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Research Article

BSA Interference in Immunoassays in Individuals with Egg Allergy

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Abstract

Aim: The aim of current study was to determine interference by bovine serum albümine (BSA) as blocking agent in enzyme-linked immunosorbent assay (ELISA) carried out in individuals with egg allergy.

Material and Methods: 14 people diagnosed with egg allergy and 7 people without allergy were included. The sample were studied with an indirect ELISA method for egg-white IgG antibody developed in our laboratory. Effect BSA on interference was studied by manipulating antigene coating (none vs. egg white extract), blocking (1% BSA vs. Tween 80), and sample diluent (PBS vs. PBS + 0.5% BSA).

Results: In wells that were blocked with 1% BSA without being coated with antigen, positive samples cross-reacted with BSA to give an optical density (OD) of 0.99 ± 0.16, while negative samples gave an OD of 0.08±0.01 ($p<0.05$). However, when the same samples were diluted with 0.5% BSA, the OD of positive samples decreased (from 0.99±0.16 to 0.08±0.01), and the statistical difference with negative samples disappeared. It was observed that tween, which was used as a blocking and diluting agent, did not cross-react with the samples. Positive samples gave an OD of 0.66±0.07 in antigen (egg white extract) coated and tween-blocked wells, and 1.01±0.11 OD in BSA blocking ($p<0.05$). When the samples were diluted with 0.5% BSA, positive samples gave 0.18±0.01 OD on the antigen coated plate, while negative samples gave 0.12±0.04 OD ($p<0.05$).

Conclusion: Ovalbumin, which is found in high levels in eggs, has a similar molecular structure to BSA, and some antibodies produced against ovalbumin in people with egg allergy may also cross-react against BSA. Therefore, it was concluded that the use of BSA in both dilution and blocking solution should be avoided if the samples of individuals with egg sensitivity are to be analyzed by ELISA method. It has been observed that Tween can be easily applied as an alternative blocking agent in allergy ELISA tests.

Keywords: Egg, allergy, BSA, IgG

INTRODUCTION

Food allergy is an important public health problem that adversely affects the lives of allergic patients and their families, may occur in adulthood or childhood, and its prevalence is increasing in the world (1,2). Allergic diseases are an immune disorder that reacts to certain types of allergens (3). Most allergens are proteins or glycoproteins with molecular weights ranging from 5000 to 100,000 Da. In addition, polysaccharides and low molecular weight substances may be allergenic (4).

All techniques using enzymes to demonstrate antigen-antibody reactions are generally referred to as the enzymatic immunoassay EIA/ELISA method (5). ELISA is a versatile, sensitive and quantitative technique that requires very little equipment (6). There are 4 different techniques when applying the ELISA test. These; direct, indirect, sandwich and competitive ELISA (7). In the indirect ELISA, the microplate is plated to the bottom of the wells. Blocking agent is used to prevent non-specific binding to spaces between antigens. Samples are diluted with dilution buffer and added to the wells. Afterwards, the secondary antibody, substrate and stop solution that recognize the antibody are added respectively and the reactions are stopped. The microplate is read on a microplate reader set to 450 nm (8).

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The most preferred blocking agents are proteins (BSA, milk powder, etc.) and Tween (9). Researchers often use BSA as a blocking agent to prevent non-specific binding of antigens and antibodies to the microtiter well (10).

ELISA is one of the approved and routinely used immunological tests in allergy research, in various industries, in allergy-related quality control, and in allergy diagnosis (11). Food allergy is a pathological, potentially fatal immune reaction triggered by normally harmless food protein antigens (12). Egg allergy is one of the most common food allergies in infants and young children, and egg whites contain more than 20 different proteins and glycoproteins. Ovomucoid (Gal d 1), ovalbumin (Gal d 2), conalbumin (ovotransferrin) (Gal d 3) and lysozyme (Gal d 4) have been identified as the main allergens in chicken eggs (13). Since ovalbumin (OVA) and BSA have some immunologically similar epitopes, the antibody produced against one of them usually cross-reacts against the other (14). Therefore, if the samples of people with egg sensitivity are to be analyzed by ELISA method, the use of BSA may be inconvenient. Because if the antibodies in the sample bind to the BSA used in the blocking, a similar result occurs as if they were bound to the antigen (Figure 1). This actually leads to non-specific high optical density. In this context, the aim of our study is; The aim of this study is to investigate the effect of BSA use on the results in the ELISA test to determine the IgG level in egg-sensitive individuals.

MATERIAL AND METHOD

Before the study, ethical approval was obtained from the Inonu University Health Sciences Non-Interventional Clinical Research Ethics Committee (No: 2022/3017).

As a positive control in our study; In our laboratory, 14 people whose samples were previously found and diagnosed with egg allergy were used. Samples of 7 people without any allergic disease were used in the negative control group.

A 96-well ELISA plate was used in the study (Thermo Scientific Maxisorp Nunc 96 well Plate) and the test protocol was designed according to the indirect ELISA method (Figure 2). Wells were coated with egg white extract as antigen (Figure 2. ”1. process”). Blocking was done with 1% BSA or 0.5% Tween 80 (Figure 2. ”2. process”). Samples were diluted 1/100 with solutions containing PBS or 0.5% BSA and added to the wells (Figure 2. ”3. process”). Then to all wells in order; secondary antibody (Biotinylated anti-human IgG,) and streptavidin peroxidase were added (Figure 2. ”4. and 5. process”). The color formed after the addition of chromogen substrate (3,3',5,5'-Tetramethylbenzidine (TMB)) was stopped with a stop solution (11% H2O2) and read at 450 nm wavelength (Figure 2.”6. process”). A total of 6 different experimental protocols studied are given in Table 1.

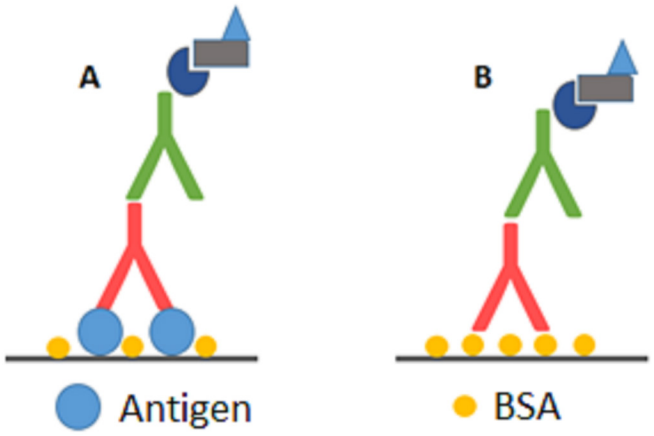

Figure 1. The binding of antibodies found in the samples of children with egg allergy to the antigen (A) and the BSA-blocked surface (B) provides similar results

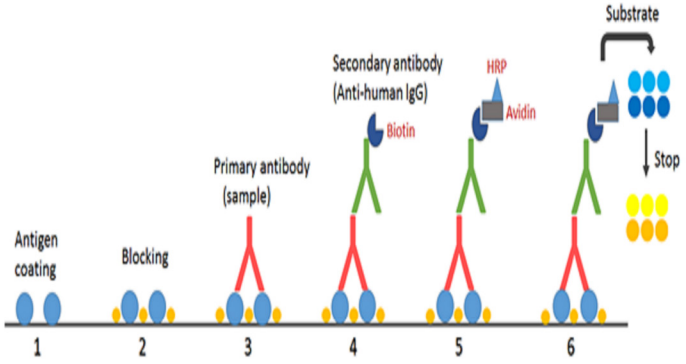

Figure 2. The indirect ELISA working principle that we used in our study

In Protocols 1, 2 and 3, direct blocking was performed without coating the wells with antigen. Protocol 1 was designed to determine the binding levels of samples to BSA. In the second protocol, the samples were diluted with BSA to measure the extent to which BSA in the dilution solution would bind the antibodies in the sample. In protocol 3, the affinity of the samples for a blocking agent other than BSA was measured by blocking the empty well with a BSA-free agent (Tween 80).

In protocols 4, 5 and 6, wells were coated with egg white extract as antigen. BSA was not used in protocol 4. Thus, IgG levels against egg white were determined in the samples. Unlike the previous experiment in protocols 5 and 6, BSA was used and how BSA affected the reference result in protocol 4 was evaluated.

Statistical Analysis

Since the data obtained did not meet the parametric conditions, Friedman test was used for dependent groups and Kruskal Wallis-H test was used for independent groups in statistical comparison. Post hoc evaluations were made with Bonferroni corrected Wilcoxon (dependent) or Mann-Whitney U test (independent). The cut off (threshold) value used to evaluate the binding adequacy of the samples to BSA or egg extract was determined by the ROC curve (Receiver Operating Characteristic Curve).

Table 1. Six different protocols of the study (The applications made after adding the samples were not included in the table since they were common in all trials)

	Protocol 1	Protocol 2	Protocol 3	Protocol 4	Protocol 5	Protocol 6
Antigen coating	-	-	-	Egg extract	Egg extract	Egg extract
Blocking	1% BSA	1% BSA	0.5% Tween80	0.5% Tween80	1% BSA	1% BSA
Sample dilution	PBS	0.5% BSA	PBS	PBS	PBS	0.5% BSA

Table 2. Descriptive statistical data of allergic and non-allergic samples (Results are given as OD)

	ALLERGİC SAMPLES		NON-ALLERGİC SAMPLES		
	Mean±S deviation	Median (min-max)	Mean±S deviation	Median (min-max)	P value
Protocol 1	0.99±0.16	0.86 (0.20-2.39)	0.08±0.01	0.08 (0.06-0.09)	0.00*
Protocol 2	0.08±0.01	0.08 (0.06-0.10)	0.08±0.01	0.08 (0.07-0.10)	0.64
Protocol 3	0.08±0.01	0.08 (0.07-0.10)	0.08±0.00	0.09 (0.08-0.09)	1.00
Protocol 4	066±0.07	0.66 (0.28-1.27)	0.13±0.04	0.12 (0.08-0.19)	0.00*
Protocol 5	1.01±0.11	0.92 (0.50-1.85)	0.12±0.04	0.12 (0.08-0.20)	0.00*
Protocol 6	0.18±0.01	0.17 (0.15–0.34)	0.12±0.04	0.11 (0.08-0.20)	0.00*

P values represent the difference between the results of allergic and non-allergic subjects for each protocol. (*) Indicates that there is a statistically significant difference

RESULTS

Determination of affinity for BSA

In our study, six different protocols were used in which allergic and non-allergic samples were tested. Binding levels of samples to BSA were analyzed in protocol 1. The cut off value was calculated as 0.146 Optical Density (OD) by ROC analysis, and samples that gave results above the cut off were considered to have affinity for BSA. All 14 allergic samples tested in protocol 1, where blocking with 1% BSA without coating with antigen, were found to be above the cut-off value (Figure 3A). In protocol 2, allergic samples were diluted with 0.5% BSA. Because BSA in the dilution solution binds antibodies with affinity to it, the antibodies did not reach the bottom of the wells and no color was formed in the wells. In protocol 3, allergic samples were tested against a BSA-free blocking agent (0.5% tween 80) and similar results were obtained with protocol 2 (Figure 3A). In the same protocols, 7 non-allergic samples were also tested and, unlike the allergic samples, showed no affinity for BSA (Figure 3B).

Determination of IgG levels of samples against egg extract

IgG levels of allergic samples against egg white extract were tested in protocol 4 without BSA. Cut off value was determined as 0.236 OD. Allergic samples gave positive results above the cut-off (0.66±0.07), while non-allergic samples remained below the threshold value (0.13±0.04) (Table 2).

Effect of BSA use on results

The data obtained from protocol 4 were accepted as the reference value and the effect of BSA on the experiment was evaluated. A statistically significant increase in OD values for allergic samples was seen in protocol 5 where blocking was done with BSA compared to protocol 4 (p=0.001). When looking at protocol 6, in which BSA is used both in blocking and dilution of the samples, a significant decrease was observed in the OD values of the allergic samples compared to protocol 4 and 5 ([p=0.001], [p=0.001]). The use of BSA did not change the results, since non-allergic samples did not show affinity for BSA.

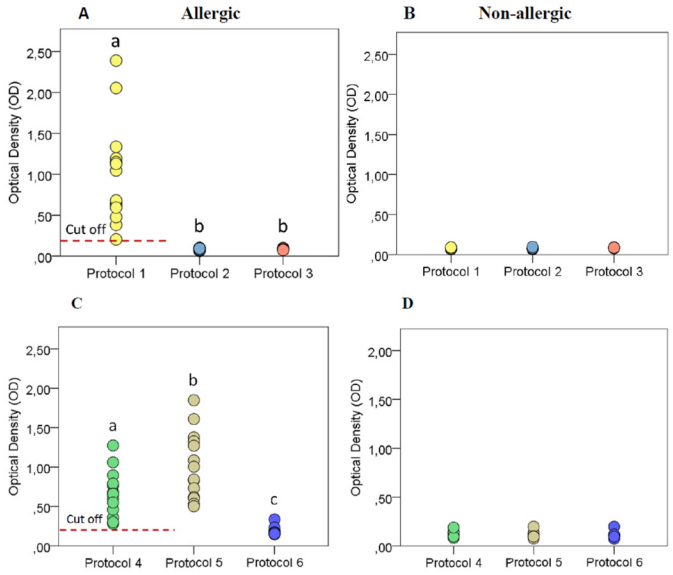

Figure 3. ELISA egg white specific IgG results of different protocols applied in allergic and non-allergic individuals

DISCUSSION

The ELISA method is a frequently used method in allergy research (11). For allergy ELISA tests to give accurate results, antibodies only need to recognize the antigens in the wells. Substances such as tween and BSA as blocking agents are used to close the gaps left after coating with antigens (9). BSA is frequently used as a blocking agent in egg allergy-specific IgG studies (15-18).

Tomicic, et al. (15) used BSA as a blocking agent for

ELISA IgG1 and IgG4 measurement against food allergens (including OVA). In another study, Zhang et al. (16) used 3% BSA as a blocking agent in the ELISA method to measure the IgG level specific to egg components Gal d 1, Gal d 2 (OVA), Gal d 3, Gal d 4 and Gal d 5. McKendry, et al. (18) in a study; used 1% BSA for the measurement of raw peanuts, egg whites and cow's milk specific IgG ELISA. However, according to the findings we obtained in our study, it was seen that the use of BSA as a blocking agent can cause false results.

Zhang and Mine (1998), on the other hand, used 3% BSA as a blocking agent for the measurement of ovomucoid IgG ELISA, but performed it with 1% BSA as serum dilution. In this study, dilution of samples from subjects allergic to ovalbumin was done with BSA. This dilution eliminated non-specific binding to BSA used as the blocking agent, resulting in ovomucoid (not ovalbumin) specific IgG measurement. This study design is compatible with the results of our study.

In some studies, although BSA was used as the blocking agent in allergy-specific IgG ELISA tests, some wells were coated with BSA as a control to eliminate specific binding. The results of the absorbances on the BSA-coated plate were subtracted from the results on the allergen-coated plates, following a different protocol (19,20). However, since antibodies formed against ovalbumin in these protocols will give a certain optical density as a result of cross-reaction in the well coated with BSA as a control, the result obtained in the applied subtraction process; will give the total optical density of those other than ovalbumin. Instead of these protocols, the use of tween 80 as a blocking agent, which we have shown not to bind specifically in our study, is thought to be a more reliable protocol.

In a study by Hermanson (2013); It has been noted that ovalbumin (OVA) and BSA have some immunologically similar epitopes, and antibodies produced against one of them will often cross-react against the other. In our study, it was seen that the antibodies formed against ovalbumin by the ELISA method cross-reacted with BSA, and this result is consistent with the statements of Hermanson (2013).

CONCLUSION

BSA is not suitable for use as a blocking agent because it cross-reacts with antibodies against ovalbumin. The use of tween as a blocking agent instead of BSA is important in terms of obtaining more accurate results. When serum dilutions of people with egg allergy are made with BSA, it is recommended not to use BSA in serum dilution, since antibodies against ovalbumin will bind to BSA and will not bind to the coated antigen.

At the same time, the present results indicate that there will be false high optical density in people with egg allergy as a result of using BSA as a blocking agent in ELISA tests other than allergy ELISA tests. Therefore, it is recommended not to use BSA as a blocking agent in ELISA tests other than allergy ELISA tests. Even if it will be used, it is recommended to take a history of past or current allergic conditions in the people who will be included in the study.

Studies have shown that ovalbumin and BSA have similar epitopes and cross-react. Whether this situation causes erroneous results in the ELISA method has not been examined. In the current study, it was observed that the use of BSA in ELISA measurements in egg allergic individuals caused interference and affected the test result. It seems essential to investigate BSA interference in individuals with allergies to different foods.

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Conflict of Interest: *The authors declare that they have no competing interest.*

Ethical approval: *Before the study, ethical approval was obtained from the Inonu University Health Sciences Non-Interventional Clinical Research Ethics Committee (No: 2022/3017).*

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