Food Analytical Methods (2021) 14:1806–1814
https://doi.org/10.1007/s12161-021-02012-5

Development of a Sandwich Enzyme-linked Immunosorbent Assay (ELISA) for the Detection of Egg Residues in Processed Food Products

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Received: 17 November 2020 / Accepted: 4 March 2021 / Published online: 20 March 2021

Abstract

Food allergy has become an important food safety concern affecting people's health and quality of life. As one of the most common allergic foods, egg poses a safety hazard to susceptible individuals. Therefore, it is crucial to develop rapid and sensitive detection methods for allergen monitoring. Sandwich ELISA detection method was developed based on two polyclonal antibodies for the detection of egg white allergens in this study. Goat antisera and rabbit antisera are used as capture antibodies and detection antibodies, respectively. The detection limit of this ELISA detection method is 0.105 ng/mL with high specificity to egg white proteins, and the linear range is between 1 and 20 ng/mL. The mean recovery rate ranges from 70.27 to 101.95% and the mean coefficients of variation of intra-assay and inter-assay are within 2.73% and 9.19%, respectively. The sandwich ELISA detection method can be effectively used for the detection of egg proteins in foods on the market and will help reduce the incidence of egg allergy.

Keywords Egg white proteins · Sandwich ELISA · Food allergen · Detection

Introduction

Egg proteins are among the most important ingredients in food industry. What's more, eggs also contain some active ingredients with antibacterial, antiviral, immune regulation, and anticancer properties (Abeyrathne et al. 2014). For these reasons, nowadays egg proteins are playing an increasingly significant role in daily life.

However, as one of the eight major food allergens, egg is the second largest allergic food after milk in allergies found in children (Eggesbo et al. 2001; Vidal et al. 2005). In some western countries, egg allergy has even surpassed milk allergy to become the first allergic food. It can induce IgE-mediated allergic reactions with urticaria, dermatitis, and anaphylactic rhinitis. Occasionally severe egg allergic reactions can cause shock or even death. About 1–2% of infants are allergic to eggs worldwide (Savage et al. 2007; Dhanapala et al. 2015).

Egg allergy is mainly caused by several major allergenic proteins including ovalbumin (OVA), ovomucoid (OVM), ovotransferrin (OVT), and lysozyme (Lys). Studies have shown that these four major allergic proteins are all present in egg white (Hoffman 1983; Holen et al. 2001; Dubois et al. 2015) and their content accounts for 54%, 11%, 11–13%, and 3.5% of the total egg white protein respectively (Fu et al. 2010). Among these four allergen proteins, OVM has the strongest allergenicity, while OVA is the most abundant (Mine and Zhang 2002; Bartnikas et al. 2013).

The threshold for allergic reactions caused by contact with allergens is very low and currently there is no effective approach to completely eliminate allergic reactions (Kobernick and Burks 2016). In addition, the only most effective way to prevent food allergies is to completely avoid contact or intake of allergic proteins in daily life (Ewan and Clark 2001). However, with the progress of society, the degree of food processing has become more and more intensive, which has potentially increased the cross-contamination between different foods. This has increased the risk of unintentional intake of allergic components by allergic individuals. Hence, in order to effectively prevent the occurrence of allergies, it is very necessary to develop a detection method with high sensitivity, specificity, reproducibility, and for allergen monitoring.

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Currently, the commonly used methods for detecting allergens include mass spectrometry, immunoblotting, biosensor, and ELISA. Although mass spectrometry has high sensitivity, it is expensive and time-consuming. Biosensor method requires high sample preparation. Western blotting method has low sensitivity and cannot be quantitatively detected. However, ELISA detection method has been preferred due to the advantages it possesses such as high sensitivity, specificity, reliable detect method, low detection cost, and ease of use and versatility (Monaci and Visconti 2010; Nguyen et al. 2019).

In previous studies, ELISA detection method was used to detect egg allergens where the monoclonal antibodies were often established based on an allergenic protein component in eggs. For example, Juan Peng et al. (2014) established a monoclonal antibody-based sELISA method to detect OVA in food. Similarly, Vidal et al. (2005) also established an indirect ELISA method based on monoclonal antibodies to detect egg white lysozyme in food. Although monoclonal antibodies can effectively select a single epitope and have the advantage of high specificity. But eggs contain 4 major allergic proteins, a specific epitope of these allergen proteins is destroyed or hidden easily during food processing, which may lead to false positives. In that case, using a polyclonal antibody-based ELISA detection method can effectively compensate for this shortcoming.

In the present study, we attempted to use 4 mixed egg white target proteins as immunogen to immunize animals to obtain polyclonal antibodies goat anti-egg white proteins IgG (goat antiserum) and rabbit anti-egg white proteins IgG (rabbit antiserum) instead of traditional monoclonal antibodies, and developed a sandwich ELISA for the detection of egg allergens with high sensitivity.

Materials and Methods

Materials and Reagents

The goat antiserum was purchased from Puluojin company (Wuhan, China) and the rabbit antiserum was obtained from Huada Innovation (Beijing, China). Horseradish peroxidase (HRP)–conjugated goat anti-rabbit IgG antibodies were obtained from Baiaotong company (Luoyang, China), while single-component substrate solution and TMB (3,3′,5,5′-tetramethylbenzidine) was obtained from Solarbio Co., China. All other chemicals were of analytical grades and food samples were purchased from local supermarkets in Qingdao.

Preparation of the Egg White Proteins

The egg white proteins were extracted from the fresh egg white following the published procedure and a slight improvement (Bernhiselbroadbent et al. 1994; Abeyrathne et al. 2014). Briefly, 4 egg white target allergen proteins OVA, OVM, OVT, and Lys were extracted from fresh chicken egg. The extraction process was divided into two steps: First, after manually isolated from fresh eggs, the egg white was homogenized with more than 5 volumes of pre-cooled acetone and stirred at room temperature for 24 h to remove fat and pigments. Then, the egg white powder was homogenized with 10 volumes of extraction buffer (0.01 M PBS, pH 7.4) at room temperature for 24 h and centrifuged at 10,000 rpm for 20 min. After the acetone has completely evaporated, the supernatant was stored at −20 °C for the subsequent steps. Secondly, the separated egg white was gently stirred with 2 volumes of extraction buffer for 1 h to destroy the ingredients in the OVA package. Then, the pH was adjusted to 6.0 for the isoelectric precipitation to remove other proteins, then followed the centrifugation steps described previously to get the supernatant. The crude protein obtained in these two steps was adjusted to the same concentration and then mixed in a suitable volume, which was the 4 mixed egg white target proteins.

Production of Rabbit and Goat Polyclonal IgG Antibodies

Goat and rabbit were used to generate IgG antiserum from the desired egg white proteins. The rabbit antiserum was produced by immunizing New Zealand rabbit with egg white proteins with Freund's adjuvant. After the first immunization and three booster immunizations, whole blood sample was taken and the rabbit antiserum was separated. The interval between immunizations was done for two weeks. The goat antiserum was produced by immunizing Boer goat with Freund's adjuvant in the first immunization. After four booster immunizations, whole blood sample was taken and the goat antiserum was isolated. (All animal experiments were performed following established guidelines and the experimental protocol in accordance with the Ethical Committee of Experimental Animal Care at Ocean University of China (OUC, China). No:SPXK20150001.)

Evaluation of Rabbit and Goat Polyclonal Antibodies

The specificity of polyclonal antibodies was assessed by Western blot and indirect competitive ELISA using various crude egg white protein and poultry meat derived from different avian sources.

SDS-PAGE

The proteins samples were analyzed by SDS-PAGE according to the method described by Laemmli (1970). A crude egg white protein extract of various poultry eggs (−6 μg of protein) was mixed with a loading buffer and heat-denatured for 7

min. After cooling, the samples were added in 5% stacking gel and 12% running gel. The electrophoresis conditions were performed at 80 V for 30 min and then at 120 V until the end. Coomassie Brilliant Blue G-250 was used to stain the electrophoresis gel and after decolorization, the electrophoresis gel was imaged using BIO-RAD gel imaging and analysis software (Tanon Science and Technology Co., Ltd., Shanghai, P. R. China).

Western Blot

The corresponding samples were separated by the abovementioned SDS-PAGE method and the pre-run gel was transferred to a polyvinylidene fluoride (PVDF) membrane by 2 Gel Transfer Device electrophoresis at room temperature for 7 min. The membrane was washed with an iBind solution kit. Then, it was incubated with rabbit antiserum (1:10000 dilution) or goat antiserum (1:10000 dilution) in an iBind western device as the primary antibody. Thereafter, HPR conjugated goat anti-rabbit IgG antibody (1:10000 dilution) or HPR conjugated rabbit anti-goat IgG antibody (1:10000 dilution) was added and kept at room temperature for 5 h. After washing with deionized water, the results were observed using a Western blot chemiluminescence substrate. The PVDF film was exposed for multiple exposure times and the images were captured by imaging systems BIO-ROD gel imaging and analysis software (Tanon Science and Technology Co., Ltd., Shanghai, P. R. China).

Indirect Competitive ELISA

Wells were coated with 100 μL well^{-1} egg white protein (20 μg/mL) in coating buffer (50 mm sodium carbonate buffer, pH 9.6) and incubated overnight at 4 °C. After washing 3 times, the wells were blocked with 150 μL of blocking solution (PBST with 2% BSA) at 37 °C for 2 h. After washing, a mixture (90 μL diluted antiserum with 10 μL samples) that has been incubated at 37 °C for 10 min was added to and incubated at 37 °C for 1 h. Then, 100 μL of HRP-labeled goat anti-rabbit diluted antibody (1:15000) or HRP-labeled rabbit anti-goat was added to each well for 1 h at 37 °C. After washing the plate, 100 μL of TMB was added to each well to develop color at 37 °C for 10 min and the reaction was stopped by adding 50 μL of 2 M sulfuric acid. The absorbance was measured at $OD_{450\text{-}630\ nm}$.

Procedures for the Development of Sandwich ELISA

Microwell plates were coated with 100 μL well^{-1} capture antibody (1:20,000 dilution of goat antisera) in coating buffer (50 mm sodium carbonate buffer, pH 9.6) and incubated overnight at 4 °C. The plate was washed 3 times with PBST (0.01 M PBS containing 0.05% Tween-20, pH 7.4) for 5 min (the next washing steps were performed as same). After washing, the plate was blocked with 150 μL of blocking solution (PBST with 2% BSA) at 37 °C for 2 h. Crude egg white extracts with different concentration gradients (PBS as blank control sample) were incubated at 37 °C for 1 h. After washing the plate, 100 μL of 1:10000 rabbit antiserum was added to each well and incubated at 37 °C for 1 h. Then, 100 μL of HRP-labeled goat anti-rabbit diluted antibody (1:15000) was added to each well for 1 h at 37 °C. After washing the plate, 100 μL of TMB was added to each well to develop color at 37 °C for 10 min and the reaction was stopped by adding 50 μL of 2 M sulfuric acid. The absorbance was measured at $OD_{450\text{-}630\ nm}$.

Evaluation of the Performance of Sandwich ELISA

In order to determine the sensitivity of the sandwich ELISA method, a detection standard curve for the method was prepared. When preparing the standard curve, the extracted egg white proteins were diluted with buffer (0.01 M PBS, pH 7.4) to make the final concentrations of egg white proteins (0, 0.5, 1, 2.5, 5,10, 15, and 20 ng/mL). The PBS buffer solution (0.01 M, pH 7.4) was used instead of the egg white immunogen as a blank control group, then measured by the sandwich ELISA method established. The measured values were calculated with reference to previous studies (Segura-Gil et al. 2019).

The precision of the method was estimated by the intra-assay and inter-assay variations (Hsin-Yi et al. 2019). The egg white proteins at different concentrations were measured in 6 replicates to calculate the precision of the intra-assay. Each group of samples was divided into 5 groups every day and the measurement was performed for 6 consecutive days to calculate the precision of the inter-assay.

The specificity of the method is reflected by cross-reaction. Three common crude extracts of poultry eggs (duck, goose, and quail eggs), 2 common crude extracts of poultry meat (chicken and duck), and other 25 common crude extracts of food were selected to perform cross-reaction test.

The recovery of the method was performed to determine the effect of matrix. Recovery due to matrix effect was estimated as the ratio of egg white proteins concentration gained from non-containing egg model foods and extraction buffer. Compressed biscuits, black sesame paste, and dove chocolate were selected as model foods.

Detection for Egg White Proteins Matrix Interference

All solid food sample was extracted according to the procedure described in the literature (Peng et al. 2014). In short, 1 g of compressed biscuits, black sesame paste, and dove chocolate were crushed and homogenized in 9.5 mL of PBS (0.01 M, pH 7.4) containing 0.5 mL egg proteins solution. After

taking homogenate for 12 h at 4 °C, the supernatant was centrifuged at 8000 *g* for 10 min and then collected as the food sample solution. Then, the egg white proteins were added to the matrix extract with a final concentration of 0.01, 0.10, and 0.2 μg/mL. Finally, the solution was determined by the established sandwich ELISA method.

Egg White Proteins Detection in Real Food Sample

Through the establishment of the sandwich ELISA, 10 real food samples were analyzed, which were divided into three categories: egg-containing products, may egg-containing product, and egg-free products. The extraction steps for these food samples were the same as described previously. Each sample was analyzed by the developed sandwich ELISA method.

Results and Discussion

Extraction of 4 Egg White Allergen Proteins

A vital requirement of the correct steps of allergen analysis in egg white is to get complete and unaltered protein extracts. In order to obtain whole allergen proteins in egg white, two different egg white pre-treatment methods were used in this study. Figure 1a shows the electrophoretic profile of egg white proteins. Whole proteins are mainly composed of OVA, OVM, OVT, and a small amount of Lys.

One-step extraction method was once used to complete the extraction of 4 kinds of egg white protein, but only one method of pre-processing the egg white to extract often caused the loss of some of the protein components, especially the loss of OVA, which is also the highest content of allergic protein in egg white. Therefore, in order to extract OVA, the two-step

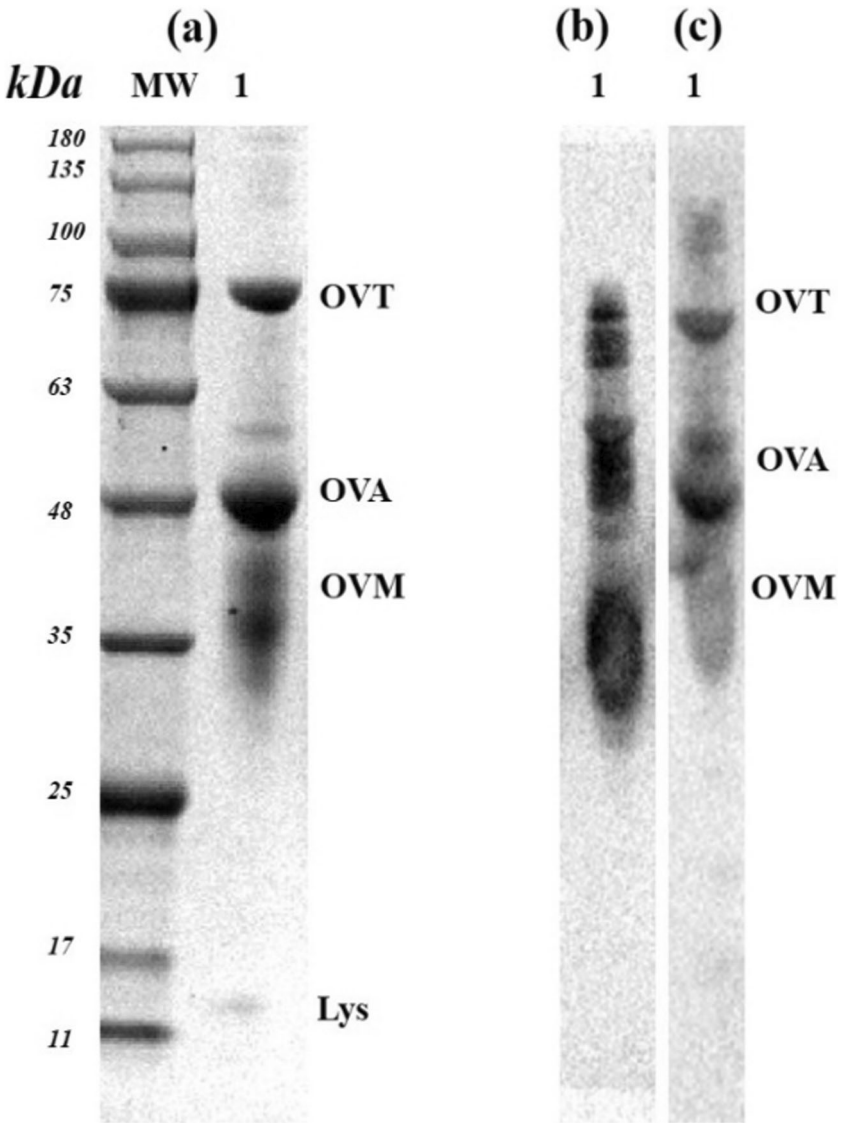

Fig. 1 SDS-PAGE in 5–12% acrylamide gel (a) and Western blotting against rabbit antiserum (b) and goat antiserum (c) to egg white proteins. MW, molecular weight marker. Lane 1, raw egg white extract

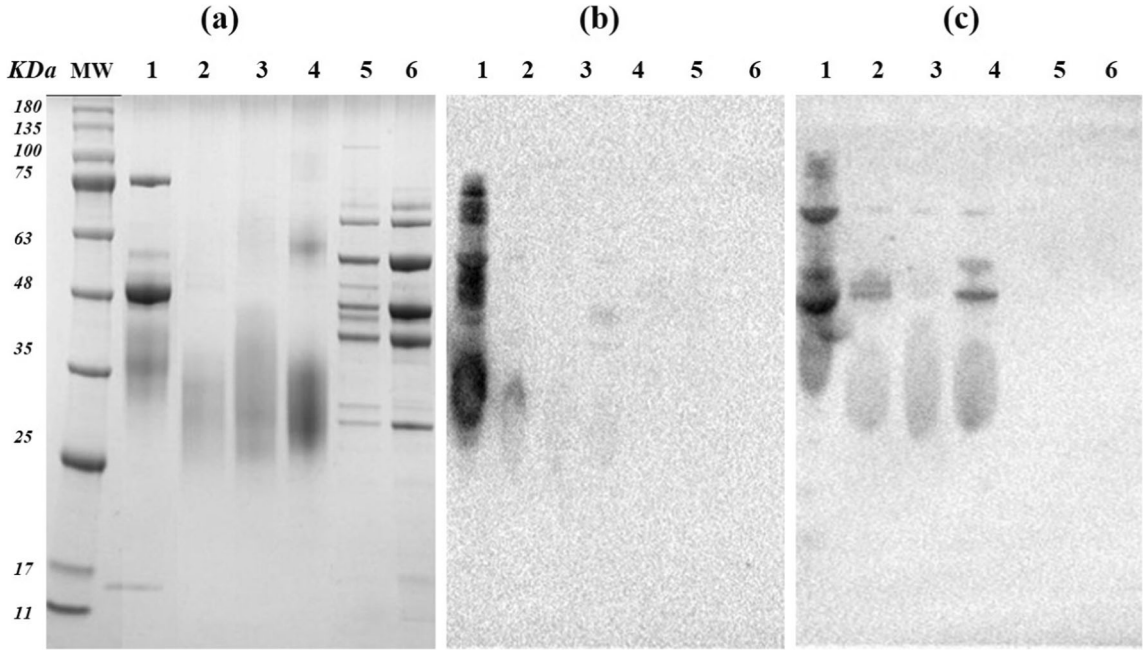

Fig. 2 SDS-PAGE in 5–12% acrylamide gel (a). Immunoreactivity and specificity of rabbit antiserum (b) and goat antiserum (c) from 5 different species. MW, molecular weight marker. Lane 1, raw egg white extract; lane 2, raw duck egg white extract; lane 3, raw goose egg white extract; lane 4, raw quail egg white extract; lane 5, raw chicken meat extract; lane 5, raw duck meat extract

method described earlier was adopted to extract the allergic protein component of whole egg white.

The reason behind the poor extraction of OVA after the egg white was processed by pre-cooling acetone might be due to two reasons: one is that some other components in the egg white wrap the OVA, and its extraction will be hindered if the wrapper is not removed; the other is that OVA may be tightly bound to the lipids in the egg white. At the time the lipids were removed, OVA was also dropped out by the way.

Titer and Specificity of Anti-egg White Proteins Antisera

In the immunological testing process, the accuracy of the testing method often depends on the quality of the antibody. Since the two antibodies in this study were prepared by different companies, they need to be evaluated as necessary.

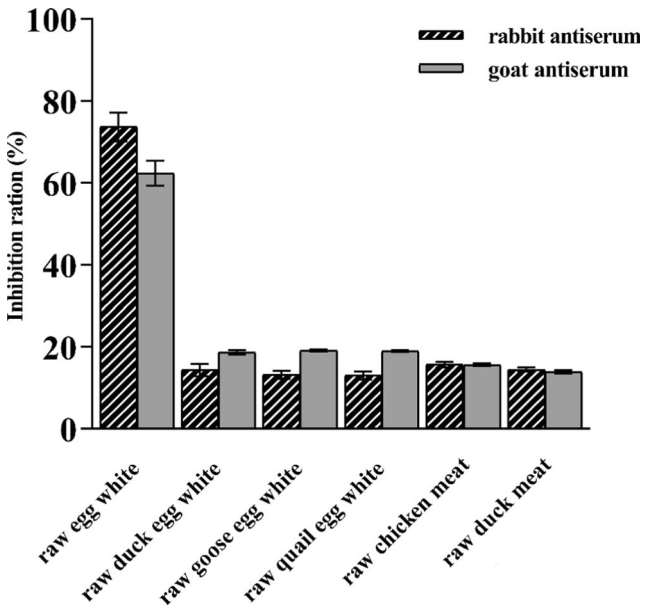

Fig. 3 Evaluation results of specificity of the antiserum by competitive ELISA

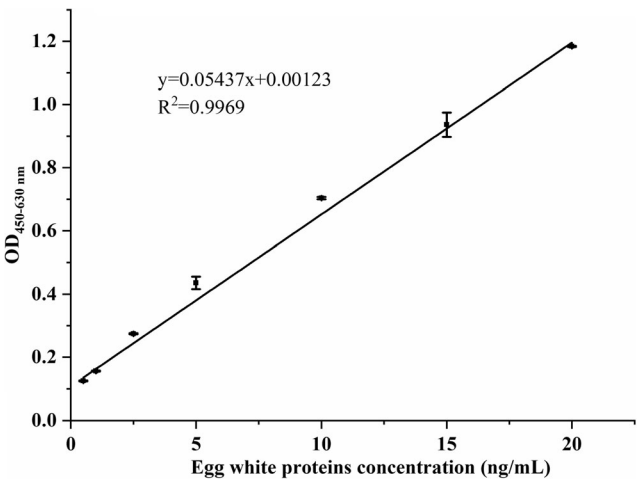

Fig. 4 Calibration curves obtained for the determination of egg white proteins by sandwich ELISA format. Standards were prepared with egg white proteins. Each data point represents the mean of 10 measurements of the absorbance at 450–630 nm

Table 1 Study of cross-reactivity using food commodities, analyzed by sandwich ELISA to determine egg white proteins. Results correspond to the average concentration of egg white proteins (μg/g food) from extraction analyzed in three assays

Egg white proteins (ng/g)	
Milk	< LOQ
Soybean	< LOQ
Wheat	< LOQ
Peanut	< LOQ
Hazel	< LOQ
Almond	< LOQ
Pistachio nuts	< LOQ
Walnut	< LOQ
Sesame	< LOQ
Fish	< LOQ
Shrimp	< LOQ
Peach	< LOQ
Tomato	< LOQ
Mango	< LOQ
Rice	< LOQ
Corn	< LOQ
Buckwheat	< LOQ
Gram	< LOQ
Ormosia hosiei	< LOQ
Pea	< LOQ
Oat	< LOQ
Barley	< LOQ
Rye	< LOQ
Pork	< LOQ
Beef	< LOQ
Chicken meat	1.12
Duck meat	1.44
Duck egg	0.46
Goose egg	0.70
Quail egg	0.73

LOQ was 0.32 ng/mL

The titer of anti-egg white proteins antisera was determined by an indirect non-competitive ELISA. And the values of goat antisera range between 2.0^5 and 8.0^5, and the values of rabbit antisera range between 4.0^5 and 16.0^5 depending on the bleeding.

The immunoreactivity of anti-egg white proteins antisera were determined by Western blotting (Fig. 1b and c). The OVT, OVA, and OVM in the electrophoresis band are the main recognition targets of the two antisera, and the recognition strength of the rabbit antiserum is better than that of the goat.

Various eggs and poultry proteins were examined to test the possible reactivity using the two antiserum. Figure 2a shows the protein profiles of extraction of 5 different kinds of proteins. Seen from the electrophoresis bands: three kinds of eggs contain bands similar to OVM, and two poultry meat contain bands similar to OVA, OVM, and Lys. As shown in Fig. 2b, the rabbit antiserum and other proteins have little binding. Figure 2c indicates the binding ability of goat antiserum. Both rabbit and goat antiserum exhibited similar binding specificities, showing weak cross-reactivity with other eggs and poultry proteins.

In addition, a competitive ELISA experiment was used to evaluate the specificity of the antiserum. Standards of egg white proteins were assayed in a concentration of 1–100 ppm. The cross-reactivity was calculated as the concentration of egg white proteins required to produce 50% inhibition of antiserum binding compared to egg white proteins, this value being less than 20% (Fig. 3), which indicates that antisera exhibited a high specificity to detect egg white proteins.

Sandwich ELISA Standard Curve and for Detection Egg White Proteins

The sandwich ELISA combines goat antisera for coating and rabbit antisera for detection. The calibration curves (Fig. 4) were generated for egg white allergen concentrations in the range of 1–20 ng/mL and its correlation

Table 2 Results of intra- and inter-assay variances performed with the sandwich ELISA prototype. Values were analyzed in six assays

Egg white proteins (ng/mL)	Intra-assay		Inter-assay[a]	
	Mean±SD (ng/mL)	RSDr[b] (%)	Mean±SD (ng/mL)	RSDr[c] (%)
15	14.63±0.54	3.21	13.67±2.32	7.83
10	10.21±0.04	0.78	11.01±1.31	11.05
5	4.89±0.87	1.33	5.46±1.77	12.16
2.5	2.28±0.22	4.01	2.04±0.16	8.36
1	1.15±0.31	4.33	0.89±0.43	6.53

[a] Assay was performed every 2 days for 12 sequential days

[b] Relative standard deviation of repeatability for intra-assay precision

[c] Relative standard deviation of reproducibility for inter-assay precision

Table 3 Recovery of egg white proteins from three blank food samples spiked with standard concentration of egg white proteins by the sandwich ELISA prototype

Spiked level (mg/g)	Compressed biscuits		Black sesame paste		Dove chocolate	
	Mean recovery (%)	CV (%)	Mean recovery (%)	CV (%)	Mean recovery (%)	CV (%)
0.01	85.55	9.13	83.34	11.38	62.89	16.37
0.10	104.97	5.24	110.48	5.63	68.55	14.21
0.20	115.32	2.12	117.41	4.89	79.37	11.56

coefficient $r^2 > 0.99$. The lowest detection limit of the established ELISA is 0.105 ng/mL, and the limit of quantification was 0.164 ng/mL.

The LOD of the sandwich ELISA in this experiment was 0.105 ng/mL, indicating that its sensitivity is particularly high and it has very high predictability. As there is no relevant report on the allergen threshold in foods, there are some studies on the prediction of allergen thresholds worthy of reference: as shown in a study with predictive threshold, the protein dosage at which about 5% of allergic people is likely to cause their allergic responses was 1.6 mg of peanut, 1.1 mg of milk, 1.5 mg of sesame, 7.4 mg of cashew nut, and 0.29 mg of hazelnut (Ballmer-Weber et al. 2015). And in the VITAL project of VSEP (VITAL® Scientific Expert panel), the safety threshold of egg was 0.2 mg (http://allergenbureau.net/vital/vital-science). In addition, the accepted threshold for crustaceans in Japan is 10 ppm (Shoji et al. 2018). Therefore, referring to these prediction thresholds it can be known that, in order to be able to achieve the labeling and monitoring of trace amounts of allergens in samples, it is better to have an established detection with lower detection limit.

Table 4 Detection of egg white proteins in the commercial foods

Declaration	Quantitative (μg/mL; μg/g)
Egg-containing product	
Egg noodles	4.93±0.01
Bread	0.52±0.01
Fish tofu	0.006±0.01
Instant noodles	0.002±0.01
May egg-containing product	
Small round cookie	ND
Potato chips	0.001±0.01
Coffee	ND
Egg-free product	
Cola drinks	ND
Beef paste	ND
Cushion	ND

ND, not detectable

Cross-reactivity of the Sandwich ELISA

The specificity of the developed sandwich ELISA formats was evaluated by testing undiluted extracts of 30 foods including common allergic food and other eggs protein. In order to express results in μg of egg white protein/g food and considering that 1 g or 1 mL of food was extracted with 10 mL of extraction buffer, values obtained in ng/mL were divided by a factor of 100 (Table 1).

There was no cross-reactivity when using milk protein, peanut protein, soy protein, wheat protein, and other common food proteins. Weak cross-reactivity was observed with chicken meat, duck meat, duck egg, goose egg, and quail egg proteins. These results may be due to the higher homology between egg white protein and other eggs protein, due to the presence of the same or similar epitopes. There have been previous reports of cross-reactions between eggs and quail eggs and duck eggs (Alessandri et al. 2005; Alcántara et al. 2019; Moghtaderi et al. 2020). In this study, there was no cross-reactivity between eggs and other common food proteins, suggesting that the developed sandwich ELISA method is highly specific.

The Reproducibility of the Sandwich ELISA for Detecting Egg White Proteins

To evaluate the reproducibility of the developed sandwich ELISA detection, an intra-assay and inter-assay coefficient of variation was obtained. The CV values of intra- and inter-assay were 0.78–4.33% and 6.53–12.16%, respectively (Table 2), which shows that the developed sandwich ELISA is usable with high accuracy and repeatability.

Analysis of Egg White Protein–Spiked Samples for Sandwich ELISA to Product Matrix Interference

The accuracy of this method was evaluated by examining the recovery of egg white protein added to different egg-free food matrices. The comparison between the detected value and the actual value was used to judge the accuracy of the method. As shown in Table 3, 0.01, 0.1, and 0.2 g/kg samples were spiked in the compressed biscuits and black sesame paste and the

results were positive. Therefore, the method has sufficient accuracy and can be used for subsequent detection of real samples. However, the spiked recovery rate in Dove chocolate showed relatively low (the maximum being only 79.37%), indicating that food matrix has a significant impact on the developed method. Similar results have been reported in previous reports by Poms et al. (2005). Since chocolate contains some interfering components such as fats, polyphenols, and tannins, during the detection process, which will reduce the sensitivity and accuracy of the assay, resulting in a low recovery rate. Therefore, if chocolate-like food substrates are encountered with this detection method in the future, the dilution method can be used to reduce the fat concentration and thereby reducing the effect of the substrate.

Egg White Proteins Detection in Real Food Samples

Table 4 further confirms the accuracy and capability of the established sandwich ELISA method, all the egg-containing products and potato chips were positive in ELISA, and other samples showed negative results. All the detection results indicated that the sandwich ELISA can be used to detect the egg white proteins in food samples.

Conclusions

In summary, a polyclonal antibody-based sandwich ELISA method was successfully developed for detection of egg white proteins. After verification, the results showed that the detection method has the characteristics of high sensitivity, specificity, precision, and repeatability. Therefore, the sandwich ELISA method is a reliable detection method that can be used to detect egg proteins in various food products on the market to prevent egg allergy.

Funding This work was supported by the National Science Foundation of China (No. 31871719).

Declarations

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Informed Consent Not applicable.

Conflict of Interest The authors declare that there are no conflicts of interest.

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