Effect of processing on the detectability of peanut protein by ELISA

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ABSTRACT

Chicken IgY was used for the detection and quantification of peanut proteins by indirect competitive ELISA. The method was optimized by using a checker board approach to determine the optimal concentration of coating antigen, primary antibody and secondary antibody. Peanut protein could be detected in foods down to levels of 10 ppm. The effect of physical (heat treatment at 80 °C and 100 °C) and chemical (acid, alkali and reducing sugar) treatments on the IgY binding of peanut proteins was investigated. The optimized assay was relatively sensitive for the roasted peanut proteins. However, the binding ability of chicken IgYs to peanut proteins was found to be significantly altered by denaturation and hydrolysis of proteins. It was also observed that the effect of Millard chemistry on the detectability of peanut protein was less pronounced at high temperatures than at low temperatures.

1. Introduction

Food allergies are an emerging public health problem in industrialized areas (~8% of population affected; Ortolani, Ispano, Scibilia, Pastorello, 2001 and Sicherer, Munoz-Furlong, Murphy, Wood, Sampson, 2003). Over the last few decades the prevalence of food allergies has increased presenting an important challenge to both clinical allergology and to the food industry. Usually food allergens are proteins or glycoproteins. Only a small percentage of proteins in foods are allergenic. These allergens belong to a variety of different protein families (Aalberse, 2000; Breiteneder Ebner, 2000). The general consensus is that the most common allergenic foods, worldwide, are peanuts, eggs, milk, fish and crustacean, soybean, wheat and tree nuts (Birmingham et al., 2007).

Food allergy denotes an immunologic mechanism represented almost exclusively by IgE-mediated reactions (Koomen, Ortolani, Aas, 1995). Allergic symptoms which could be induced by ingestion of foods ranging from a mild rash to anaphylactic shock and even death. Of the major food causing allergic reactions, peanut allergens are of particular interest because of the reported life threatening consequences due to anaphylactic shock. Peanut is reported to be responsible for more than 50% of food allergy fatalities (Bock, Munoz-Furlong, Sampson, 2001). In most cases peanut allergy persists throughout the person’s life and may increase over time (Sicherer et al., 2002). Threshold doses for peanut allergenic reactions have been found to range from as low as 100 μg to 1 g of peanut protein (Hourihane et al., 1997; Wensing et al., 2002), which equates to 400 μg to 4 g peanut meal.

The problem of hidden allergens from peanut in food is a very serious one for people with peanut allergies. The food industry is confronted with the need for reliable analytical methods which are able to detect and quantify particular allergens in food. Different techniques have been adopted to achieve this goal for a variety of important allergens as recently reviewed by Poms, Klein, and Anklam (2004). Immuno-based techniques are frequently used and in particular enzyme-linked immunosorbent assays have gained a lot of attention in recent years (Poms, Lisi, Summa, Stroka, Anklam, 2003).

The design and development of an immunoassay format with a combination of sensitivity, specificity and cost effectiveness in terms of analytical performance, for the detection and quantification of peanut proteins has become a necessity for the laboratory and field use. The aim of the present study was to optimize an indirect competitive immunoassay to detect peanut protein allergens. In the second part of this study the effect of thermal and chemical treatment of peanut allergens on the IgY binding and detectability of the peanut proteins by indirect competitive ELISA was examined.

2. Material and methods

Anti-chicken IgG developed in rabbit (Horse Raddish peroxidase- conjugated) was bought from ICN Biomedicals, Inc. and Bovine serum albumin was purchased from Sega Chem. Co.
Primary antibodies (polyclonal) and blank were obtained from the immunized and non-immunized chicken eggs, Gent University. ELISA plates (Microlon 96W) were purchased from Greiner bio-one.

2.1. Optimization of ELISA protocol

In order to obtain an optimized ELISA, a checker board titration was carried out, using different concentrations of coating antigen, competing antigens, primary antibody and secondary antibody.

2.2. Optimized indirect competitive ELISA

Ninety-six well flat bottom high binding ELISA-plates were coated with the coating antigen (100 µl/well). Roasted peanut aliquot was used as an antigen (10 µg/ml). The plates were placed inside a large Petri plate with wet cotton in order to restrict evaporation from the wells during incubation at 4 °C overnight. The Petri plate is also covered with aluminium foil to remove any light. The following day the plates were washed three times with Tween20 as washing solution (200 µl/well) and blocked with sodium caseinate in phosphate buffer solution (PBS) as blocking solution (200 µl/well), followed by 30 min of incubation in the dark at room temperature. The plates were washed twice as before. The competing antigen (peanut protein) was added (50 µl/well). This was followed by adding the primary antibody (50 µl/well), ensuring that the ionic strength was maintained for each dilution. They were incubated at 37 °C for 1 hour in the dark. This was followed by three washings as before. The secondary antibody was added (100 µl/well) at 1/10,000th dilution in the dilution buffer and again incubated at 37 °C for 1 h in the dark. The plates were washed three times and freshly prepared substrate solution was added (100 µl/well) and incubated for 1 h at 37 °C in dark. The stop solution (conc. H₂SO₄) was then added (25 µl/well). After this the absorbance at 450 nm was immediately recorded using an ELISA reader (Titertek Multiskan plus; MK II). Absorbance was corrected for blank readings obtained by using immunoglobulins isolated from eggs of non-immunized chicken. Measurements were performed in duplicate.

2.3. Detectability of peanut protein after heat treatment

A peanut protein solution (1 mg/ml) was prepared in PBS buffer (pH 7.4). From the solution 0.5 ml was aliquoted into capped plastic tubes in duplicate. The samples were then heated in a water bath at 80 °C and removed at various time intervals (0, 12, 24, 30, 36, 42, 48 and 54 min). The rest of the procedure was identical to that mentioned previously.

2.4. Detectability of peanut protein after heat treatment in PBS of various pH

A peanut protein solution (1 mg/ml) was prepared in PBS buffer at various pH (pH 4.0, pH 7.4 and pH 10.0). From each of these solutions, 0.5 ml was aliquoted into capped plastic tubes in duplicate. The samples were then heated in a water bath at 100 °C and removed at various time intervals (0, 6, 12, 18, 38 and 58 min). The samples were then kept in an ice bath for 5 min. Finally 4.5 ml of distilled water was added to the tubes.

An indirect competitive assay was carried out, following the same procedure as mentioned above. Absorbance was also corrected for blank readings obtained by using immunoglobulins isolated from eggs of non-immunized chickens. Measurements were performed in duplicate. Detectability of peanut protein after heat treatment in reducing sugar solution.

A peanut protein solution (1 mg/ml) was prepared in a solution containing a reducing sugar (glucose; 20 mM) in PBS buffer (pH 7.4). From each of the solution, 0.5 ml was aliquoted into capped plastic tubes in duplicate. The samples were then heated in a water bath at 80 °C and 100 °C and removed at various time intervals (0, 6, 12, 18, 38 and 58 min). The rest of the procedure was identical to that mentioned previously.

2.5. Data processing

Competitive curves were obtained in duplicate. For statistical evaluation a 95% confidence interval was applied. The competition curves obtained were fitted to the four parameter logistic function corresponding to the equation below using a commercial software package (SPSS 12.0 for windows). The competition curves were normalized by expressing the experimental absorbance level (B) as (B/B₀)max, where B₀max is the maximal absorbance in the absence of analyte (Englebienne, 2000).

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B = \frac{B_0 - S}{1 + (\frac{S}{I_{50}})^p} + S
\]

where B = absorbance, B₀ = maximum absorbance, S = lower asymptote value, almost zero, I₅₀ = point of inflection of curve, X = Concentration of peanut antigen (µg/ml), P = exponent (slope).

3. Results and discussion

3.1. Optimization of ELISA

In order to obtain an optimized ELISA, a checker board titration was carried out, using different concentrations of coating antigen, competing antigens, primary antibody and secondary antibody. At lower concentrations of 1/20,000th of the secondary antibody, very low absorbance values were observed where as at a higher concentration (1/5000th and 1/10,000th) little difference was found (results not shown). Therefore a secondary antibody dilution of 1/10,000th was chosen. Similarly, a concentration of 1/10,000th of the primary antibody gave low absorbance, while a concentration of 1/5000th gave a good absorption value. By using concentrations of 10 µg/ml for the coating antigen and of 1/5000th for the primary antibody, a good curve was obtained with a maximum absorbance of approximately 1 and I₅₀ of almost 10 µg/ml ± 0.017 (Fig. 1). This protocol was followed as an optimized ELISA for further detection of peanut proteins undergoing different processing conditions (i.e. heating, treatment in acidic and alkalic conditions and in the presence of reducing reducing sugar).

The results obtained for the optimization of ELISA is in fair agreement with the results of De Meulenaer, De La Court, and Huyghebaert, (2002); Kiio and De Meulenaer (2012). Both of them found the same effect of different concentrations of primary antibody and coating antigen on assay performance.

3.2. Effect of different processing condition on the detectability of peanut protein by indirect competitive ELISA

3.2.1. Effect of heat treatment on binding of IgY

The effect of two different heat treatments on the detectability of peanut proteins by Chicken IgY was investigated. Fig. 2 shows
that the assay is very sensitive towards peanut proteins in PBS buffer before a heat treatment. The effect of denaturation of peanut protein was observed with heating at 80 °C and 100 °C. The gradual decrease in the peanut allergens concentration at 80 °C was due to slow kinetics and could be correlated to the loss of IgY binding due to conformational changes of peanut proteins. After a heat treatment at 80 °C for approximately 54 min the peanut protein concentration reached an almost constant value of 43.56 µg/ml. The remaining may possibly represent the heat stable fraction, which is an indication that certain fractions of the epitopes were not affected by heat and hence retained their IgY binding capacity. The effect of heat treatment at 100 °C was much more severe than that at 80 °C (Fig. 2). The loss in concentration of peanut proteins was reached approximately at 84.88 µg/ml within 12 min and remained constant for longer heating times. This may be due to the fact that some of the epitopes are heat resistant and are still available for detection by IgY using Indirect Competitive ELISA. The epitopes which are recognized by antibodies were linear epitopes while those which were not recognized by antibodies were conformational epitopes. These results are in good agreement with those of Kiio and De Meulenaer (2012), who also observed that at 100 °C approximately 90% of the peanut antigens were not available for detection by IgY antibodies.

3.2.2. Effect of pH on the detectability of peanut proteins

The effect of heating peanut antigens at 100 °C in normal, acidic and alkali conditions on IgY binding of peanut proteins was recorded at pH 4, pH 7.4 and pH 10. From the results in Fig. 3, it can be concluded that pH has an effect on the IgY binding which may be due to the conformational changes of the protein molecules because both antibodies and antigens are proteins and can be affected by the changes in pH. Both alkali and acidic conditions have almost the same effect on the binding of IgY. There was a large almost instantaneous change in the protein concentration, which may be due to the fast hydrolysis of peanut proteins in alkaline conditions. The concentration of peanut protein decreased by 86.02 µg/ml within 6 min of heat treatment in alkaline conditions, and by 84.88 µg/ml in acidic conditions, and then remained constant for up to 1 h. The denaturation of proteins in alkaline conditions was marginally faster which may be due to the fact that hydrolysis of proteins occurs faster in alkaline conditions than in acidic conditions. The effect of a heat treatment at neutral pH was also significant but took longer to reach a loss of 80 µg/ml of peanut proteins, which means that alkali and acid conditions may affect the kinetics of reaction and will greatly decrease the detectable concentration of peanut proteins when compared to heating in neutral pH. These results agree with those of De Meulenaer, Baret, Lanckriet, and Huyghebaert, (2001), Kiio and De Meulenaer (2012).

3.2.3. Effect of a reducing sugar on the assay performance

Fig. 4 shows that the effect of heating in 20 mM glucose at 80 °C was less than heating in 20 mM of glucose at 100 °C. With 20 mM at 100 °C the concentration of peanut proteins decreased to 5 µg/ml within 12 min while with 20 mM at 80 °C the concentration decreased to 42 µg/ml within 12 min. A possible explanation for this may be Maillard chemistry, a reaction between an amino acid and a reducing sugar, will proceed faster at higher temperatures. The Maillard products formed may also interfere with the IgY binding by altering the protein resulting in the antibody not being able to recognize the protein anymore. It was however observed that the Maillard reaction had a more pronounced effect at 80 °C than at 100 °C (Figs. 2 and 4), which may be due the fact that at lower temperature some of the epitopes are more vulnerable to change by Maillard chemistry.

Different processing conditions can therefore denature, alter or destroy proteins so that they are no longer detectable by assays using antibodies. Thus for processes that can completely change the protein’s immunochemical characteristics, the risk of masking the antigen is high. This is much more likely to happen when using
monoclonal antibodies which are specific for a particular protein. By using polyclonal antibodies, this risk is reduced.

4. Conclusion

Over the last few years, there has been an increasing demand for sensitive, relatively simple assays for detecting food allergens for use in basic research and clinical diagnosis. We can conclude that the indirect competitive ELISA assay format meets all of the desired criteria in many situations. Although ELISA fulfils the above mentioned requirements, optimization of the method is necessary before performing an assay on a sample. In ELISA formats there are several parameters that are common and critical to assay performance and should be considered during the optimization process, including antibody concentration, antigen concentration, incubation time of blocking, substrate solution and washing solution. In this research the optimized ELISA was obtained by fixing the concentration of primary antibody (1/5000th), secondary antibody (1/10,000th), coating antigen (10 µg/ml) and blocking incubation times to 30 min at room temperature.

Although immunoassays offer a specific, sensitive and rapid method to detect and quantify even trace amounts of allergens in foodstuffs, certain processes may denature, alter or destroy proteins so that they are no longer detectable by assays using antibodies. When using monoclonal antibodies, there is a great chance of obtaining false negatives in the detection. Polyclonal antibodies are recommended for ELISA techniques as the risk of obtaining false negatives is reduced.

There is a need to carry out more work on the biochemical and physical stability of allergenic epitopes after food processing. Interactions of proteins with other food components could lead to the masking of epitopes and hence preventing their recognition by the antibodies during immunoassay. Further work should be carried out to study the effect of different processing parameters on the detectability of peanut proteins both in a wet and in a dry food matrix. The influence of some important ingredients and additives that are of great interest include reducing sugars (both glucose and fructose), non reducing sugars, SO2, phenols and other proteins (e.g. potato powder).

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References


Fig. 4. Detectability of peanut protein after heat treatment at 80 °C in 20 mM glucose solution and 100 °C in 20 mM glucose solution. Data points are mean ± standard deviation, n = 2.