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Immunochemical detection of peanut (*Arachis hypogaea* L.) proteins using chicken antibodies

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ELISA is a cheap, fast and simple method for the detection of food allergens. Use of chicken antibodies in the analysis of food allergens and specifically the effect of the immunisation frequency on antibody generation is not well documented. This study aimed at comparing the activity of the immunoglobulins generated using a three-week and a six-week immunisation protocol. Isa brown chicken were immunised with 500 µg of roasted peanut protein extract using varying booster injection intervals. After induction, antibody production was sustained during the immunisation period in the six-week protocol with a marked drop in the three-week regime. The activity of antibodies was higher in the six-week protocol than the three-week protocol. An assay was optimised in the detection range of 1 ppm. Upon heating, the peanut protein binding by IgYs decreased with subsequent fluctuations. This was associated with denaturation of proteins and unmasking of new epitopes and stable fractions persisting.

Keywords: immunisation; immunoglobulins; peanut; protein

Introduction

Peanut is responsible for more than 50% of the food allergy induced fatalities (Bock, Morrow-Furlong, & Sampson, 2001). Peanut allergy is the most common and serious in terms of persistence and severity of reactions (Sampson, 2002). Hidden allergens in foods are of concern because up to now, allergenic avoidance is the only therapy (Schappi, Konrad, Imhof, Etter, & Wuthrich, 2001). Due to the risk of inadvertent contamination of foods with allergenic products during manufacturing processes, the food industry and regulatory agencies require tools to monitor foods for the presence of allergens. One aspect of prevention is the development of analytical tools to support the food industry in controlling their products. Enzyme-linked Immunosorbent Assays (ELISA) have proved to be fast and simple methods for the detection of food allergens.

Studies have reported the possibility of inducing substantial amounts of specific chicken antibodies (IgY) against a single antigen (De Meulenaer, De La Court, Acke, De Meyere, & Van De Keere, 2005; Schade et al., 2005). The availability of a large amount of IgY from egg yolks makes it feasible to use this antibody in many applications (Schade et al., 2005). Using chicken antibodies (IgYs) offers a number

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of advantages. An egg may contain 50–100 mg of IgY, of which 2–10% are specific antibodies (Schade & Hlinak, 1996) and no bleeding of the animal is necessary. However, the use of chicken antibodies in the analysis of food allergens is not so well documented compared to the use of other antibodies, despite of the ease of immunisation and antibody isolation and purification. Various immunisation protocols for raising antibodies in chickens' eggs have been reported in the literature, but these are difficult to compare with respect to the type and dose of antigen used and frequency of injection.

The vaccination frequency and interval depend on the immunogenic potential of antigen itself (Leenars et al., 1999). A booster can be considered after the antibody titer has reached a plateau or begins to decline. The boosting protocol can have a decisive impact on the result of the immunisation. The often reported interval is two to four weeks (Camenisch et al., 1999; Matsuda et al., 1999; Tini, Jewell, Camenisch, Chilov, & Gassmann, 2002). Different immunising protocols using different vaccination frequency and interval for each antigen and for each animal species need to be tested to find out which method induces the highest antibody titer. Few chicken immunisation protocols with peanut proteins raising specific IgY have been reported. There is need to determine an optimum primary-booster interval in the generation of immunospecific IgYs. In a previous trial of a three-week immunisation protocol using peanut proteins, the optimum levels of IgY production was attained after 3 weeks of immunisation followed by a decline in IgY production (De Meulenaer et al., 2005). The aim of the present study was to generate IgYs using a six-week immunisation protocol and compare the activity of IgY generated to the three-week immunisation protocol.

Different processing method can denature, alter or destroy proteins so that they are no longer detectable by antibodies used in the assays (Whitaker, Williams, Trucksess, & Slate, 2005). This study further sought to investigate the influence of heat treatments on the IgY binding activity of peanut proteins. In this study, an assay for the detection of peanut proteins was optimised and the effect of heat treatment on the detection of peanut proteins determined.

Methodology

Animals

Two Isa Brown hens (*Gallus domesticus*), 43 weeks old at the beginning of the experiment, were kept in pens in a standard animal room. The experimental protocol was approved by the Official Animal Care and Use Committee of the Gent University before the initiation of the research and followed the Belgian guidelines approved by the committee on care, handling and sampling of the animals.

Chicken immunisation

Two Isa Brown chicken were injected intramuscularly with 1 ml (chicken 5) and 0.5 ml (chicken 6) of 1:1 (v/v) mixture of Freud complete adjuvant and PBS containing 500 µg/ml of immunising antigen; a protein extract obtained from roasted peanuts, type Virginia USA. After six weeks, a supplementary injection of 1 ml of 1:1 (v/v) mixture of Freud incomplete adjuvant and PBS containing 500 µg/ml of immunising

antigen was given to both chickens, followed by a booster injection of 500 µg/ml of immunising antigen in PBS after another additional six weeks. The eggs were collected daily, labelled accordingly. Every one week, from day 1 till day 84, one egg per hen was taken, labelled accordingly, and stored in a refrigerator at 4°C until immunoglobulins were isolated. Eggs from non-immunised chicken were also obtained from Delhaize Supermarket, Belgium for the extraction of blank IgYs.

Isolation of immunoglobulins from eggs

The IgY separation from eggs was done using the method described by Akita and Nakai (1992). The eggs were broken carefully and the intact egg yolk separated from the albumen by washing several times with distilled water. The yolk was then patted dry on a filter paper. The yolk sac was pierced and the yolk broken through a fine wire mesh and the filtered volume noted. The extraction was done from individual egg yolk. The extracted yolk was diluted eight times its volume with distilled water. The pH of the diluted yolk was set in the range of pH 5.0 and 5.2, using 1N HCl. The extract was incubated for 16 hours at 4°C. This was followed by centrifugation at 9500 g for 1 hour at 4°C (Sorvall RC-5B, Refrigerated Superspeed Centrifuge from Dupont Instruments). Purification of IgY was done using the salt precipitation method (Svendsen, Crowley, Ostergaard, Stodulski, & Hau, 1995). The final residue was dissolved in 1/6 (the volume of the original yolk) ml of PBS and the aliquot stored in small plastic vials at -18°C from which it was further diluted for further use. To determine of the activity of the IgYs, one egg per week was extracted initially and after good response was established, combined egg extraction per week followed. This was done by combining two egg yolks at the second salt precipitation step and finally, a combination of all the collective week's IgYs in the final step of dissolving in PBS. IgYs from a previous three-week immunisation protocol extracted using the same procedure were used for comparison of the two protocols. From the previous study (De Meulenaer et al., 2005), the produced antibodies showed low to very low cross reactivity towards proteins from real nuts and other major food proteins hence for the present protocol it was not done.

Assay optimisation

Ninety-six well flat bottomed high binding ELISA-plates (Micro-colon 96W) from Greiner bio-one were coated with the coating antigen (100 µl/well) starting with 100 mg/ml for the first column and a series of half dilutions for the subsequent columns using coating buffer solution as the diluting solution. Roasted peanut aliquot was used as the antigen. The plates were sealed with plastic film, wrapped in aluminium foil and stacked in between two other plain plates to reduce the edge effect. These were incubated overnight at 4°C. The plates were washed three times with the wash solution (200 µl/well) and blocked with the blocking solution (200 µl/well), followed by 30 minutes incubation in the dark at room temperature. The plates were washed twice as done previously. To each row, different concentrations of the primary antibody (100 µl/well) were added, ensuring that the ionic strength was maintained for each dilution. Incubated at 37°C for 1 hour in the dark followed by three washing steps. The secondary antibody (Horse Raddish Peroxidase-conjugated antibody) was added (100 µl/well) at 1:10,000 dilution in the dilution buffer and the plates

incubated at 37°C for 1 hour in the dark. The plates were washed thrice and freshly prepared substrate solution added (100 ml/well) and incubated for 1 hour at 37°C in the dark. The stop solution was added finally (25 ml/well). Immediately the absorbance was read twice at 450 nm using an ELISA reader (Titertek Multiskan plus; MK II). For the competitive assay, 6.25, 3.125 and 1.5625 mg/ml of peanut proteins were used. Absorbance was also corrected for blank readings obtained by using immunoglobulins isolated from eggs of non-immunised chicken. Measurements were performed in duplicate.

Assay validation

The validation of the ELISA was based on assay linearity, repeatability and reproducibility. Because there was no uniform peanut protein standard available, own peanut protein extracts as were used as standards to generate a standard curve.

Effect of heat treatment on immunochemical detection of peanut proteins

Peanut solution (10 µg/ml) was prepared in PBS and 500 µl of the solution pipetted in to capped plastic tubes in duplicate. The samples were then heated in a water bath at 100°C and removed at intervals of 1 hour and then cooled immediately with 4.5 ml of distilled water. A competitive assay was carried out as per the optimised protocol to determine the peanut proteins concentration.

Data processing

Competitive curves were obtained in duplicates and fitted to the four parameter logistic function (Englebiene, 2000) using a commercial software package (SPSS 11.0). The competition curves were normalised by expressing the experimental absorbance levels (B) as $(B/B_{o, \max})$, where $B_{o, \max}$ is the maximal absorbance in absence of the analyte. For statistical evaluation a 95% confidence interval was applied.

Results and discussion

Chicken immune response and IgY production

Two Isa brown chickens were immunised with varying initial immunisation dose of peanut proteins; 500 µg for chicken 5 and 250 µg for chicken 6 respectively. Peanut proteins specific antibodies in egg yolks were present as early as two weeks of immunisation and increased to plateau at week 3 and week 6 for chicken 5 and chicken 6 respectively (Figure 1). The slow induction in immune response of chicken 6 could be associated with the lower initialisation dose administered as compared to chicken 5. After the plateau, there was a slight decrease in activity for 4 weeks in both chickens, followed by an increase in IgY activity to reach the optimum. In the three-week immunisation protocol however, optimum levels were observed at 3 weeks followed by a marked decline during the 12 weeks observation period (De Meulenaer et al., 2005).

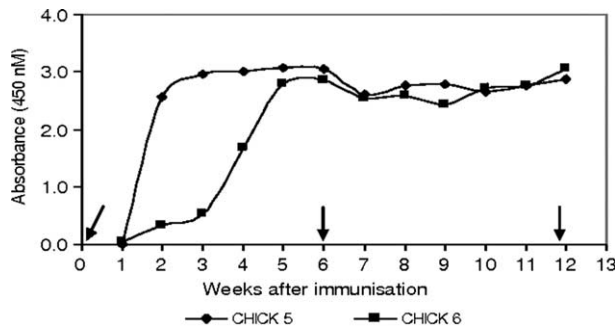


Figure 1. Activity of IgY's generated using six-week immunisation protocol.

The lag time which varies in different animals, can be explained by the time it takes for specific antibodies produced in chicken serum to be transferred and accumulated in egg yolk as reported by Li et al. (1998). Other studies have shown that the immune response varies in different chicken (Mayo et al., 2005). Mayo reported that some chicken are better responders than other individuals of the same genetic lineage and that there is a significant difference in immune response between individual lines of chicken. The increase of IgY production observed after the second immunisation in the six-week immunisation regime is not due to a boosting effect but may be due to the fact that some chicken respond throughout the immunisation period. Other studies have reported high specific immune responses in the egg yolk from hens inoculated over a long period (Bollen & Hau, 1998; Chalhouni, Théwis, Portetelle, & Beckers, 2008; Pauly et al., 2009; Schade et al., 2005).

Comparison of the three-week and six week immunisation protocols on IgY activity

IgYs from Chicken 5 at week 5 and week 6 for the six week immunisation protocol were chosen because of demonstrating a better activity than Chicken 6. The activity of the weekly combined-yolk IgY produced using the 3-week (old) and 6-week (new) immunisation protocol were compared (Figure 2). IgYs produced using the six-week protocol showed a higher activity at dilutions of more than 1:10,000 when compared to the three-week protocol. However, the results of this study are not conclusive

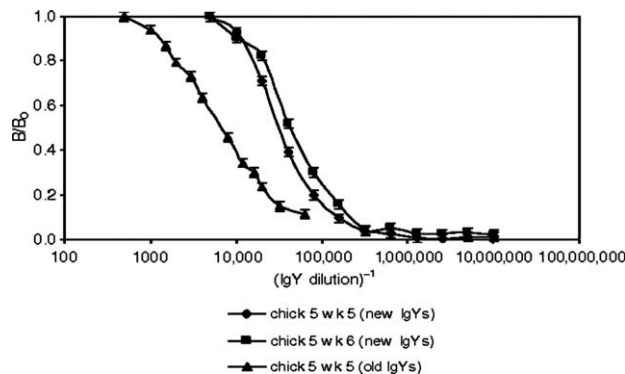


Figure 2. IgY activity: three-week (old IgY) and six-week (new IgY) immunisation protocols.

because of the small sample size of chicken used and the difference could similarly be attributed to the varied immune response of individual chicken.

Optimisation of ELISA

To obtain absorbance values of 1.0 of the assay in detection range of 1.0 µg/ml, the ELISA was optimised. The optimum plate coating concentration for peanut proteins was tested by coating the plate with a series of peanut protein concentration range from 0.78–100 µg/ml. The optimum peanut protein concentration for plate coating was found to be 1.5 µg/ml, where binding of IgY to peanut proteins was a linear function from 0.1–10 µg/ml peanut protein concentration. The optimal primary and secondary antibody dilutions were found at 1:5000 and 1:10,000 respectively. Competition curves within the detection range were obtained by using the optimised ELISA (Figure 3). The optimised competitive assay was used to evaluate the affinities of the different IgYs in the detection of peanut proteins (Figure 4). The IgYs produced from the new protocol displayed a higher affinity to peanut proteins than those produced using the three weeks immunisation protocol as the I_{50} values were 1.156, 1.411 and 2.197 for week 5 (new), week 6 (new) and week 5 (old) IgYs respectively.

Assay validation

The assay had a linearity of $r^2 > 0.9997$ for the peanut protein concentration range from 0.1 to 200 µg/ml. The repeatability (within day precision) was calculated by the coefficient of variation (CV) as measured by using three locally prepared standards of the peanut proteins that were run four times on the same day, whereas reproducibility (between day precision) was calculated from the average of the within assay precision in three runs on three different days (Table 1). The assay repeatability was <5% CV and assay reproducibility was <6% CV.

Effect of heat treatment on immunochemical detection of peanut proteins

Changes in IgY binding activity of food proteins and glycoproteins could be induced by various technological processes and treatments (Sanchez & Fremont, 2003). The modification of epitopes by conformational changes, thermal or

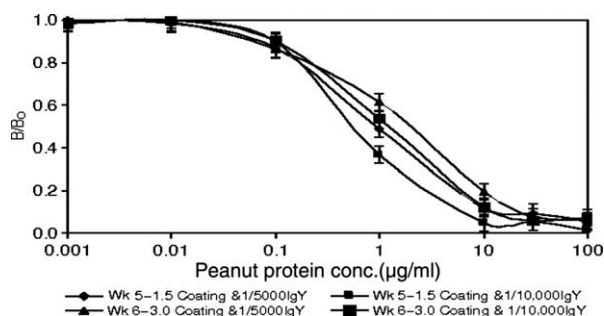


Figure 3. Competitive assay for the detection of peanut proteins.

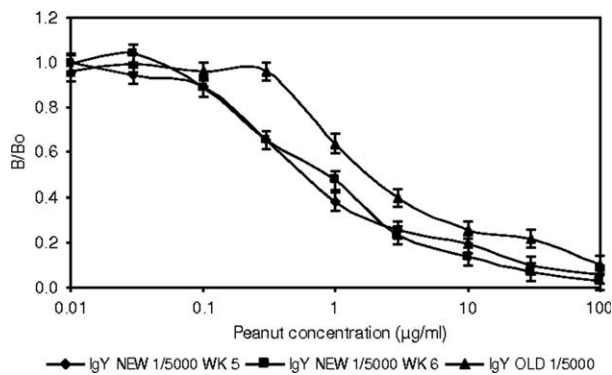


Figure 4. Optimised competitive assay comparing the sensitivity of new and old IgY's.

hydrolytic fragmentation of proteins is possible. Figure 5 show the effect of heat treatment on the detection of peanut proteins by chicken IgYs. There was a marked decrease in peanut concentration in the first two hours of boiling which could be associated with the loss of IgY binding due to the conformational changes of the peanut proteins. After boiling for 2 hours, the protein concentration remained at a relatively low level, an indication that certain fractions of the epitopes were not affected by heat and hence retained their IgY binding capacity and possibly representing the heat stable fraction. A subsequent increase and decrease in the concentration of proteins concentration followed after 3 hours of heating, which could be attributed to the unmasking and masking of epitopes respectively. Other studies have reported similar findings (Beyer et al., 2001; Koch et al., 2003; Poms, Lisi, Summa, Stroka, & Anklam, 2003; Wigotzki, Steinhart, & Paschke, 2000). Due to the masking of epitopes, the risk of false negatives in the detection is increased.

Table 1. The repeatability and precision of the assay.

	Peanut protein concentration (µg/ml)		
	Sample 1	Sample 2	Sample 3
Repeatability (within day precision)			
1	10.43	32.50	100.25
2	11.23	30.47	98.30
3	10.87	31.40	99.10
4	11.45	30.92	98.76
Average	11.00	31.32	99.10
Standard deviation	0.45	0.87	0.83
Coefficient of variation (CV)	4.06	2.78	0.84
Reproducibility (between day precision for 3 days)			
Day 1	11.45	30.92	98.76
Day 2	12.49	31.48	100.42
Day 3	11.21	29.95	96.45
Average	11.72	30.78	98.54
Standard deviation	0.68	0.77	1.99
Coefficient of variation (CV)	5.81	2.51	2.02

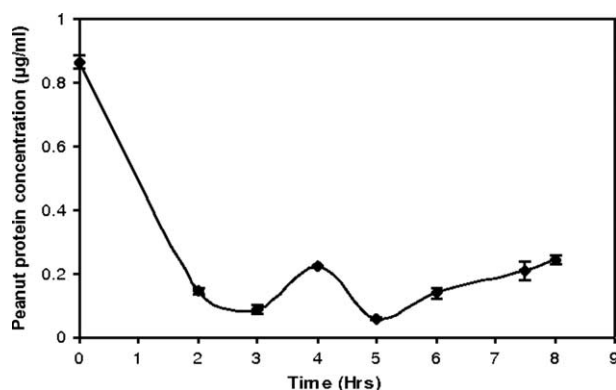


Figure 5. Effect of heat treatment of peanut proteins on IgY detection.

The risk could be magnified if monoclonal antibodies are used in assays, a reason why the detection of allergens in the food industry should not target specific allergenic proteins.

Conclusions and recommendations

The generation of substantial amounts of peanut specific chicken antibodies is relatively easy, cheap and fast. ELISA can reliably be used to detect peanut proteins, which once detected, are associated with the allergenic proteins in foodstuffs. The choice of immunisation protocol is important in IgY generation. In this study, the timing of booster injections is associated with induction and activity of the generated antibodies. A dose-response-effect of peanut proteins was also associated with the immune response in chicken. Other genetic factors could also have played a role in the immune response. However, there is need to pursue other immunisation protocols using larger samples of study animals to establish whether the immunisation frequency has an effect on the generation and activity of chicken IgYs. Food processing methods affects the detection of peanut proteins. The integrity of the target proteins could be changed during food processing leading to a change in their detection. Heat processing induces conformational changes to peanut protein with subsequent masking and unmasking of epitopes and consequently decreasing the sensitivity of the IgYs to peanut proteins. However, heat stable fractions are likely to persist. There is a need to determine further the extent to which other processing techniques affect the extent of detection of allergens in foods.

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