

Matrix-Aware ELISA Analysis of Soluble Ovotransferrin in Egg White Subjected to Household Cooking and Gradient Heating

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Abstract

Ovotransferrin (OVT) is the second most abundant protein in egg white and an important indicator of the nutritional and functional quality of egg products. In thermally processed egg-white matrices, however, sandwich enzyme-linked immunosorbent assay (ELISA) reflects the relative abundance of ELISA-detectable soluble OVT rather than the absolute amount of total OVT. In this study, egg white samples obtained from five common household cooking conditions, namely raw egg white, boiled egg, steamed egg, egg custard, and fried egg, were used to establish matrix-adapted pretreatment and extraction procedures prior to ELISA analysis. The effects of home cooking methods and gradient heating (25, 60, 75, and 95°C for 0, 10, and 20 min) on the relative abundance of OVT in the ELISA-detectable soluble fraction were then systematically evaluated. Fully cooked samples showed significantly lower relative OVT content in the ELISA-detectable soluble fraction than raw egg white ($P < 0.05$), whereas no significant differences were observed among the four cooked groups. In the gradient-heating experiment, temperature was the dominant factor ($P < 0.0001$), and both holding time and the temperature-time interaction were also highly significant ($P < 0.0001$). The apparent increase in OVT signal at elevated temperature was interpreted as relative enrichment of soluble OVT caused by preferential denaturation, aggregation, and precipitation of more heat-labile egg-white proteins, together with matrix-dependent extraction and epitope accessibility effects. These results provide a more rigorous analytical framework for evaluating functional protein retention during domestic egg processing.

1 Introduction

Eggs are among the most widely consumed animal-derived foods worldwide, as they provide high-quality protein, balanced amino acids, and diverse micronutrients [1, 2]. In China, household cooking accounts for more than 60% of total egg consumption, with boiling, steaming, and frying being the most common daily processing methods [3, 4]. Egg white makes up approximately 60% of the whole egg mass and contains more than 40 bioactive proteins [5]. Ovotransferrin (OVT), a 78 kDa monomeric glycoprotein belonging to the transferrin family, is the second most abundant protein in egg white, accounting for about 12% of total egg-white protein. It is therefore a core functional indicator for evaluating the nutritional and functional quality of eggs and egg products [5, 6].

Beyond its classical function of reversible iron binding and transport, OVT exhibits well-documented antimicrobial, antioxidant, anti-inflammatory, and immunomodulatory activities [6–8]. These bioactivities endow OVT with broad application prospects in functional foods, pharmaceutical excipients, and cosmetic additives [9, 10], further highlighting the importance of accurate quantification of OVT in processed egg products. Thermal processing is an indispensable step to ensure the microbial safety and sensory acceptability of egg products [11, 12]: moderate heating can improve the digestibility and sensory quality of eggs, while excessive heating will cause irreversible unfolding, aggregation, and functional loss of egg-white proteins [13–15]. However, the variation pattern of OVT under realistic home cooking conditions remains poorly understood.

Current studies on the thermal stability of OVT have some notable research gaps and application limitations. Most studies focus on purified OVT, investigating its thermal denaturation kinetics and structural changes in isolated systems [16–19], while the findings from pure protein systems cannot be directly applied to the

complex multi-component egg white matrix; the existing thermal treatment studies mostly use laboratory-simulated heating with single temperature-time combinations, and systematic quantitative studies on OVT in egg white under realistic home cooking scenarios are still scarce; and the egg white matrix varies significantly with cooking methods: raw egg white is a viscous hydrocolloid, boiling, steaming and custard preparation form dense protein gel networks [20], while frying introduces a high-fat matrix with intense protein denaturation [15]. For the analysis of OVT, conventional extraction methods often fail to achieve consistent and efficient OVT recovery from differently cooked samples [21, 22], and commercial double-antibody sandwich ELISA kits for chicken OVT are only validated in serum and tissue homogenates, their reliability in high-protein egg white matrices were not systematically confirmed.

Therefore, sandwich ELISA results for heated egg white samples should be interpreted cautiously, as they may reflect matrix-dependent extraction efficiency, altered epitope accessibility, and compositional shifts of the soluble fraction, rather than absolute changes in total OVT content. In this study, based on the matrix characteristics of egg white under five common household cooking conditions (raw egg white, boiled egg, steamed egg, egg custard, and fried egg), we established and optimized matrix-adapted pretreatment and extraction procedures for OVT quantification. Meanwhile, we systematically evaluated the effects of the above five cooking methods, as well as gradient heat treatments (25, 60, 75, and 95°C for 0, 10, and 20 min), on the relative content of OVT in the ELISA-detectable soluble fraction of egg white. This study aims to fill the research gap of OVT variation under realistic home cooking conditions, provide data support for consumers to choose cooking methods that maximize the retention of egg functional nutrients, and offer a technical reference for OVT quality control and thermal process optimization during egg deep processing.

2 Materials and Methods

2.1 Experimental Materials and Reagents

Fresh eggs were purchased from a local Sam's Club (produced by Sichuan Sundaily Eco-Food Co., Ltd., China). After purchase, the eggs were immediately stored at 4°C, and all sample processing was completed within 24 h. A commercial one-step double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit specific for chicken ovotransferrin (OVT) (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China; Catalog No. CB10103-Ch) was used for quantitative determination of OVT in the ELISA-detectable soluble fraction of egg white extracts. The kit had a validated linear detection range of 0.25–8 g/L, a limit of detection (LOD) < 0.1 g/L, an intra-assay coefficient of variation (CV) < 10%, and an inter-assay CV < 15%. Phosphate-buffered saline containing 0.05% Tween 80 (PBST, 0.01 mol/L, pH 7.4) was prepared in-house. The phosphate-buffered saline (PBS) powder (calcium- and magnesium-free) was purchased from Hunan BiKeMan Holding Co., Ltd. (Changde, Hunan, China), and Tween 80 was obtained from Shanghai Macklin Biochemical Technology Co., Ltd. (Shanghai, China; catalog No. T6336-100 mL). The 75% (v/v) ethanol solution was prepared as needed using analytical-grade absolute ethanol produced by Millipore Chemical Reagent Co., Ltd. (Shanghai, China).

2.2 Experimental Methods

2.2.1 Preparation of Egg White Samples Subjected to Different Cooking Methods

All cooking treatments were performed in triplicate. For parallel samples within the same group, the heating medium, containers, and operation procedures were kept consistent. Egg white was first separated from the yolk, and the chalazae along with other impurities were removed prior to subsequent pretreatment. After cooking was completed, the samples were immediately placed in an ice-water bath and cooled to below 4°C to terminate the thermal reaction. The specific procedures for each group were as follows: (1) Raw egg white (control group): Egg white was aseptically separated from fresh eggs, stored at 4°C, and subjected to pretreatment without heat treatment; (2) Boiled egg group: Whole eggs in their shells were immersed in boiling water bath and heated until the egg white and yolk were fully coagulated (10 min), then removed and cooled in an ice-water bath; (3) Steamed egg group: Egg white was separated and placed in a sterile covered container, then heated under atmospheric pressure with saturated steam from boiling water until fully coagulated (10 min), followed by cooling in an ice-water bath. (4) Egg custard group: Egg white was mixed with ultrapure water at an approximate volume ratio of 1:1, heated to solidification under atmospheric pressure with saturated steam generated by boiling water (15 min), then the sample was left to stand and cooled in an ice-water bath; (5) Fried egg group: A non-stick pan was preheated to a constant high temperature. Egg white was poured into the pan and fried on one side. After removing any burnt portions, the edible egg white was collected and cooled in an ice-water bath.

2.2.2 Gradient Heat Treatment of Egg White

Fresh raw egg white was used to investigate the effect of thermal stress alone on the relative content of OVT in the ELISA-detectable soluble fraction while minimizing structural differences introduced by distinct household cooking procedures. Fresh egg white (0.5 g) was placed into sterile centrifuge tubes, and heated separately in a constant-temperature water bath (HH-6, Changzhou Langyue Instrument Manufacturing Co., Ltd.) at 25°C (room-temperature control without thermal stress), 60°C (initial denaturation range for heat-labile egg-white proteins), 75°C (accelerated denaturation range), and 95°C (high-temperature treatment approaching extensive coagulation) [13, 18]. Three holding times (0, 10, and 20 min) were applied at each temperature. For the 0 min group, samples were removed immediately after the centrifuge tubes were fully immersed in the water bath at the designated temperature to simulate brief high-temperature exposure. After heating, all samples were immediately placed in an ice-water bath to terminate the thermal reaction. Each treatment group was prepared in triplicate.

2.2.3 Preparation of Test Solutions for Ovotransferrin ELISA Detection in Egg White Samples

All utensils were thoroughly disinfected with 75% ethanol and allowed to air dry before use. The entire procedure was conducted in an ice bath. For egg white samples subjected to different cooking methods, the samples were firstly cut into smaller pieces using sterile scissors based on their degree of doneness and gel strength. Detailed parameters are provided in Table 1. Subsequently, accurately 0.5 g portions of the cut egg white samples were placed into centrifuge tubes, with the ice bath maintained throughout the process. For the gradient heat treatment groups, the samples consisted of small volumes of egg white in uniform centrifuge tubes. After heat treatment, no large gel blocks were present, so cutting was not required. These samples were processed simultaneously with the cooked samples for ice-bath homogenization. During homogenization, pre-chilled PBST extraction buffer was added, and the samples were gently mashed along the tube wall using a

sterile glass rod until a homogeneous emulsion was formed with no visible solid particles. The final system was standardized to 0.5 g of egg white sample plus 4.5 mL of pre-chilled PBST extraction buffer.

Table 1
Chopping degree of egg white samples cooked by different methods

Cooking method	Degree of chopping
Raw egg white	Chopped into uniform flocculent pieces
Boiled egg	Chopped into uniform rice-grain-sized pieces
Steamed egg	Chopped into uniform fine fragments
Egg custard	Gently chopped into uniform small pieces
Fried egg (without burnt portions)	Chopped into uniform fine granules
After the system was prepared, differentiated vortexing and ultrasonic treatments were performed according to the matrix characteristics of the samples, with the entire process maintained in a low-temperature ice-bath environment:	

- (1) Liquid and weak gel matrix samples (raw egg white, egg custard, and instantaneous heat-stressed samples): Samples were vortexed using a vortex mixer at maximum speed in an ice bath for 1–2 min until no visible protein aggregates remained. Ultrasonic disruption was not applied.
- (2) Heat-induced gel matrix samples (boiled egg, steamed egg, and gradient heat-treated samples): Samples were first pre-mixed via vortexing under ice-bath condition for 1 min, followed by ultrasonication treatment under consistent ice-bath cooling using an ultrasonic cell disruptor (SCIENTZ-950E, Ningbo Xinzhi Biotechnology Co., Ltd.). The ultrasonic parameters were set as follows: power 200 W, operating mode with 3 s of sonication followed by 2 s of interval, and total sonication duration of 1 min.
- (3) High-fat matrix samples (fried egg): Samples were first vortexed in an ice bath for 1 min to form a homogeneous emulsion, then centrifuged at 8000 r/min for 5 min at 4°C for defatting. The lower aqueous phase was collected and gently vortexed in an ice bath for 30 s to mix, with no additional ultrasonic disruption applied.

After the above treatments were completed, all sample solutions were placed on a decolorizing shaker with ice packs continuously placed at the bottom of the shaker tray to maintain a low-temperature extraction environment throughout the process. Extraction was carried out with shaking at 150 rpm for 30 min. After extraction, the sample solutions were centrifuged at 11000 rpm for 15 min at 4°C (H2-16KR, Hunan Kecheng Instrument Equipment Co., Ltd.). The supernatant was carefully aspirated to obtain the crude egg white extract.

The crude egg white extract was serially diluted at 10-, 50-, 100-, and 500-fold using the sample diluent provided with the ELISA kit. Based on preliminary experimental validation, the dilution factor that yielded absorbance values within the linear range of the kit's standard curve was selected to accurately dilute the crude extract to a fixed volume, preparing the test solution for ELISA detection. The test solutions were temporarily stored at 4°C and analyzed within 12 h.

2.2.4 Quantitative Determination of OVT in Egg White by Sandwich ELISA

The ELISA signal was determined strictly following the kit instructions. In this study, it was interpreted as the relative content of OVT in the ELISA-detectable soluble fraction, not as the absolute total OVT. After removal from refrigerated storage at 2–8°C, the kit was allowed to equilibrate at room temperature (20–25°C) for 20 min before use. Disposable pipette tips used throughout the experiment were sterile and nuclease-free, and a new tip was used for each pipetting step to avoid cross-contamination. The 20× wash buffer was diluted with ultrapure water at a volume ratio of 1:19 to prepare 1× wash working solution, which was freshly prepared before use. The assay was implemented within the manufacturer-specified analytical range (0.25–8 g/L; minimum detection limit < 0.1 g/L) and interpreted with reference to the manufacturer-reported intra- and inter-assay precision (< 10% and < 15%, respectively). Only sample dilutions yielding absorbance values within the linear range of the standard curve were used for quantitative interpretation. The experimental procedure was carried out according to the manufacturer's instructions and analyzed using a microplate reader (Feyond-A300, Hangzhou Allsheng Instruments Co., Ltd).

2.2.5 Sequence- and structure-based comparative analysis of major egg-white proteins

A lightweight sequence and structure-based comparison was performed to provide supportive evidence for interpreting the experimentally observed differences in the detectable soluble fraction of major egg-white proteins after heating. Reviewed *Gallus gallus* protein entries were obtained from UniProtKB, including ovotransferrin (P02789), ovalbumin (P01012), lysozyme C (P00698), and ovomucoid (P01005) [23]. Basic physicochemical parameters, including sequence length, molecular weight, theoretical isoelectric point (pI), instability index, aliphatic index, and grand average of hydropathicity (GRAVY), were calculated using ExPASy ProtParam [24]. Representative three-dimensional structures were obtained from experimentally resolved structures available in the RCSB PDB and/or predicted models from AlphaFold DB [25, 26] and visualized using UCSF ChimeraX [27]. Because the present comparison was intended as rapid supportive evidence rather than as a full bioinformatics study, reported disulfide-bond information was summarized together with sequence-derived cysteine numbers and potential N-glycosylation motifs. This analysis was used only as supportive structural evidence for interpretation of the heating results and was not intended as a direct simulation of thermal denaturation behavior.

2.2.6 Data Processing and Statistical Analysis

All experimental data are expressed as mean ± standard deviation (mean ± SD). For the effects of different household cooking methods on the relative content of OVT in the ELISA-detectable soluble fraction of egg white, one-way analysis of variance (one-way ANOVA) followed by Tukey's multiple comparison test was used to assess the significance of differences between groups, with $P < 0.05$ considered statistically significant. For the changes in the relative content of ELISA-detectable soluble OVT under gradient heat treatments, two-way analysis of variance (two-way ANOVA) followed by Tukey's multiple comparison test was performed to evaluate main effects, interaction effects, and simple effects, with $P < 0.05$ considered statistically significant. The analytical purpose of the present workflow was comparative evaluation across treatment groups under a standardized extraction-to-assay procedure rather than full formal validation of absolute recovery across all

cooked matrices. Accordingly, the resulting values should be interpreted as matrix-aware comparative measurements of the ELISA-detectable soluble fraction. All statistical analyses and graphical representations in this study were completed using Microsoft Excel 2019, GraphPad Prism 9.5, and Microsoft PowerPoint 2019.

2.2.7 Human and Animal Rights Statement

This article does not contain any studies with human participants or live vertebrate animals performed by any of the authors. Fresh hen eggs were purchased from a commercial retailer and used as food materials.

3 Results

3.1 Effects of Different Home Cooking Methods on the Relative Content of OVT in the ELISA-Detectable Soluble Fraction of Egg White

This study investigated the effects of different household cooking methods on the relative content of ovotransferrin (OVT) in the ELISA-detectable soluble fraction of egg white firstly. Significant differences were observed among the different cooking methods ($P < 0.05$) (Fig. 1). Raw egg white exhibited the highest relative OVT content in the ELISA-detectable soluble fraction, which was significantly higher than that of all heat-treated groups ($P < 0.05$). No significant differences were found among the boiled, fried, steamed, and egg custard groups ($P > 0.05$), and the values in all these groups were significantly lower than that in the raw egg white group.

The highest relative OVT content in the ELISA-detectable soluble fraction of raw egg white can be attributed to the native state of the egg-white matrix without heating, in which major proteins remain in their naturally folded soluble conformation. Under these conditions, OVT remains in the soluble extract and its antigenic epitopes are readily accessible, enabling accurate immunoassay-based quantification. In contrast, after household cooking treatments such as boiling, steaming, custard preparation, and frying, the relative OVT content in the ELISA-detectable soluble fraction decreased significantly. This reduction is more reasonably explained by heat-induced denaturation, aggregation, and structural rearrangement of thermosensitive proteins in the egg-white matrix, together with conformational changes in OVT itself or altered heteroprotein interactions, rather than by direct evidence of absolute OVT depletion [20, 28–30].

The absence of significant differences among the various fully cooked groups suggests that, under the conventional fully cooked household conditions established in this study, different heating media and cooking modes exert broadly comparable effects on the relative OVT content in the ELISA-detectable soluble fraction of egg white. A measurable proportion of ELISA-detectable soluble OVT was retained across all household cooking treatments. These findings provide practical evidence for evaluating OVT retention behavior in cooked egg products prepared under household conditions.

3.2 Effects of Gradient Heat Stress on the Relative Content of OVT in the ELISA-Detectable Soluble Fraction of Egg White

A completely randomized factorial design was employed to systematically investigate the effects of heat stress conditions on the relative content of OVT in the ELISA-detectable soluble fraction of egg white, using

four treatment temperatures (25, 60, 75 and 95°C) and three holding times (0, 10, and 20 min). The results are presented in Fig. 2 Two-way analysis of variance revealed that the main effect of temperature on the relative content of ELISA-detectable soluble OVT was highly significant ($F(3,60) = 5468$, $P < 0.0001$), accounting for 86.22% of the total variation and establishing temperature as the dominant factor. The main effect of treatment time was also highly significant ($F(2,60) = 413.6$, $P < 0.0001$), and a highly significant temperature $\times$ time interaction was observed ($F(6,60) = 289.0$, $P < 0.0001$). The underlying rationale for this statistical pattern is consistent with differences in thermal behavior between OVT and other major egg-white proteins. As an iron-binding glycoprotein of the transferrin family, OVT shows relatively high thermal robustness compared with more heat-labile proteins such as ovalbumin [17–19], so temperature acts as the primary driver of changes in the ELISA-detectable soluble fraction, whereas the effect of holding time becomes evident only after the relevant denaturation threshold is approached or exceeded.

Simple-effect tests for different treatment times at each fixed temperature were consistent with the thermal response of egg-white proteins. At 25°C, no significant differences were observed in the relative content of ELISA-detectable soluble OVT across 0–20 min ($P > 0.05$), because major egg-white proteins remained in a native soluble state and neither OVT epitope accessibility nor the soluble-phase distribution changed appreciably. At 60°C, the relative OVT content in the ELISA-detectable soluble fraction increased significantly with treatment time, and pairwise differences between all three time points were significant ($P < 0.05$). At 75°C, a similar increasing trend was observed; the 0 min group was significantly lower than the 10 min and 20 min groups ($P < 0.05$), whereas no significant difference was found between the 10 min and 20 min groups ($P > 0.05$). Because 60–75°C lies within the critical denaturation range of heat-labile egg-white proteins, prolonged heating preferentially promotes denaturation and insolubilization of those proteins, while a larger proportion of OVT remains measurable in the soluble extract; dissociation of native protein associations may also improve the accessibility of OVT epitopes to the sandwich ELISA [28–31].

Under high-temperature treatment at 95°C, no significant difference was observed between the 0 min and 10 min groups ($P > 0.05$). However, when the treatment time was extended to 20 min, the relative content of OVT in the ELISA-detectable soluble fraction was significantly higher than that in both the 0 min and 10 min groups ($P < 0.001$). This phenomenon is more appropriately interpreted as the consequence of extensive denaturation, aggregation, and precipitation of non-OVT proteins from the soluble phase, which results in relative enrichment of ELISA-detectable soluble OVT in the measured extract. Within the initial 0–10 min period, this process had already progressed substantially, so the measurable signal changed little; after 20 min, further removal of other proteins from the soluble phase likely increased the relative contribution of OVT even more. Accordingly, the higher signal at 95°C should not be regarded as evidence of de novo OVT generation, but as matrix-dependent relative enrichment shaped by differential protein thermal behavior.

4 Discussion

The matrix-adapted pretreatment developed in this study is central to the interpretation of the ELISA results. Existing extraction strategies for egg-white proteins are often designed for relatively simple or weakly structured systems [21, 22], whereas raw egg white, heat-induced gels, and fried high-fat matrices differ substantially in rheology, water distribution, and extractability. By matching chopping degree, vortexing, sonication, defatting, and low-temperature extraction conditions to each matrix type, the present workflow

improved the analytical comparability of the ELISA-detectable soluble fraction across differently cooked samples without changing the underlying measured signal.

Taken together, the household-cooking and gradient-heating experiments indicate that the ELISA signal should be interpreted as the relative content of OVT in the ELISA-detectable soluble fraction, not as direct evidence of absolute OVT abundance in the whole egg-white matrix. Raw egg white yielded the highest signal because major proteins remained soluble and OVT epitopes were readily accessible. With progressive heating, especially once the denaturation range of more heat-labile egg-white proteins was reached, matrix-dependent extraction, selective insolubilization of competing proteins, and changes in epitope accessibility jointly reshaped the detectable soluble fraction.

Unlike purified protein systems, the native egg-white matrix contains multiple proteins with different structural constraints and thermal behaviors. Denaturation, co-aggregation, and precipitation of non-OVT proteins can alter both the composition of the soluble extract and the immunochemical accessibility of OVT. Accordingly, the relatively high signal observed after severe heating is best understood as relative enrichment of ELISA-detectable soluble OVT in the measurable extract, driven by differential thermal behavior of egg-white proteins together with matrix-dependent extraction efficiency, rather than as proof of an absolute increase in OVT [20, 28, 29].

4.1 Sequence- and structure-based rationale for the differential thermal behavior of major egg-white proteins

To further support the interpretation of the heating results, a lightweight sequence- and structure-based comparison was conducted for major egg-white proteins, including OVT, ovalbumin, lysozyme C, and ovomucoid. As summarized in Tables 2 and 3 and Fig. 3, these proteins differ markedly in molecular size, disulfide-bond pattern, potential N-glycosylation motifs, and overall structural organization. In particular, OVT shows a bilobal iron-binding glycoprotein architecture, whereas ovalbumin represents a structurally distinct major storage protein in egg white; lysozyme C is a compact disulfide-constrained globular protein, and ovomucoid is a disulfide-rich domain-based inhibitor. These differences provide structural context for why major egg-white proteins do not respond uniformly to heating and extraction.

Notably, the present sequence- and structure-based comparison should be regarded as supportive evidence rather than direct proof of thermal stability under the current experimental conditions. When interpreted together with the ELISA results, however, these structural features support a cautious mechanism in which, at 60–75°C, preferential denaturation and insolubilization of more heat-labile proteins progressively increase the relative contribution of OVT to the measurable soluble fraction, whereas at 95°C, further depletion of non-OVT proteins from the soluble phase promotes stronger relative enrichment of ELISA-detectable soluble OVT in the measurable extract. Because the present study did not directly determine the absolute recovery of each individual protein after heating, these interpretations should remain mechanistic but conservative.

Table 2

Sequence-derived physicochemical features of major egg-white proteins potentially related to their differential behavior during heating

Protein	UniProtKB	Length (aa)	MW (kDa)	pI	Instability index	Aliphatic index	GRAVY	References
Ovotransferrin (OVT)	P02789	705	77.78	6.85	35.85	73.87	-0.364	[23, 24]
Ovalbumin (OVA)	P01012	386	42.88	5.19	37.11	89.95	-0.001	[23, 24]
Lysozyme C (LYZ)	P00698	147	16.24	9.36	19.86	81.70	-0.150	[23, 24]
Ovomucoid (OVM)	P01005	210	22.59	4.75	30.67	63.57	-0.208	[23, 24]
Note: Sequence length, molecular weight, theoretical pI, instability index, aliphatic index, and GRAVY were calculated from reviewed UniProtKB sequences using ExPASy ProtParam-based sequence analysis [23, 24].								

Table 3

Structural features of major egg-white proteins and possible implications for the detectable soluble fraction after heating

Protein	Cys residues (n)	Annotated / reported disulfide bonds (n)	Potential N- glycosylation motifs (n)	Representative structural features	Possible implication	References
OVT	32	15	2	Bilobal iron-binding glycoprotein; representative metal-binding regions and structurally stabilized lobes	May partially explain the relatively high proportion of ELISA-detectable soluble OVT after heating	[19, 23, 25, 26, 32]
OVA	6	1	2	Major egg-white storage protein with a compact single-chain architecture distinct from OVT	Structurally consistent with a different heat response and contribution to matrix aggregation	[20, 23, 25, 26, 30]
LYZ	9	4	0	Small compact globular protein with multiple stabilizing disulfide bonds	Indicates that proteins with different architectures may show distinct thermal and solubility behaviors	[23, 25, 26]
OVM	19	9	6	Disulfide-rich protease inhibitor with domain-based organization	Supports the idea that the behavior of non-OVT proteins under heating depends on structural constraints	[21–23, 25, 26]
Note: Cysteine residues and potential N-glycosylation motifs were derived from reviewed UniProtKB sequences [23], whereas annotated / reported disulfide bonds and representative structural features were compiled from reviewed UniProtKB annotations and publicly available structural resources, including the PDB and AlphaFold DB, together with the literature [19–23, 25, 26, 30, 32]. The “Possible implication” column reflects interpretation based on sequence/structure characteristics together with the present experimental results and should not be regarded as direct proof of thermal denaturation behavior.						

Representative structures and sequence-derived features of major egg-white proteins, including OVT, ovalbumin, lysozyme C, and ovomucoid, were compared to provide supportive structural evidence for interpretation of the heating results. The figure highlights the bilobal architecture and representative metal-binding regions of OVT, together with the distinct structural organizations of ovalbumin, lysozyme C, and

ovomucoid. These differences are consistent with the view that major egg-white proteins exhibit different thermal responses and contribute differently to the measurable soluble fraction after heating. This figure is intended to support interpretation of the experimental data rather than to serve as direct evidence of thermal denaturation behavior.

4.2 Proposed mechanism underlying the higher ELISA signal after heating

The apparent increase in OVT observed under some heat treatment conditions should be interpreted cautiously. Based on the present results, the sandwich ELISA readout more appropriately reflects the relative content of OVT in the ELISA-detectable soluble fraction than the absolute amount of OVT in the whole egg-white sample. Under raw conditions, major egg-white proteins remain largely soluble, and OVT epitopes are readily accessible for antibody recognition. When the temperature reaches 60–75°C, more heat-labile proteins preferentially denature and aggregate, which reduces their contribution to the soluble fraction and increases the relative contribution of OVT to the measurable extract. At 95°C, depletion of non-OVT proteins from the soluble phase becomes more extensive, while a measurable proportion of OVT remains detectable after matrix-adapted pretreatment; consequently, the measured signal is further shifted toward relative enrichment of ELISA-detectable soluble OVT.

This interpretation indicates that the higher signal observed at severe heating should not be taken as evidence of de novo OVT generation or a demonstrated absolute increase in OVT. Instead, it reflects the combined effects of differential thermal behavior of egg-white proteins, matrix-dependent extraction efficiency, and epitope accessibility. Framed in this way, the present workflow provides an analytically comparable approach for discussing OVT behavior across raw, gelled, and high-fat cooked egg-white systems while acknowledging that the measured outcome remains extract- and assay-dependent. The proposed interpretive framework for the relative increase in ELISA-detectable soluble OVT after heating is summarized in Fig. 4.

Under raw conditions, major egg-white proteins remain largely soluble and OVT epitopes are accessible. At 60–75°C, heat-labile proteins preferentially denature and aggregate, increasing the relative proportion of OVT in the measurable soluble fraction. At 95°C, more non-OVT proteins precipitate from the soluble phase, resulting in relative enrichment of OVT in the extract. In combination with matrix-adapted pretreatment and extraction, sandwich ELISA therefore reflects the relative abundance of detectable soluble OVT rather than absolute generation of OVT.

5 Conclusion

In this study, matrix-adapted pretreatment combined with sandwich ELISA was used to evaluate changes in OVT in the ELISA-detectable soluble fraction of egg white subjected to representative household cooking methods and gradient heat treatment. Fully cooked samples, including boiled, steamed, fried, and egg-custard preparations, showed significantly lower relative OVT content in the ELISA-detectable soluble fraction than raw egg white, whereas no significant differences were observed among the four cooked groups.

The gradient-heating experiment showed that temperature was the dominant factor affecting the analytical signal ($P < 0.0001$), while holding time and the temperature-time interaction were also highly significant ($P <$

0.0001). The higher signal observed under more severe heating is most appropriately interpreted as relative enrichment of ELISA-detectable soluble OVT caused by preferential denaturation and precipitation of more heat-labile egg-white proteins, together with matrix-dependent extraction and epitope accessibility effects, rather than as an absolute increase in total OVT. These findings highlight the importance of matrix-aware pretreatment for realistic cooked systems and provide practically relevant evidence for egg-quality assessment and thermal-processing optimization.

Declarations

Conflict of interest

The authors declare that they have no conflict of interest.

Clinical trial number

Not applicable.

Ethical Statement

Not applicable.

This study used commercially purchased shell eggs and did not involve human participants, identifiable human data, or live vertebrate animals. Formal ethics approval, informed consent to participate, and consent for publication were therefore not required.

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Author Contribution

Jingyi Zhang: Investigation, methodology, data curation, writing - original draft. Anjia Huang: Methodology, software, visualization. Shujie Chen: Software, validation. Chenggang Cai: Conceptualization, supervision, writing - review and editing. Dongrong Zhu: Resources, supervision. All authors read and approved the final manuscript.

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Data availability

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Figures

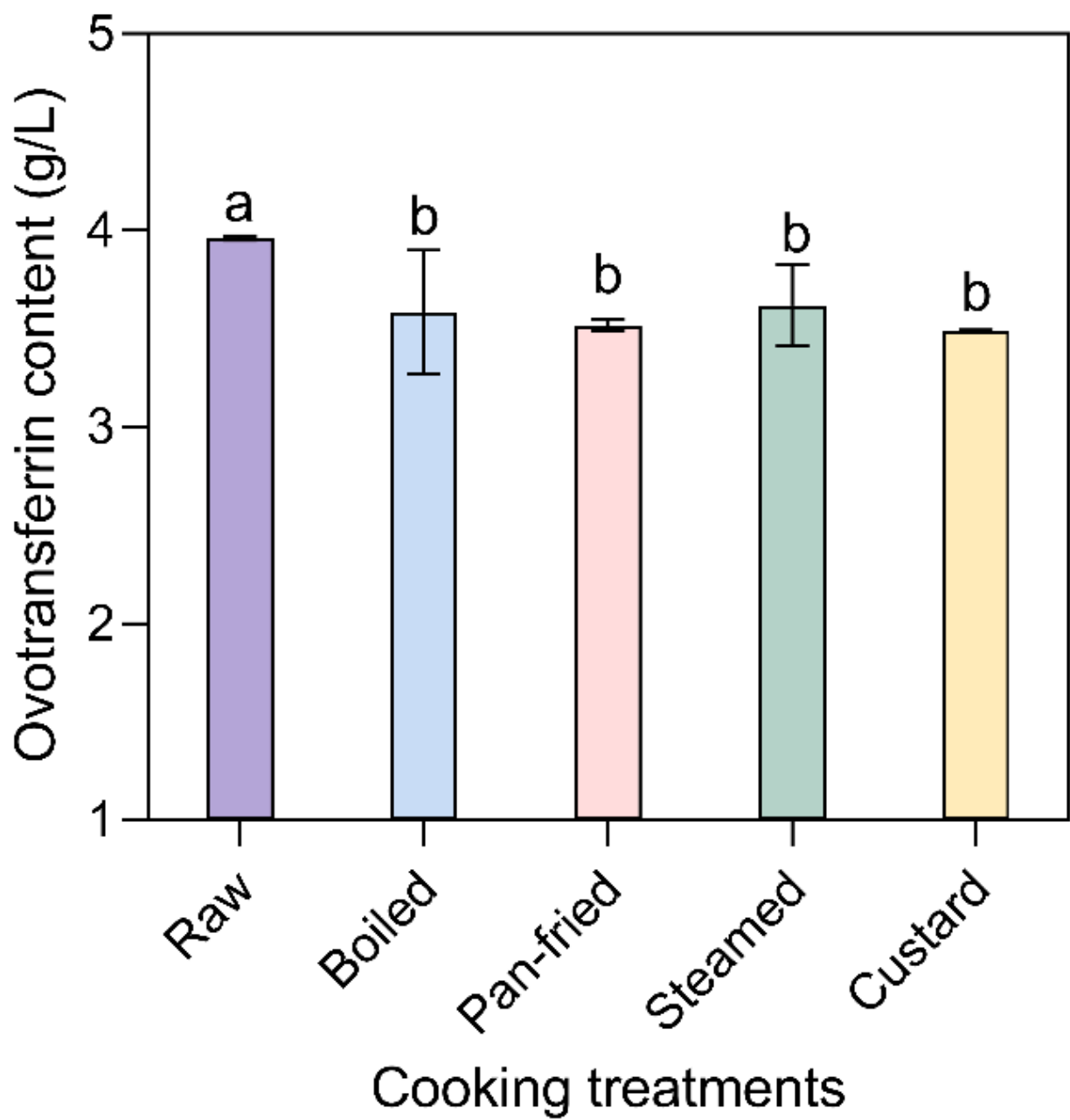


Figure 1

Effects of different home cooking methods on the relative content of OVT in the ELISA-detectable soluble fraction of egg white

Note: Different lowercase letters indicate statistically significant differences between groups (one-way ANOVA followed by Tukey's multiple comparison test, $P < 0.05$), while the same letter indicates no significant difference ($P > 0.05$). Data are presented as mean $\pm$ standard deviation, $n = 3$.

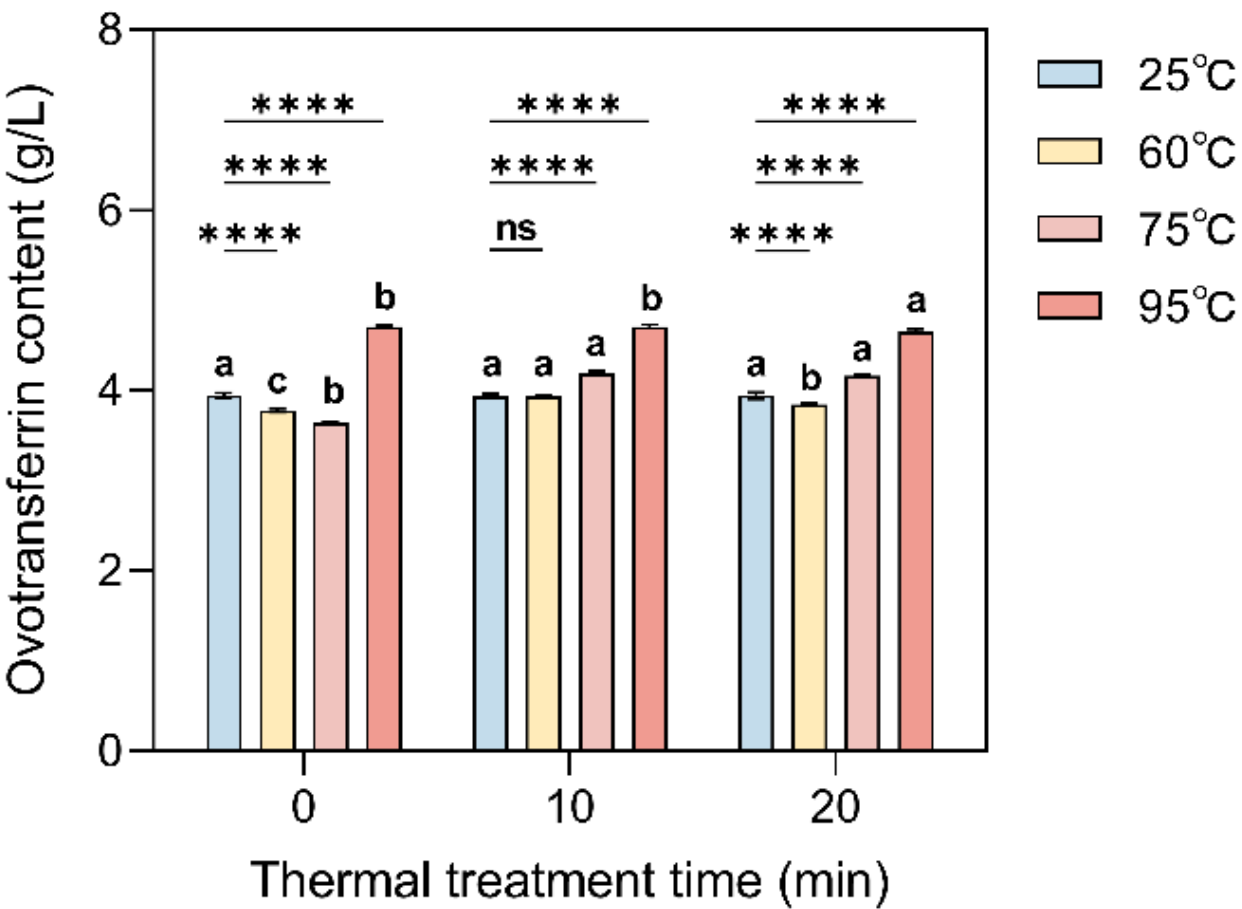
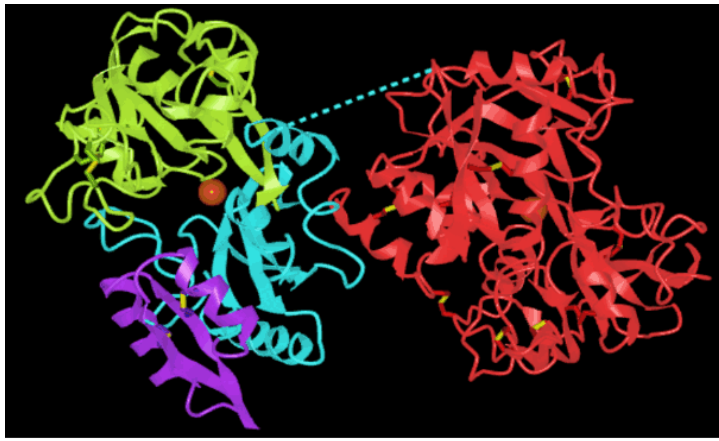


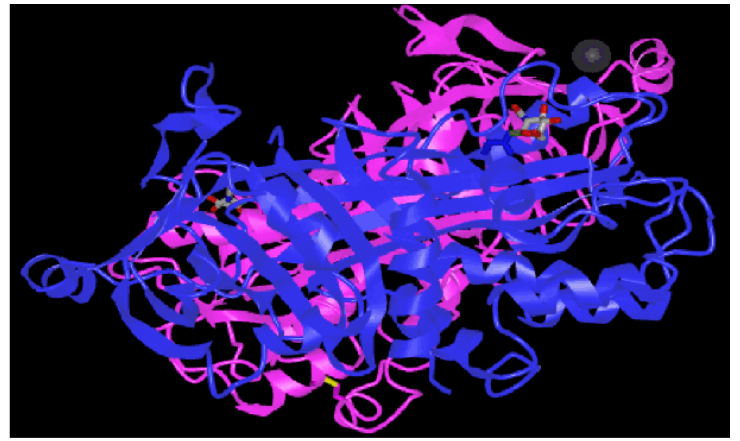
Figure 2

Effects of different gradient heat stress conditions on the relative content of OVT in the ELISA-detectable soluble fraction of egg white

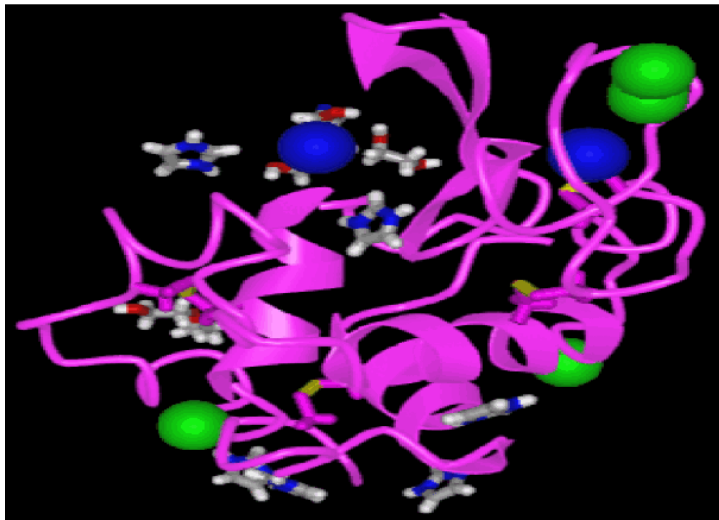
Note: Under the same heat treatment temperature, different lowercase letters indicate statistically significant differences in the relative content of OVT in the ELISA-detectable soluble fraction among groups with different treatment durations (two-way ANOVA followed by Tukey's multiple comparison test, $P < 0.05$), whereas groups sharing the same letter show no statistically significant difference. Under the same treatment duration, **** indicates a highly significant difference compared with the 25°C control group at the corresponding duration ($P < 0.0001$), and ns indicates no statistically significant difference between the two groups ($P > 0.05$). Data are presented as mean $\pm$ standard deviation, $n = 3$.



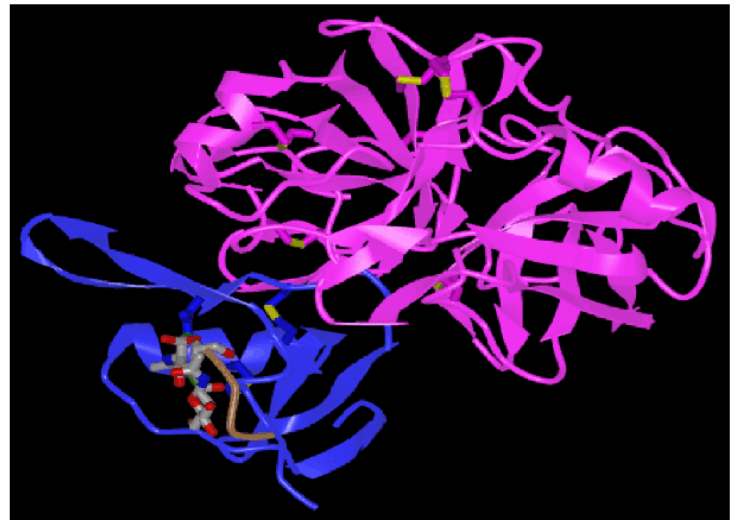
OVT



OVA



LYZ



OVM

Figure 3

Sequence- and structure-based comparison of major egg-white proteins (from *Gallus gallus*) related to their differential behavior during heating

Note: **OVT**, PDB ID 8FEI: CryoEM structure of Conalbumin from chicken egg white (sigma-Cas 1391-06-6); **OVA**, PDB ID 1OVA: Crystal Structure of Uncleaved Ovalbumin at 1.95 Angstroms Resolution; **LYZ**, PDB ID 9H7W: Hen Egg White Lysozyme in Complex with Imidazole; **OVM**, PDB ID 1Z7K: Crystal Structure of Trypsin-Ovomucoid turkey egg white inhibitor complex.

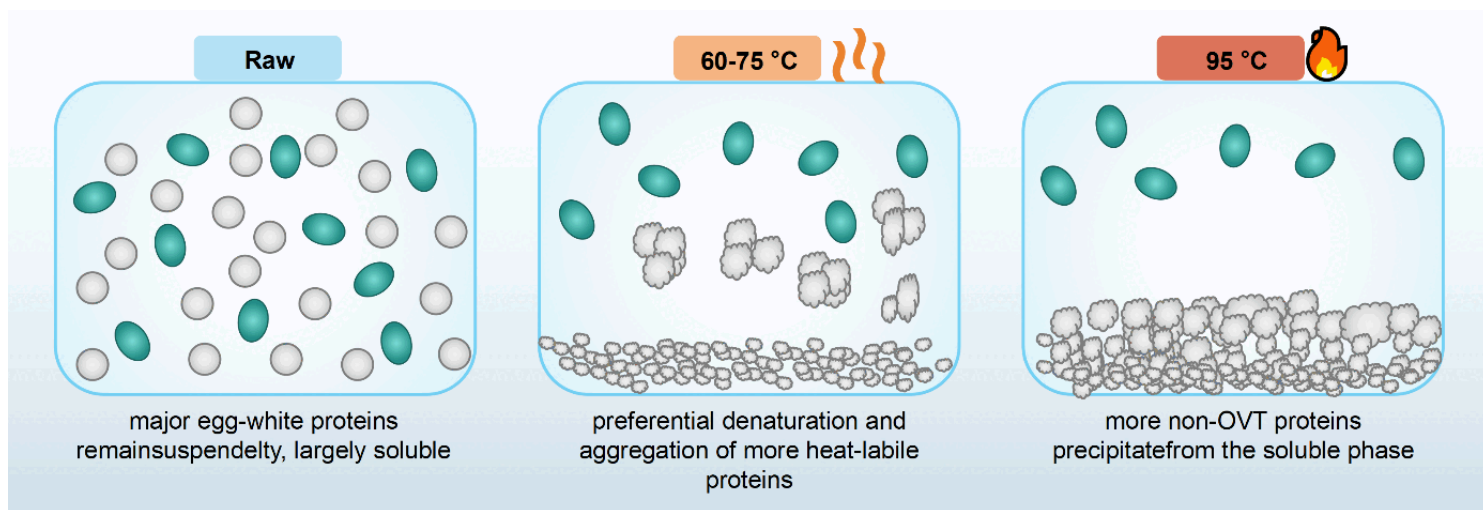


Figure 4

Proposed mechanism underlying the relative increase in ELISA-detectable soluble OVT after heating

Supplementary Files

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