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Purification of peanut proteins for further use in affinity chromatography and as immunogens

Chickens were immunised with peanut protein extracts. Because of the high cross reactivity, the IgY antibodies were purified by means of an affinity column. An ion chromatographically purified peanut protein fraction was therefore coupled to the affinity support material and this column was used for further IgY purification.

Key Words: Peanut proteins; Chicken antibodies IgY; Ion chromatography; Affinity chromatography; Immuno blot

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1 Introduction

Peanuts and hazelnuts are widely used in the food industry. These nut proteins are responsible for allergic reactions [1] in a small group of the population yet often with fatal consequences. As the only effective measure for these individuals is avoidance, specific analytical methods are required for the screening of trace amounts of potentially allergenic proteins in food samples [2]. Immunoassays offer the tools for selective determination of specific target proteins [3–8]. Normally polyclonal antibodies from rabbits or monoclonal antibodies are used. As an alternative approach, not only providing a different antibody source but also constituting an animal friendly method, antibodies can be developed in chickens. In general antibodies of all animal species often show cross-reactivities with other proteins, e.g. anti-peanut immunoglobulins cross-react with hazelnut proteins. For further use of the antibodies in ELISA or dipstick test development they have to be purified. Anti-peanut and anti-hazelnut IgY were prepared. The anti-hazelnut IgY was used to find the specific peanut protein fraction which shows no cross-reactivities. This specific peanut protein fraction was purified by ion chromatography and coupled to a column for the affinity purification of the anti-peanut antibodies.

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Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; IgY, immunoglobulin Y; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PEG, polyethylene glycol; IC, ion chromatography; SDS, sodium dodecyl sulphate; Tris, tris(hydroxymethyl)aminomethane.

2 Materials and methods

2.1 Immunisation of (Derco brown laying) hens with peanut proteins

The protein extracts (1 g peanuts, 1 g hazelnuts extracted with 20 mL PBS, 0.01 M K_2HPO_4 , 1 M NaCl, pH 7.4 for 1 h, centrifuged, stored in aliquots) were dialysed against 0.9% NaCl overnight. The protein concentration was measured according to Bradford and diluted to 1 mg protein/mL. A 250- μ L volume of the 0.9% NaCl-protein solution was mixed with 250 μ L of Freund's complete adjuvant. The resulting emulsion was applied by intramuscular injection. Two more injections were carried out in intervals of six weeks each, this time using Freund's incomplete adjuvant. Eggs were collected (one batch = five eggs).

2.2 Preparation of antibodies

Antibodies were isolated from egg yolk by polyethylene glycol (PEG 8000, Sigma) precipitation based on a modification of Polson's method [9]. The eggs were usually used within two to three weeks after their collection, but they sustain longer storage periods at 4°C. Yolks were separated from whites, rinsed with distilled water, mixed with an equivalent volume of 0.1 M PBS, 1 M NaCl, and 0.025% NaN_3 and precipitated with 3.5% (w/v) PEG. The solution was stirred for 15 min and centrifuged (10000 g, 15 min). The supernatant (nearly clear) was filtered over several layers of gauze, the volume determined and stored frozen (–70°C) in 1-mL aliquots, whereas the precipitate was discarded.

2.3 Ion chromatography

Peanuts (10 g) were extracted with 200 mL of 20 mM K_2HPO_4 , 1 M NaCl, and 8 M urea at pH 7.0. The solution was dialysed against buffer A (mentioned below) over-

night at 4°C. The protein content was determined according to Bradford [10]. A 30-mL volume of protein solution was applied on a Q-Sepharose column (Q-Sepharose, High Load 16/10, No. 17-1064-01, Amersham Pharmacia Biotech). As starting buffer (A) 20 mM tris-bis-propane and 8 M urea at pH 7.2 was used and the elution buffer (B) was 20 mM tris-bis-propane, containing 8 M urea and 1.5 M NaCl at pH 7.2. A step gradient from 0, 5, 10, 15 to 100% buffer B was run at a flow rate of 1 mL/min; 2-mL fractions were collected.

2.4 Electrophoresis and blotting

SDS electrophoresis was performed with 15% acrylamide gels. The gels contained no SDS but the running buffer contained 0.5 g SDS in addition to TRIS and glycine. As standard, SDS-6H from Sigma was used which contains proteins with molecular weights between 30 000 and 200 000 Da. Separation gel buffer: 1.5 M Tris/HCl pH 8.8; sampling gel buffer: 0.5 M Tris/HCl pH 6.8; SDS separation buffer: 1.5 g Tris, 7.2 g glycine, 0.5 g SDS to 1 L; silver-staining according to Pharmacia.

2.5 Affinity chromatography

Three runs from the IC-fractionated (see Section 2.3) peanut proteins (dialysed against water and freeze-dried) were coupled to 2 g epoxy-activated Sepharose (Epoxy-activated Sepharose® 6B, Amersham Pharmacia Biotech) according to the procedure issued by Pharmacia and packed into a glass column (8 mL, Merck 1-19828.0001). Whole anti-peanut IgY solution (500 µL) was applied onto the column at a flow rate of 0.1 mL/min. The non-bound antibodies were washed from the column with 0.1 M K₂HPO₄ buffer pH 7.2. The bound antibodies were eluted with 0.1 M glycine at pH 2.5. 1 M Tris-solution pH 8.5 (1–2 mL) was used for immediate neutralisation of the eluted antibody.

2.6 Immunoblot

Nut extracts (2 µL; extractions as described in Section 2.1; peanut: 9.35 mg protein/mL, hazelnut: 6.89 mg protein/mL determined according to Bradford) were spotted in different dilutions on the nitrocellulose membrane with a Hamilton syringe, dried for 10 min, and then blocked with 3% milk powder in PBS. The membrane was washed three times for 10 min with 0.1% Tween20 in PBS. After washing the membrane was incubated with anti-nut antibodies (in PBS with 0.1% BSA) for 30 min, washed again three times, and then incubated with the rabbit-anti-chicken-IgG-HRP (1 : 10 000 with 0.1% BSA in PBS, VWR international). The staining procedure was done according to Vieths et al. [11] with tetramethylbenzidine.

3 Results and discussion

Because of cross-reactivities, the anti-peanut IgY cannot be used directly for further ELISA development. The strategy adopted was to extract peanut protein in high quantities, separate different protein fractions by ion chromatography, couple a protein fraction to an affinity gel, and purify the antibodies with this column. For further use in ion chromatography the peanut proteins were extracted with a PBS buffer with addition of 8 M urea [12, 13]. The addition of urea to the peanut extraction procedure improved the solubility of the proteins. It was possible to extract about 15 mg protein/mL solution. This protein solution was used for the ion chromatographic separation. Q-Sepharose was used for further separation of the proteins. On running a step gradient from 0–15% NaCl, 6 different fractions appeared (**Figure 1**). The peaks were collected and characterised with SDS-Page. The collected peaks from the ion chromatographic separation step were tested for cross-reactivities with an anti-hazelnut IgY antibody (prepared as described for the peanut antibody in Section 2.1). All fractions were spotted onto a nitrocellulose membrane (data not shown) and no cross reactivities occurred for fraction D, E, and F with the anti-hazelnut IgY. Subsequently fraction E was chosen for further processing. Fraction E from 3 runs was pooled, dialysed against water, lyophilised (262 mg protein after dialysis and lyophilisation), and used for further coupling to epoxy-activated Sepharose 6B. The anti-peanut IgY antibodies were purified with this immunoaffinity column. After eluting the antibodies at pH 2.5 from the column, neutralising with 1 M Tris pH 8.5, the purified anti-peanut IgY antibodies were compared to whole anti-peanut IgY in an immuno dot blot experiment with peanut and hazelnut protein (protein extracts prepared as mentioned in Section 2.1). **Figure 2** shows the used ELISA format. A comparison of the whole anti-peanut IgY and the affinity-purified

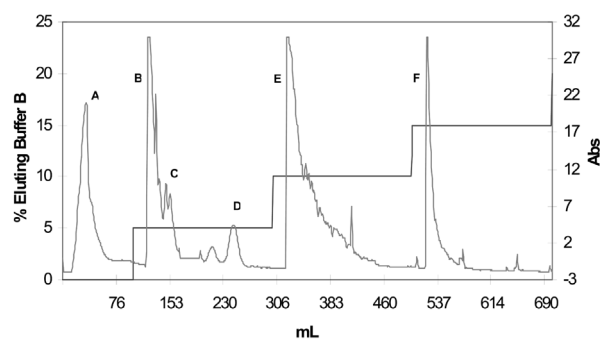


Figure 1. Ion chromatographic separation of a peanut extract. Column: High Load 16/10 Q-Sepharose; starting buffer A: 20 mM bis-tris-propane, 8 M urea, pH 7.2; elution buffer B: 20 mM bis-tris-propane, 1.5 M NaCl, 8 M urea, pH 7.2; flow rate: 1 mL/min; step gradient: 0%, 5%, 10%, 15%, and 100% B [not shown, for column cleaning]; detection: UV 280 nm; fraction collection: 2 mL; A–F collected peaks.

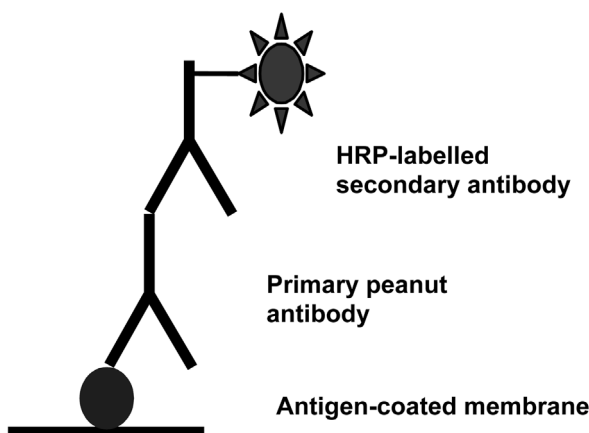


Figure 2. The ELISA format used for the dot blot experiment. Nut protein was dotted onto the nitrocellulose membrane. Whole anti-peanut IgY and affinity-purified anti-peanut IgY were used as primary antibodies. HRP-labelled rabbit anti-chicken IgG was used as secondary antibody.

anti-peanut IgY showed that the purified anti-peanut IgY is still active although the antibodies were subjected to a more or less very harsh purification procedure. The cross-reactivities to hazelnut protein were completely removed. This purified antibody can be used for further ELISA or dipstick test development.

4 Concluding remarks

Two different strategies are now followed. On the one hand, the already available cross-reactive antibodies will be purified with this immunoaffinity column and used for the development of, e. g., a dipstick test. And on the other hand, new immunisations of chickens will be done with IC-

purified peanut protein fraction. It should therefore be possible to exclude cross-reactivities already at the immunisation stage.

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