A Simple AGPT-Based Method for Preliminary Stability Assessment of Chicken Immunoglobulin Y (IgY)

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Abstract

Immunoglobulin Y (IgY) is a promising alternative antibody with broad applications in immunotherapy, diagnostics, and passive immunization due to its low production cost, non-invasive extraction, and high yield from egg yolk. In addition, IgY offers several biological advantages over mammalian IgG, including the absence of interaction with mammalian Fc receptors and the inability to activate the human complement system, making it safer for biomedical and veterinary applications. However, the functional stability of IgY can be compromised by environmental stressors such as elevated temperature, extreme pH conditions, and exposure to digestive enzymes, which may limit its effectiveness in various formulations and delivery routes. This study aimed to evaluate IgY stability using the Agar Gel Precipitation Test (AGPT), a simple and low-cost qualitative method capable of detecting antigen–antibody interactions through visible precipitin line formation. Purified IgY was exposed to different temperatures (room temperature to 70°C), pH conditions (3–9), and digestive enzymes (pepsin at pH 3 and trypsin at pH 8) for 15, 30, and 60 minutes prior to AGPT evaluation. The intensity and clarity of precipitin lines were used as indicators of preserved antigen-binding activity. The results demonstrated that IgY maintained strong precipitin intensity at temperatures up to 60°C, under neutral to alkaline pH conditions, and following exposure to trypsin. In contrast, exposure to 70°C and pepsin at pH 3 resulted in markedly reduced precipitin intensity. Physical coagulation of IgY was also observed at 70°C, indicating heat-induced denaturation and loss of functional integrity. Overall, this study demonstrates that AGPT is an effective and practical preliminary screening tool for assessing IgY stability under various physicochemical and enzymatic stress conditions, particularly in laboratories with limited access to advanced analytical techniques, before further quantitative or structural analyses are conducted.

1. Introduction

Immunoglobulin Y (IgY) has emerged as a promising alternative antibody for therapeutic and prophylactic applications due to its non-invasive production, rapid generation time, low cost, and high yield from egg yolk. In addition, IgY does not bind to mammalian Fc receptors, does not activate the human complement system, and does not trigger inflammatory responses, making it advantageous for biomedical use [1]. Chickens immunized with specific pathogens can produce large quantities of antigen-specific IgY that is subsequently deposited into the egg yolk, allowing scalable and economical antibody production [2]. IgY has been evaluated in various delivery formats, including injectable formulations [3], aerosol administration [4], oral delivery [5], and other applied dosage forms [6].

Despite its therapeutic potential, IgY is susceptible to physicochemical degradation when exposed to environmental stressors such as high temperatures, extreme pH, or digestive enzymes [7]. These conditions can induce conformational changes, aggregation, or proteolysis, ultimately reducing antigen-binding activity [5, 8]. As a glycoprotein, IgY is highly sensitive to denaturation, particularly at temperatures exceeding 70°C, where irreversible structural alterations may occur [9]. Acidic environments, such as those found in the stomach, can also lead to partial or complete degradation unless protective formulations are used [10]. Maintaining IgY stability is therefore essential for its development into reliable therapeutic or prophylactic agents, especially for oral or mucosal application where harsh physiological conditions may significantly compromise its activity.

Conventionally, IgY stability is assessed using enzyme-linked immunosorbent assay (ELISA) to quantify antigen-binding capacity or sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to evaluate structural integrity [9, 11]. Although sensitive and informative, these techniques require specialized equipment, trained personnel, and relatively high operational costs. Laboratories with limited resources may therefore benefit from an alternative approach that is simpler, more economical, and suitable for preliminary stability screening.

The Agar Gel Precipitation Test (AGPT) is a classical immunodiffusion technique that visualizes antigen–antibody interactions through precipitin line formation within an agar matrix [12]. While AGPT has traditionally been used in serological identification, its applicability as a functional stability assay for antibodies has not been extensively explored. Because precipitin line intensity reflects the presence of intact antigen-binding domains, AGPT may serve as a rapid and low-cost method for detecting early loss of IgY activity before more detailed quantitative or structural analyses are performed.

Therefore, the objective of this study was to evaluate the functional stability of IgY under variations in temperature, pH, and digestive enzyme exposure, and to demonstrate the applicability of AGPT as a simple, low-cost preliminary screening method for IgY stability assessment compared with conventional techniques such as ELISA and SDS-PAGE.

2. Materials and methods

2. 1. IgY Preparation

The IgY used in this study was obtained from previously produced and purified stock material as described by Rizal et al. (2026). Briefly, IgY was isolated from the yolk of Leghorn chickens immunized with *Escherichia coli* and purified using a stepwise Polyethylene Glycol (PEG-6000) fractionation method. The purified IgY was stored at 4°C until use and applied directly in all stability assays.

2. 2. Preparation of Soluble Antigen

Escherichia coli was cultured in Brain Heart Infusion (BHI; Thermo Fisher Scientific®) and incubated at 37°C for 18 hours. The bacterial culture was centrifuged at 20,000 rpm for 10 minutes to obtain a cell pellet, which was then washed with PBS. The pellet was resuspended in 0.5 mL of 0.2 N HCl and centrifuged again under identical conditions. After discarding the supernatant, the pellet was mixed with another 0.5 mL of 0.2 N HCl and heated at 52°C for 1 hour. Following heating, the homogenate was titrated with 1 N NaOH using phenol red as an indicator until a color change from yellow to red occurred. The mixture was centrifuged at 10,000 rpm for 10 minutes, and the resulting supernatant was collected as the soluble antigen (HCl extract) for AGPT.

2. 3. Treatment of IgY and Stability Testing

Thermal Treatment of IgY. To evaluate thermal stability, IgY samples were aliquoted into microtubes and exposed to five temperatures: room temperature (~25°C), 37°C, 50°C, 60°C, and 70°C (Figure 1). Temperature exposure was conducted using a rotary incubator to ensure uniform heating. Samples were incubated for 15, 30, and 60 minutes. After incubation, samples were returned to room temperature before AGPT analysis.

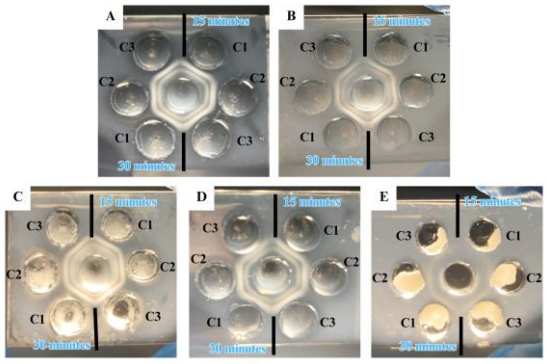

Figure 1. Thermal Treatment of IgY room temperature (~25°C) **(A)**, 37°C **(B)**, 50°C **(C)**, 60°C **(D)**, and 70°C **(E)**. All treated samples were subsequently used directly in the AGPT assays.

Enzymatic Treatment of IgY. The stability of IgY against digestive enzymes was tested by mixing IgY with pepsin (pH 3) or trypsin (pH 8) at a 1:1 ratio. Enzymes were prepared in pre-adjusted buffers to maintain the original IgY concentration. Samples were incubated at room temperature for 15, 30, and 60 minutes (Figure 2). Enzymatic reactions were terminated by stopping the incubation time, and samples were directly used for AGPT without additional neutralization. Enzymes were supplied by Shaanxi Fonde Biotech Co., Ltd.

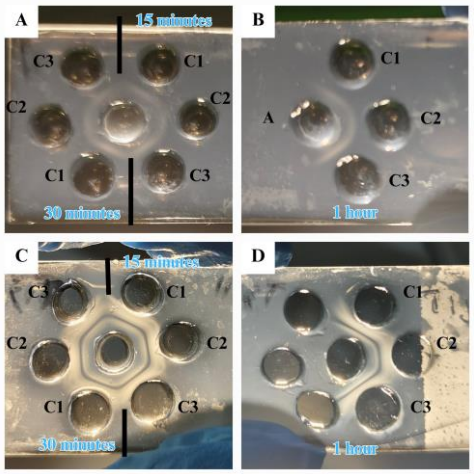

Figure 2. Enzymatic Treatment of IgY. Mixing IgY with pepsin (pH 3) incubated at room temperature for 15 and 30 minutes **(A)**. Mixing IgY with pepsin (pH 3) incubated at room temperature for 1 hour **(B)**. Mixing IgY with trypsin (pH 8) incubated at room temperature for 15 and 30 minutes **(C)**. Mixing IgY with trypsin (pH 8) incubated at room temperature for 1 hour **(D)**. All treated samples were subsequently used directly in the AGPT assays.

pH Treatment of IgY. IgY samples were exposed to pre-prepared buffers at pH 3, 5, 7, and 9 to assess the effects of acidic and alkaline conditions. Samples were incubated at room temperature for 15, 30, and 60 minutes and were subsequently used directly in AGPT assays (Figure 3).

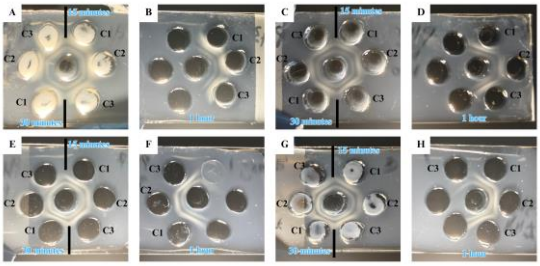

Figure 3. pH Treatment of IgY pH 3 for 15 and 30 minutes **(A)** and for 1 hour **(B)**, pH 5 for 15 and 30 minutes **(C)** and for 1 hour **(D)**, pH 7 for 15 and 30 minutes **(E)** and for 1 hour **(F)**, and pH 9 for 15 and 30 minutes **(G)** and for 1 hour **(H)**. All treated samples were subsequently used directly in the AGPT assays.

2. 4. Agar Gel Precipitation Test (AGPT)

A 1% agarose–PEG gel was prepared in 0.5 M PBS (pH 7.4) and poured onto glass slides to solidify. Wells were punched in a circular pattern consisting of one central well surrounded by six peripheral wells. Treated IgY samples from each stability assay (temperature, pH, and digestive enzymes) were dispensed into the peripheral wells, while the HCl-extracted *E. coli* antigen was placed in the central well. The plates were incubated at 25°C for up to 18 hours to allow antigen–antibody diffusion. A positive AGPT reaction was indicated by the formation of distinct white precipitin lines between IgY and the antigen, whereas the absence of such lines was interpreted as a negative reaction.

3. Results

IgY exhibited strong and consistent precipitin lines at room temperature, 37°C, 50°C, and 60°C across all incubation periods (15, 30, and 60 minutes). These findings indicate preserved antigen-binding activity under these thermal conditions. However, exposure to 70°C resulted in a noticeable reduction in precipitin intensity, characterized by weaker and irregular lines compared to lower temperature groups. Additionally, IgY at 70°C showed visible physical alteration; the solution became turbid and coagulated, losing its fluid consistency. This macroscopic change likely interfered with IgY diffusion within the agar matrix, contributing to diminished precipitin formation.

Trypsin exposure at pH 8 maintained strong precipitin intensity, demonstrating that IgY remained stable in alkaline enzymatic conditions. Conversely, pepsin exposure at pH 3 generated very weak precipitin lines, indicating substantial degradation under acidic proteolytic stress. pH treatment alone showed that IgY was stable within pH 5–9, while pH 3 caused a significant decline in precipitin intensity—mirroring the effect observed with pepsin.

Overall, AGPT effectively visualized differences in IgY functional stability by demonstrating variations in precipitin intensity under different physicochemical conditions. These results confirm that the assay can be used not only to assess antigen–antibody interactions, but also to evaluate the impact of temperature, pH, and enzymatic activity on antibody integrity. Therefore, AGPT provides a reliable qualitative platform for screening IgY robustness prior to advanced analytical or therapeutic applications.

4. Discussion

The findings of this study demonstrate that IgY possesses good functional stability when exposed to moderately elevated temperatures, neutral-to-alkaline pH, and trypsin digestion. IgY maintained strong precipitin intensity at temperatures up to 60°C, indicating that antigen-binding activity remained intact under mild thermal stress. However, exposure to 70°C caused a marked decline in precipitin intensity, accompanied by visible coagulation of the IgY solution. This coagulation is consistent with heat-induced denaturation, where protein unfolding leads to irreversible aggregation. Such aggregation likely reduces the accessibility of antigen-binding sites and impairs IgY diffusion within the agar, resulting in weaker and inconsistent precipitin line formation. Previous reports similarly indicate that IgY begins to lose structural integrity above 65–70°C [14].

The stability patterns observed under pH and enzymatic treatments further reinforce the vulnerability of IgY under acidic conditions. While IgY remained functionally stable between pH 5 and 9, exposure to pH 3 significantly reduced precipitin intensity. Pepsin digestion at pH 3 produced the same effect, demonstrating that IgY is highly susceptible to proteolysis and conformational disruption in an acidic environment, as also described by Hodek et al. [15] and Gandhi and Alshehri [10]. In contrast, trypsin at pH 8 did not reduce IgY activity, consistent with its natural stability under alkaline intestinal conditions.

From a methodological perspective, this study highlights the practicality of AGPT as a preliminary screening tool for IgY stability testing. Although less sensitive than ELISA and lacking structural resolution compared to SDS-PAGE, AGPT provides clear visual differentiation of functional antibody activity based on precipitin intensity. Its low cost, minimal reagent requirements, reproducibility, and suitability for laboratories with limited resources make it a valuable first-line assay before proceeding to more advanced analyses.

Overall, the results align with the conclusions of Capota et al. [16] and Tabll et al. [17], reinforcing the importance of assessing IgY stability to support its development for therapeutic and diagnostic applications. The present findings confirm that high temperature and acidic environments are the principal factors contributing to IgY degradation, while neutral–alkaline conditions and trypsin exposure are comparatively well tolerated.

5. Conclusions

AGPT proved to be an effective, simple, and economical preliminary method for evaluating the functional stability of IgY under various physicochemical and enzymatic conditions. IgY remained stable at temperatures up to 60°C, under neutral-to-alkaline pH, and during trypsin exposure, but exhibited significant loss of activity at 70°C and pH 3, particularly under pepsin digestion. The observed coagulation at 70°C further confirms the onset of thermal denaturation. These findings support the use of AGPT as a practical screening assay prior to applying more sensitive or quantitative techniques such as ELISA or SDS-PAGE.

Acknowledgment

The authors would like to express their sincere gratitude to the management and the entire team of PT. Biomol Nusantara Mandiri, Tbk, for providing laboratory facilities and financial support, which enabled the successful completion of this study.

The authors also acknowledge the use of generative AI (ChatGPT, OpenAI), employed solely for language refinement and improvement of the manuscript's structural clarity. All scientific content, data analysis, and conclusions remain entirely the responsibility of the authors.

Authors' Contribution

M.F.R. designed the study, performed the experiments, and drafted the manuscript. I.W.T.W. and H.S.D. supervised the research and provided scientific guidance. R.L.B. and J.P. advised on laboratory methodology and technical validation. B.S. and Y.F.A. assisted with laboratory procedures and sample analysis. D.S. critically reviewed the manuscript, and S.A. finalized and edited the manuscript for journal submission.

Ethics

This study was conducted in accordance with institutional ethical guidelines. The IgY used was obtained from previously approved immunization procedures (Ethical Approval No. 260/KEH/SKE/X/2024), and no new animal experiments were performed. All work was carried out in vitro without the use of live animals.

Conflict of Interest

The authors declare no conflict of interest regarding the conduct, analysis, or publication of this research.

Data Availability

All data are available in the manuscript

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