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Effect of Milk Powder as A Protective Agent on the Stability of Anti-Hp-IgY

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ABSTRACT

Currently, multiple antibiotic combination therapy is predominantly used in clinical practice for the treatment of related infections. Although the cure rate is approximately 80%, drug resistance is increasing annually, the eradication efficacy is gradually declining, and long-term antibiotic use can readily lead to intestinal flora dysbiosis and functional disorders. Egg yolk immunoglobulin (IgY) has the potential to serve as an alternative to traditional antibiotic therapies due to its high specificity, ease of production, and lack of toxic side effects. In this study, milk powder was added as a protective agent to specific IgY lyophilized powder to investigate its storage stability, tolerance to pH changes and pepsin, with IgY titer changes measured using ELISA. The results showed that specific IgY retained stable titers within the pH range of 3–7.8. At pH 1, the residual activity of IgY was 43.0%, while the addition of milk powder under the same conditions increased the antibody activity retention rate to 72.3%. IgY exhibited relatively high resistance to pepsin, with a retention rate of 79.6% after 1 hour of pepsin treatment; the addition of milk powder further increased the retention rate to 88.0%. In stability experiments using milk powder as a protective agent, antibodies stored at 4°C or −20°C retained approximately 50% activity after 360 days. The results showed that Milk powder, as a natural and low-cost protective agent, effectively improves the pH stability, pepsin tolerance, and long-term storage activity of IgY. This study provides robust theoretical and data support for the development of highly effective and stable oral IgY formulations, thereby promoting their clinical translation and application in the prevention and treatment of related infections.

KEYWORDS

Igy (Immunoglobulin of yolk); Milk powder; Titer

1. INTRODUCTION

Egg yolk immunoglobulin (IgY) is an avian-specific immunoglobulin that shares functional similarities with mammalian IgG. However, it lacks a hinge region between the CH1 and CH2 domains of the heavy chain [1], rendering its structure relatively unstable. IgY exhibits potent agglutination, opsonization, and lysis capabilities. It does not bind to human Fc receptors nor activate the complement system; consequently, it elicits no adverse immune reactions and does not induce drug resistance, offering distinct advantages over conventional antibiotics [2-3]. In 1893, Klemperer first demonstrated that egg yolk from immunized hens could neutralize tetanus toxin, confirming the presence of protective antibodies. Currently, specific IgY can be induced in poultry using various antigens, including viruses, bacteria, proteins, and polysaccharides [4-5].

IgY has demonstrated clear efficacy in the prevention and control of livestock and poultry diseases. IgY targeting the outer membrane proteins of Salmonella significantly inhibits bacterial adhesion to intestinal epithelial cells in vitro, and feeding eggs containing specific IgY reduces bacterial shedding and cecal colonization in infected chickens [6]. Anti-enterotoxigenic Escherichia coli (ETEC) IgY alleviates clinical symptoms induced by enterotoxins, reduces intestinal permeability and inflammatory responses, and improves intestinal mucosal morphology. In the prevention and treatment of Helicobacter pylori, IgY acts by inhibiting bacterial enzyme activity, neutralizing toxins, and blocking adhesion. Polyclonal IgY, in particular, can simultaneously target multiple antigens, thereby enhancing therapeutic efficacy and reducing the risk of drug resistance [7-8].

Despite the wide-ranging applications of IgY, its stability within the gastrointestinal tract and its biosafety following oral administration remain largely unclear. This represents a critical bottleneck in evaluating the efficacy of oral IgY formulations and advancing the development of passive immune intervention products.

2. MATERIAL

Reagents: Potassium chloride, concentrated hydrochloric acid, sodium carbonate: Chengdu Kelong Chemical Co., Ltd.; Pepsin: Phygene Biotechnology; TMB single-component chromogenic solution, 30% acrylamide: Beijing Solarbio Science & Technology Co., Ltd.; Goat anti-chicken antibody: Sigma-Aldrich, USA; Sodium hydroxide: Chengdu Jinshan Chemical Reagent Co., Ltd.; Unstained protein marker: Thermo Fisher Scientific, USA; Antigen protein Anti-Hp-IgY, eggshell powder: Prepared in our laboratory.

Instruments: pH meter (TCPI): Shanghai INESA Scientific Instrument Co., Ltd.; Microplate reader (800TS): BioTek Instruments, Inc., USA; Microvolume spectrophotometer (NanoDrop 2000c): Thermo Fisher Scientific, USA; Electrophoresis apparatus with regulated power supply (DYY-6C): Nanda Biotechnology Company; Electronic analytical balance (JT1003D): Lichen Technology Co., Ltd.

Coating buffer: 0.5 mol/L carbonate buffer, pH 9.6. Dissolve 1.59 g of sodium carbonate and 2.94 g of sodium bicarbonate in purified water, adjust pH to 9.6 with 4% sodium hydroxide solution, and bring to a final volume of 1000 mL.

Simulated gastric fluid: Dissolve 2.0 g of sodium chloride and 3.2 g of pepsin in purified water, adjust pH to 2.0 with 1 mol/L HCl, and bring to a final volume of 1000 mL.

3. METHODS

3.1. Determination of Relative Protein Content by Bradford Method

Standard Curve Construction: Serial dilutions of 0.1 mg/mL BSA (0, 0.2, 0.4, 0.6, 0.8, 1.0 mL) were brought to 1.0 mL with distilled water. Coomassie Brilliant Blue G-250 (5 mL) was added, mixed, and left for 2 min before measuring OD_{595}. Triplicate measurements were performed. The standard curve (OD_{595} vs. BSA concentration) and regression equation were generated for Anti-Hp-IgY concentration calculation.

3.2. Determination of Titer

The antibody lyophilized powder stored in an ultra-low temperature freezer was dissolved in antibody dilution buffer to prepare a 10 μg/mL stock solution, which was then serially diluted at ratios of 1:10,000, 1:20,000, 1:40,000, 1:80,000, and 1:160,000 for use as test samples.

Three proteins—Helicobacter pylori blood group antigen-binding adhesin (BabA2), urease B subunit (UreB), and flagellin A subunit (FlaA)—were each diluted to 10 μg/mL with coating buffer. A 96-well microplate was coated with 100 μL of the diluted antigen solution per well and incubated at 4°C for 12 h. The plate was then washed three times with PBST. Each well was blocked with 100 μL of 5% non-fat milk powder solution at 4°C for 12 h, followed by three additional washes with PBST. The serially diluted test samples were added to the wells (100 μL/well) and incubated at 37°C for 1 h. After three washes with PBST, 100 μL of horseradish peroxidase (HRP)-conjugated goat anti-chicken IgY (diluted 1:30,000 in antibody dilution buffer) was added to each well and incubated at 37°C for 1 h. The plate was washed again three times with PBST. Subsequently, 100 μL of TMB single-component chromogenic substrate solution was added to each well and allowed to develop in the dark for 5 min. The reaction was terminated by adding 100 μL of 1 M sulfuric acid per well. The optical density was measured at 450 nm (OD_{450}) using an ELISA microplate reader.

The P/N ratio was calculated for each well (P/N = OD value of test well / OD value of negative control well). A well was considered positive when the OD value exceeded 0.1 and the P/N ratio was greater than 2.1. The antibody titer was defined as the highest dilution of the sample that yielded a positive result.

3.3. Effect of pH on Anti-Hp-IgY in the Presence of Milk Powder Protectant

Anti-Hp-IgY lyophilized powder was thoroughly mixed with milk powder at a ratio of 1:9 (w/w). The mixture was then divided into individual 1 g aliquots as test samples, with each aliquot containing 100 mg of Anti-Hp-IgY. The test samples were dissolved in phosphate buffers of varying pH values (pH 1.0, 3.0, and 7.8) to achieve a final antibody concentration of 10 mg/mL. After incubation at 37°C for 2 h, the pH of each solution was immediately adjusted to 7.0 using an appropriate volume of 1.68 mol/L sodium carbonate buffer. The antibody titer was subsequently determined by indirect ELISA. Untreated test samples served as blank controls, and the Anti-Hp-IgY concentration of all test samples was adjusted to 10 μg/mL prior to measurement. The antibody titer was evaluated based on the OD_{450} values.

3.4. Effect of Pepsin on Anti-Hp-IgY with Milk Powder Protectant

The test samples were added to a specified volume of simulated gastric fluid containing pepsin to achieve a final antibody concentration of 10 mg/mL. In the mixture, the substrate mass ratio of enzyme to antibody was 1:20. The mixture was incubated at 37°C with shaking for digestion. At time points of 0 min, 20 min, 40 min, and 60 min, 1 mL aliquots were withdrawn and transferred to separate EP tubes. The reaction was immediately terminated by adjusting the pH to 7.0 using an appropriate volume of 1.68 mol/L sodium carbonate buffer. A parallel set of samples incubated in simulated gastric fluid without pepsin served as blank controls. Finally, the antibody titer was determined by indirect ELISA. Prior to measurement, the Anti-Hp-IgY concentration of all test samples was adjusted to 10 μg/mL, and the antibody titer was evaluated based on the OD_{450} values.

3.5. Effect of Temperature and Packaging on Anti-Hp-IgY Titer

Anti-Hp-IgY lyophilized powder was thoroughly mixed with milk powder at a ratio of 1:9 (w/w). Half of the mixture was vacuum-packaged, while the other half was packaged conventionally, with each sample weighing 1 g. For test samples containing milk powder or eggshell powder as protective agents, portions were stored at 37°C, 24°C, 4°C, and −20°C, respectively. The Anti-Hp-IgY titer was measured at 30-day intervals. Anti-Hp-IgY lyophilized powder without any protective agent stored in an ultra-low temperature freezer served as the positive control, and a blank sample served as the negative control. The Anti-Hp-IgY concentration of all test samples was adjusted to 10 μg/mL prior to measurement. The group designations were as follows: c: 24°C, milk powder; d: 24°C, milk powder, vacuum packaging; g: 4°C, milk powder; h: 4°C, milk powder, vacuum packaging; k: −20°C, milk powder; l: −20°C, milk powder, vacuum packaging; n: −20°C, eggshell powder, vacuum packaging.

4. RESULTS

4.1. Antibody Titer and SDS-PAGE Results

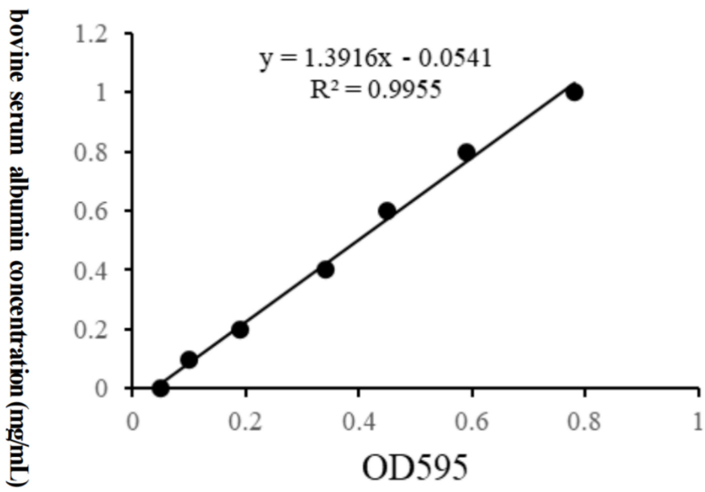

Figure 1. Standard curve for determination of protein content

By measuring the OD_{595} values of different BSA concentrations, a linear regression equation between absorbance and BSA concentration was established: Y = 1.3916X − 0.0541, with an R^2 value of 0.9955 (Fig. 2-1). Therefore, this regression equation can be used to determine the protein concentration of Anti-Hp-IgY lyophilized powder.

The SDS-PAGE results showed that under denaturing conditions, the disulfide bonds of Anti-Hp-IgY were cleaved, yielding two distinct bands corresponding to the heavy chain and light chain, located at approximately 70 kDa and 25 kDa, respectively (Fig. 2-2/I). These molecular weights are consistent with those reported in the literature for IgY heavy and light chains.

As the dilution factor of the Anti-Hp-IgY lyophilized powder increased, the OD_{450} values exhibited a continuous decreasing trend. At a dilution of 1:160,000 (Fig. 2-2/II), corresponding to an antibody concentration of 73 pg/mL, a significant signal was still observed, and the P/N ratio remained greater than 2.1. The result was determined to be positive, indicating an antibody titer of 1:160,000.

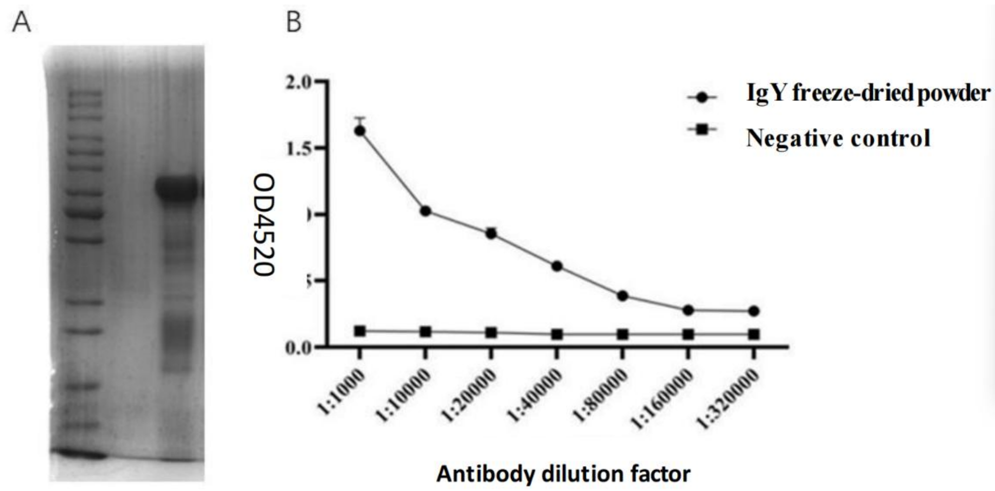

Figure 2. Anti-Hp-IgY SDS-PAGE results and titer determination

Figure 2. Panel A shows the SDS-PAGE results of Anti-Hp-IgY. Lane 1 contains the protein marker, and Lane 2 contains Anti-Hp-IgY. The heavy chain band is located at approximately 75 kDa, and the light chain band is at approximately 25 kDa. Panel B presents the results of the Anti-Hp-IgY titer determination.

4.2. Effect of pH on Anti-Hp-IgY in the Presence of Milk Powder

The OD_{450} values of Anti-Hp-IgY lyophilized powder with different protective agents after treatment at various pH levels for 2 h were comparatively analyzed to evaluate changes in antibody titer. The OD_{450} value of the blank control group was 1.38 ± 0.018 (Fig. 2-3), and its antibody retention rate was defined as 100%. The antibody retention rates of the other groups were calculated as the ratio of their OD_{450} values to that of the blank control group.

At pH 1.0, the OD_{450} value of Anti-Hp-IgY lyophilized powder decreased to 0.593 ± 0.087, corresponding to an antibody retention rate of 43.0%. In the milk powder group, the OD_{450} value was 0.997 ± 0.076, with an antibody retention rate of 72.3%.

At pH 3.0, the OD_{450} value of Anti-Hp-IgY lyophilized powder decreased to 0.993 ± 0.071, corresponding to an antibody retention rate of 72.0%. In the milk powder group, the OD_{450} value was 1.169 ± 0.061, with an antibody retention rate of 84.7%.

At pH 7.8, the OD_{450} value of Anti-Hp-IgY lyophilized powder decreased to 1.335 ± 0.077, corresponding to an antibody retention rate of 96.7%. In the milk powder group, the OD_{450} value was 1.281 ± 0.037, with an antibody retention rate of 92.8%.

At pH 1.0, both the Anti-Hp-IgY lyophilized powder alone and the milk powder group showed significant differences compared with the blank control group ($p < 0.05$). At pH 3.0, both the Anti-Hp-IgY lyophilized powder alone and the milk powder group also showed significant differences compared with the blank control group ($p < 0.05$). At pH 7.8, no significant differences were observed between the Anti-Hp-IgY lyophilized powder group, the milk powder group, and the blank control group ($p > 0.05$).

In summary, after 2 h of treatment under strongly acidic conditions (pH 1.0), the titer of Anti-Hp-IgY lyophilized powder was significantly reduced ($p < 0.05$). However, milk powder exhibited a pronounced protective effect on the titer of Anti-Hp-IgY lyophilized powder.

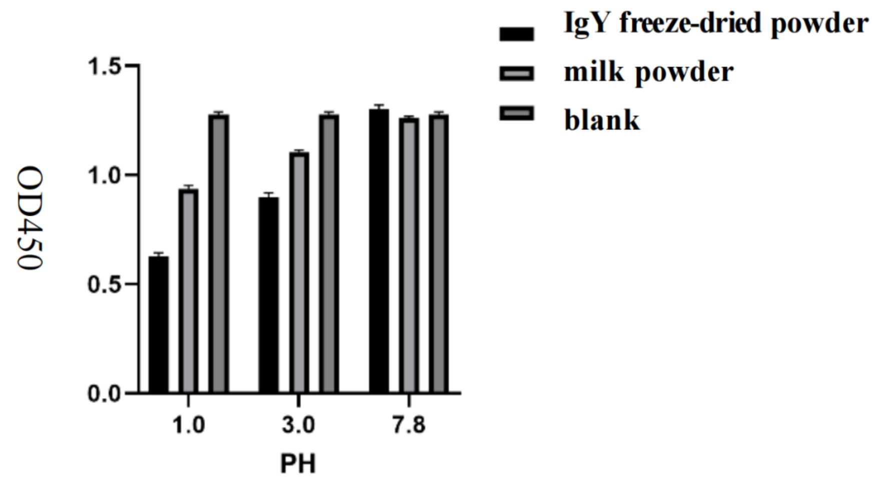

Figure 3. The effect of different pH on the titer of Anti-Hp-IgY under different protective agents

4.3. Effect of Simulated Gastric Fluid on IgY in the Presence of Different Protectants

Anti-Hp-IgY lyophilized powder with different protective agents was treated with pepsin, and samples were collected at 0, 20, 40, and 60 min for antibody titer determination (Fig. 2-4). For the blank control group without pepsin, the OD_{450} values at 0, 20, 40, and 60 min were 1.191 ± 0.017, 1.111 ± 0.004, 1.085 ± 0.016, and 0.993 ± 0.071, respectively (Fig. 2-4). The antibody titer at 0 min was defined as 100% retention, and the retention rates of the other groups were calculated as the ratio of their OD_{450} values to that of the blank control group at 0 min. In the blank control group, the antibody retention rate at 60 min was 83.3%.

For Anti-Hp-IgY lyophilized powder without additional protectant, the OD_{450} values at 0, 20, 40, and 60 min were 1.173 ± 0.051, 1.100 ± 0.026, 1.015 ± 0.061, and 0.949 ± 0.015, respectively (Fig. 2-4). The antibody retention rate at 60 min was 79.6%.

For the milk powder group, the OD_{450} values at 0, 20, 40, and 60 min were 1.195 ± 0.080, 1.104 ± 0.014, 1.067 ± 0.044, and 1.048 ± 0.012, respectively (Fig. 2-4). The antibody retention rate at 60 min was 88.0%.

As the incubation time increased, the OD_{450} values of all groups gradually declined; however, the addition of milk powder attenuated this decreasing trend. After 60 min of treatment, the antibody retention rate of Anti-Hp-IgY lyophilized powder alone was lower than that of the blank control group, whereas the retention rate of the milk powder group was higher than that of the blank control group.

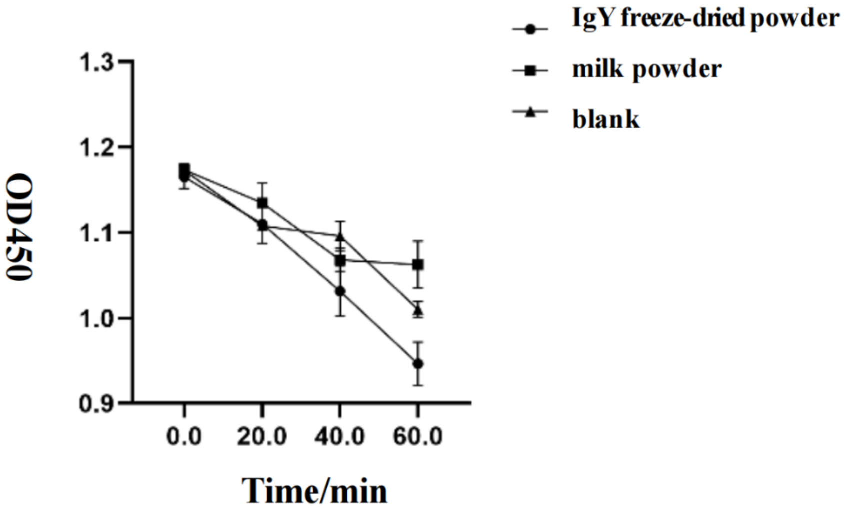

Figure 4. The effect of simulated gastric juice on the titer of Anti-Hp-IgY under different protective agents

4.4. Anti-Hp-IgY Titer Changes Under Different Temperatures and Packagings

The stability of Anti-Hp-IgY lyophilized powder with milk powder as the protective agent was evaluated. Test samples were stored under conventional packaging or vacuum packaging at 24°C, 4°C, and −20°C, respectively. The OD_{450} values were measured by ELISA every 30 days to monitor changes in antibody titer.

For the milk powder protectant group, the OD_{450} value at day 0 was 1.853 ± 0.061 (Fig. 2-5/II). The antibody titer at day 0 was defined as 100% retention, and the retention rates of all other groups were calculated as the ratio of their OD_{450} values to that at day 0.

Conventional packaging with milk powder protectant:At 24°C, the OD_{450} value at day 360 was 0.879 ± 0.220, corresponding to an antibody retention rate of 47.4%.At 4°C, the OD_{450} value at day 360 was 0.967 ± 0.245, corresponding to an antibody retention rate of 52.2%.At −20°C, the OD_{450} value at day 360 was 0.961 ± 0.060, corresponding to an antibody retention rate of 51.9%.

Vacuum packaging with milk powder protectant:At 24°C, the OD_{450} value at day 360 was 0.841 ± 0.145, corresponding to an antibody retention rate of 45.4%.At 4°C, the OD_{450} value at day 360 was 0.968 ± 0.095, corresponding to an antibody retention rate of 52.2%.At −20°C, the OD_{450} value at day 360 was 1.018 ± 0.045, corresponding to an antibody retention rate of 54.9%.

Comparison of packaging methods at each storage temperature:At 24°C, the antibody retention rate at day 360 was 47.4% for conventional packaging and 45.4% for vacuum packaging.At 4°C, the antibody retention rate at day 360 was 52.2% for both conventional and vacuum packaging.At −20°C, the antibody retention rate at day 360 was 51.9% for conventional packaging and 54.9% for vacuum packaging.

With milk powder as the protective agent, the antibody titers of samples stored under conventional packaging decreased gradually over time at all temperatures, showing a significant reduction compared with day 0 ($p < 0.05$). Similarly, samples stored under vacuum packaging also exhibited a gradual and significant decline in titer compared with day 0 ($p < 0.05$). The lowest antibody retention rate was observed in samples stored at 24°C. No significant difference in antibody retention rate was found between the two packaging methods.

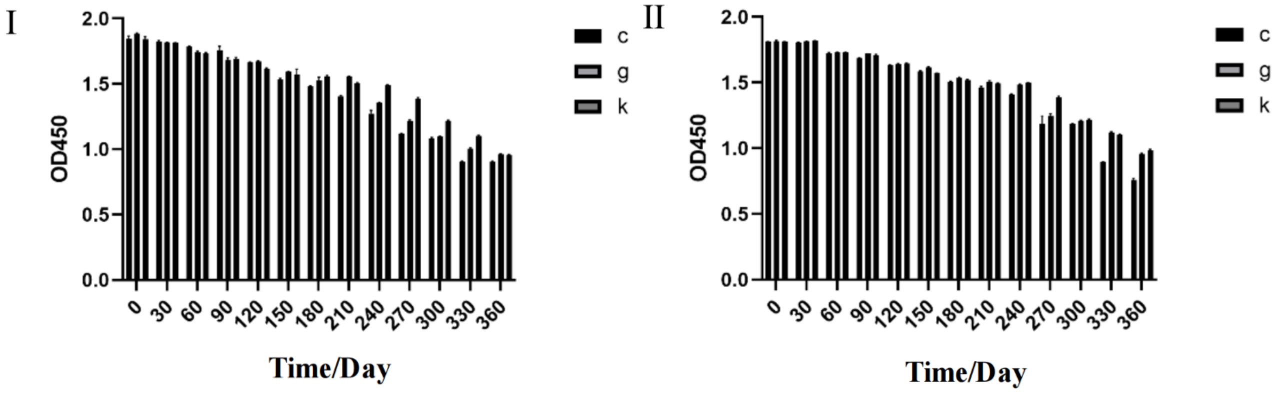

Figure 5. The changes of Anti-Hp-IgY titer of milk powder under different storage methods

Figure 5. Panel I shows the changes in antibody titer of samples with milk powder as the protective agent under conventional packaging at different storage temperatures. (c: 24°C, milk powder; g: 4°C, milk powder; k: −20°C, milk powder.) Panel II shows the changes in antibody titer of samples with milk powder as the protective agent under vacuum packaging at different storage temperatures. (d: 24°C, milk powder, vacuum packaging; h: 4°C, milk powder, vacuum packaging; l: −20°C, milk powder, vacuum packaging.)

5. DISCUSSION

This study analyzed the tolerance of Anti-Hp-IgY lyophilized powder to acidic/alkaline environments and pepsin digestion, and evaluated its stability under various storage conditions. The addition of protective agents could mitigate stability-related issues to a certain extent. Notably, a positive signal was still detectable when the lyophilized powder was diluted to 1:160,000.

After treatment under strongly acidic conditions (pH 1.0) for 1 h, the antibody activity retention rate was relatively low. However, the addition of milk powder as a protective agent improved the retention rate. Within the pH range of 3.0 to 7.8, antibody activity remained largely unaffected, indicating that Anti-Hp-IgY lyophilized powder possesses a certain degree of acid–base tolerance, which was further enhanced by the addition of protective agents. Comparative experimental results demonstrated that the inclusion of milk powder significantly improved IgY stability in strongly acidic environments. We hypothesize that the underlying mechanism involves the formation of an emulsion by milk powder in the aqueous phase, which reduces direct contact between the antibody and the solution, thereby exerting a protective effect. Similarly, Hu Ruihong et al. [9] reported that the final retention rate of antibodies under strong acid exposure was approximately 30%. Shao Min et al. [10] noted that antibody activity remained relatively stable within the pH range of 4–7, while Kyong Ae Lee et al. [11] found that antibody titers declined rapidly at pH < 2 or > 11, and that sorbitol could enhance IgY stability at pH 3. Furthermore, IgY in its native state is protected by yolk granules, and encapsulation by components such as lecithin can enhance its resistance to pH changes and digestive enzymes [12].

Following pepsin treatment for 1 h, the antibody activity retention rate of Anti-Hp-IgY lyophilized powder was 77.5%, which further increased with the addition of milk powder. The present findings differ from those reported by Zang Yuting et al. [13], who observed no significant change in yolk antibody titer during pepsin treatment (pH 4) over a 6 h period. We attribute this discrepancy to the experimental conditions: the present study was conducted at pH 2, where pepsin activity is higher and exerts a stronger degradative effect on the antibody. Nevertheless, the addition of milk powder still effectively improved antibody retention, suggesting that the protective agent can partially counteract pepsin-mediated degradation.

Marked differences were observed in the titer changes of Anti-Hp-IgY lyophilized powder under different storage conditions. After 360 days of storage, the antibody inactivation rate under frozen conditions was significantly lower than that observed at 37°C. Low-temperature environments contribute to prolonging both titer stability and shelf life, while the addition of protective agents further helps maintain titer stability. Although vacuum packaging offered a slight advantage over conventional packaging in preserving titer, the effect was not statistically significant. Half-life is a critical pharmacokinetic parameter, defined as the time required for the antibody titer to decrease by half. IgY has a relatively high sialic acid content, and purified IgY can have a half-life of up to six months, remaining stable for one month at 37°C. In this study, Anti-Hp-IgY lyophilized powder with milk powder as the protectant retained approximately 50% of its original titer after 360 days of storage at −20°C. Therefore, to ensure adequate potency at the time of use, the storage period of this product should be limited to within 360 days.

6. CONCLUSION

This study investigated the effects of simulated pH environments, pepsin treatment, and long-term storage conditions. The results demonstrate that milk powder as a protective agent significantly alleviates antibody damage under strongly acidic conditions (pH 1.0), delays pepsin-mediated degradation, and maintains higher antibody activity within 60 min of exposure. Moreover, during long-term storage (360 days), low temperature (4°C or −20°C) was identified as the critical factor for preserving antibody titer, whereas the influence of packaging method was not significant. These findings indicate that milk powder is an effective and practical protective agent for Anti-Hp-IgY, providing experimental evidence for the formulation optimization and application development of this IgY-based preparation.

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