY retained only 81.3% of its activity (OD_{450}: 0.947±0.021), significantly lower than the enzyme-free blank control (87.0%), confirming pepsin-induced degradation. In contrast, the eggshell powder (ESP)-protected group exhibited superior stability, with a retention rate of 95.6% (OD_{450}: 1.139±0.017). These results demonstrate that ESP effectively mitigates IgY loss in gastric conditions, providing significant protection against digestive enzymes.

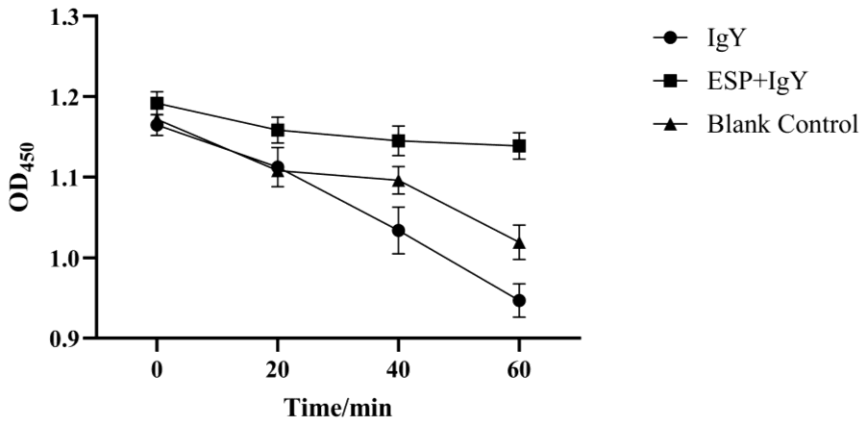

Figure 5. Stability of IgY in simulated gastric fluid over time: Effects of eggshell powder protection

4. DISCUSSION

As a protein, IgY is sensitive to temperature, pH and pepsin, which poses challenges to its application. Therefore, this chapter investigated the tolerance of IgY lyophilized powder to acid-base conditions and pepsin, as well as its storage stability under different conditions. SDS-PAGE analysis revealed distinct characteristic bands corresponding to the heavy chain (~70 kDa) and light chain (~25 kDa) of IgY, which was completely consistent with the IgY structure reported by E.P.V. Pereira et al [6]. A detectable signal was still observed when IgY lyophilized powder was diluted up to 1:160000. After treatment under strong acid condition (pH = 1.0) for 1 h, the antibody activity retention rate of IgY lyophilized powder was relatively low, which was in accordance with the conclusion that the binding capacity of IgY decreases under acidic conditions [7]. The antibody activity was almost unchanged within the pH range of 3–7.8, indicating that IgY lyophilized powder possesses certain acid-base resistance. This result was consistent with the findings of Saeed Yakhkeshi et al. that IgY remains stable in the pH range of 4–7, while its titer decreases at pH<3.5 or pH>9 [8, 9]. The addition of eggshell powder as a protective agent increased the antibody retention rate, suggesting that the protective agent could enhance the resistance of IgY to acid and alkali, which was consistent with the results of Kyong Ae Lee, Yuan Ang et al. that the activity of IgY under acidic conditions can be maintained by adding stabilizers such as sorbitol, trehalose and maltose [2, 3]. It is preliminarily hypothesized that eggshell powder forms a suspension in aqueous solution, thereby reducing the contact between IgY and the aqueous solution [8]. Meanwhile, calcium carbonate in eggshell powder neutralizes acidity in the solution, exerting a protective effect on IgY and thus enhancing its acid tolerance. In addition, the addition of eggshell powder elevated the solution pH and maintained it at neutrality. Since the optimal pH for pepsin is 1.5–2.0, pepsin was inactivated in the neutral solution, resulting in almost unchanged activity of IgY lyophilized powder [7, 10]. The titer of IgY lyophilized powder varied significantly under different storage conditions. At 360 d, the antibody inactivation rate under cryopreservation was significantly lower than that at 37℃, indicating that low temperature can prolong the titer and validity period of IgY lyophilized powder, and the addition of a protective agent contributes to better titer maintenance. Compared with conventional packaging, vacuum packaging could maintain the titer of IgY to a certain extent, but the effect was not significant.

5. CONCLUSION

In this study, immunoglobulin Y (IgY) was used as the research object to systematically investigate the protective effect of eggshell powder on the titer stability of IgY under different pH values, temperatures, storage durations and packaging methods, and to evaluate its anti-digestive ability in simulated gastric fluid. The results showed that eggshell powder could significantly improve the titer

retention rate of IgY under strong acid conditions; during long-term storage, the titer of IgY decreased faster with the increase of temperature, and vacuum packaging slightly improved the antibody stability, but the difference was not significant; after treatment in simulated gastric fluid for 60 min, the antibody titer retention rate of the eggshell powder group reached 95.6%, showing excellent acid resistance and anti-enzymolysis effects. In summary, eggshell powder can effectively maintain the titer stability of IgY in various in vitro environments and has the application potential as a natural oral carrier.

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