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Analytical Methods

Comparison of different extraction solutions for the analysis of allergens in hen's egg

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Abstract

An important requirement for the correct procedure of allergen analysis in hen's egg is to obtain complete and unaltered protein extracts. Besides the aim of a quantitative extraction of the allergens from the matrix, it is equally important not to alter their allergenic potential during the extraction process. This paper describes and compares six extraction solutions for the analysis of whole-egg proteins and allergens. These requirements were examined via protein determination according to Bradford [Bradford, M. M. (1976). Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein–dye binding. *Analytical Biochemistry, 72*, 248–254] and Kjeldahl [Meyer, A. H. (2006). Lebensmittelrecht, Verlag C.H. Beck München, Stand: 1. February 2006, § 64, Lebensmittel- und Futtermittelgesetzbuch, Amtliche Sammlung von Untersuchungsmethoden, Nr. L 06.00-7] as well as the EAST-inhibition method. It could be demonstrated that the extraction with a urea solution (8 M) led to significant interferences during the protein determination, and substantially reduced the allergenic potential of egg proteins. With all other extraction solutions adequate protein contents could be extracted. The highest protein content was achieved by the extraction with phosphate buffered saline followed by a Tween 20 solution, physiological saline, water, and acetate buffer. The results show that none of these extracts – except for the urea solution (8 M) – was altered in its' allergenic potential.

Keywords: Protein extraction; Hen's egg; Food allergy; IgE binding; EAST-inhibition

1. Introduction

Hen's egg is one of the most frequent causes of adverse reactions to food for children (Bernhisel-Broadbent, Dintzis, Dintzis, & Sampson, 1994; Crespo, Pascual, Burks, Helm, & Esteban, 1995) and adults (Nørgaard & Bindslev-Jensen, 1992; Wüthrich, 1993). Major allergenic proteins are located in egg white. These are ovalbumin (OA), conalbumin (CA), ovomucoid (OM), and lysozyme (LY), which represent about 80% of egg white proteins. However, the allergenicity of these major allergenic proteins is not known in all detail. The first requirement for the correct research procedure is to obtain complete and unaltered protein extracts. For the analysis of allergens in egg it is inevitable not only to extract the allergens out of the matrix quantitatively and reproducibly, but also not to alter their allergenic potential during the extraction process. In the case of major egg allergens several extraction methods have been developed to date. In the case of animal foods good extracts have often been obtained with the simple incubation of the respective food in a buffer solution to extract proteins contained in the raw material (Pastorello & Trambaioli, 2001). As reported by Langeland (1982a,b) hen's egg antigen solution was prepared by stirring crude egg with an equal volume of physiological saline (0.15 M) for 4 h at room temperature; after centrifugation, the supernatant was stored and used for immunological examination. The method was substantially confirmed by the study of Bernhisel-Broadbent et al. (1994), in which egg was extracted by overnight incubation at room temperature with phosphate

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buffered saline (PBS) followed by a centrifugation. A protocol from Wittemann, Akkerdas, van Leeuwen, van der Zee, and Aalbersee (1994) included the extraction at room temperature in water maintained at pH 8 for 4 h, followed by dialysis and lyophilization of extracted proteins. A further method for protein extraction was described by Berkelmann and Stenstedt (1998). For this, proteins were extracted with a urea solution (8 M) at <37 °C, followed by a centrifugation. Equal volumes of the sample and acetate buffer (0.1 M) were used in a study by Hirose, Kitabatake, Kimura, and Narita (2004) for the protein extraction. This was followed by an over night dialysis. Fernández, Padilla, and Mucciarelli (1999) described a protein extraction method using Tween 20 (0.2%) as the extraction solution. A further method for the isolation of proteins that is based on an ion-exchange column chromatography is described by Martinez, Fernandez-Rivas, Martinez, and Palacios (1997). Since this method results in the loss of proteins with an isoelectric point (p*I*) higher than 8.8, it is not applicable for hen's egg allergen analysis, because lysozyme has a p*I* of 10.7. Precipitation methods for the isolation of the whole proteins with high salt concentrations or with organic solvents are also described (Désormeaux, Blochet, Pézolet, & Marion, 1992). A consistent loss of material is typical for this kind of separation of proteins out of the matrix (Vieths, Schöning, & Petersen, 1994). Within previous studies (Bearden, 1977; Compton & Jones, 1985; Merck, 2006; Pierce & Suelter, 1977; Sedmak & Grossberg, 1977; Spector, 1978) the following concentrations of extraction solutions caused protein assay interferences: sodium chloride (>5 M), Tween 20 (>0.5 M), sodium acetate (>0.5 M), and urea (>6 M). Some chemicals are known to have the ability to denature protein structures (Tal, Silberstein, & Nusser, 1980). Denaturation of allergens affects their conformational structure, which may destroy or lay open IgE-binding epitopes and thus alter the allergenic potential of the egg white proteins (Besler, Steinhart, & Paschke, 2000).

Although the determination of the allergenic potential of the extracted proteins was the primary aim, it was also a goal of this study to isolate the whole egg protein fraction with a sufficiently high protein concentration, so that it could be used as the starting material for further egg allergen research. In this work, six different extraction methods for the analysis of egg allergens were modified and compared. In order to make the obtained results more comparable, the method from Langeland (1982a,b) was used with the six different extraction solutions described above. Quantitative results were assured by using an egg/extraction solution volume ratio of 1:10 and repeating every extraction with the residues. The protein content and the allergenic potential of these extracts were studied. All concentrations of the extraction solutions used within this study were lower than the concentrations that caused assay interferences in further studies described above, except for the urea. As urea (8 M) is recommended by Berkelmann and Stenstedt (1998) especially for allergen extraction it was included in this study.

2. Experimental

2.1. Reagents and materials

Physiological saline was prepared from 0.1 M NaCl (Merck KGaA, Darmstadt, Germany). PBS was prepared from 0.15 M NaCl and 0.01 M K_2HPO_4 (Merck KGaA, Darmstadt, Germany) at pH 7.4. Bidestilled water was brought to pH 8 with 0.1 M NaOH (Merck KGaA, Darmstadt, Germany). Urea solution was prepared from 8 M urea (Merck KGaA, Darmstadt, Germany). Acetate buffer was prepared from 0.1 M sodium acetate (Merck KGaA, Darmstadt, Germany) and brought to pH 3.8 with 4 M HCl (Merck KGaA, Darmstadt, Germany). Tween 20 solution was prepared with 0.2% Tween 20 (Serva GmbH, Heidelberg, Germany) in bidestilled water. If not otherwise mentioned, all chemicals were of analytical grade.

2.2. Patient sera

Patient sera were collected and pooled from 11 patients with an egg allergy and a positive EAST (enzyme allergosorbent test, Spez. IgE ELISA RV 5, Allergopharma, Reinbek, Germany), class 2–5 for whole egg.

Patients were procured by the Technical University of Munich (Department of Dermatology and Allergology), the University Hospital of Zurich (Allergiestation, Dermatologische Klinik), the Macedonio Melloni Hospital of Milan (Department of Pediatrics), and the University of Vienna (Department of Pediatrics and Juvenile Medicine).

2.3. Egg extraction methods

Ten whole hen's (*gallus domesticus*) eggs were homogenized by stirring on a magnetic stirrer for 10 min. The homogenate was suspended in six different extraction solutions (physiological saline, PBS, water, urea, acetate buffer and Tween 20) (see Section 2.1, Table 1 and Section 1) at a 1:10 volume ratio, respectively, stirred for 4 h at room temperature, and insoluble residues were removed by centrifugation at 1000*g* for 30 min (extraction 1). The residues were resuspended and extracted again in the same manner to assure a quantitative extraction of the egg proteins (extraction 2). Every extraction process was performed in triplicate. As a control a suspended and not extracted egg/water homogenate (1:10 v/v) was used.

2.4. Protein determination

Protein concentrations were determined relatively according to the method of Bradford (1976) using bovine serum albumin as the standard and Bradford reagent consisting of Coomassie Brilliant Blue G-250 and phosphoric acid. In addition, absolute protein concentrations were determined with the method of DIN EN 25663 – H 11 (Kjeldahl) (Meyer, 2006) by analysing the nitrogen content. The conversion factor from nitrogen to protein was 6.25.

Table 1
Methods for protein extraction out of raw material

Nr.	Extraction solution	Volumes	Extraction time	Extracting temperature	Additional preparation	Literature
1	Physiological saline (0.15 M)	1:1	4 h	Room temperature	Centrifugation (30 min, 2500 r/min)	Langeland (1982a,b)
2	PBS (pH 7.4)	1:1	Overnight	Room temperature	Centrifugation (10 min, 2500*g*)	Bernhisel-Broadbent et al. (1994)
3	Water (pH 8)	–	4 h	Room temperature	Dialysis and lyophilization	Wittemann et al. (1994)
4	Urea (8 M)			<37 °C	Centrifugation	Berkelmann and Stenstedt (1998)
5	Acetate buffer (0.1 M, pH 3.8)	1:1	–	–	Dialysis (over night) centrifugation	Hirose et al. (2004)
6	Tween 20 (0.2%)	–	–	–	–	Fernández et al. (1999)
	Extraction solutions 1–6	1:10	4 h	Room temperature	Centrifugation (30 min, 1000*g*)	This study

2.5. EAST-inhibition

IgE-binding capacity was analysed with EAST-inhibition using sera from 11 patients with hen's egg-specific IgE. As a control an unextracted hen's egg-protein solution in water was used. For the EAST-inhibition assay an egg protein control solution (see Section 2.3) was linked to bromcyane activated paper discs (Schleicher & Schüll, Dassel, Germany) using a modified method from Ceska and Lundkvist (1972). 50 µl of patients serum pool (see Section 2.2, diluted 1:2), previously incubated with different concentrations of protein extracts of the particular extraction solution or control solution (without protein content) were subsequently added to the discs and incubated for three hours at room temperature in cavities of a microtiter plate (Minisorb, 96 cavity, Nunc™, Roskilde, Denmark). An Allergopharma (Reinbek, Germany) test kit (enzyme allergosorbent test, Spez. IgE ELISA RV 5) was used for the EAST-inhibition according to the manufacturer's recommendations with modifications. For this, free binding sites were blocked with ethanolamine for 1 h. A dilution series of the inhibitor-extracts (containing egg proteins extracted with the six different extraction solutions) was prepared in seven steps (undiluted, 1:10, 1:100, 1:1000, 1:10,000, 1:100,000, 1:1,000,000). Potato protein was used to check non-specific inhibition. A total of 50 µl of diluted pool serum was added and incubated for 1 h at 37 °C in the dark. After three washes with 1% Tween 20 in PBS, 50 µl of anti-human IgE alkaline phosphatase conjugate (Allergopharma, Reinbek, Germany) diluted 1:200 in incubation buffer were added and incubated for 1.5 h at 37 °C in the dark. The plates were washed again and the bound enzyme activity was stained with 200 µl of staining solution (containing *p*-nitrophenylphosphate (PNPP)) for 1 h at 37 °C in the dark. After the addition of 100 µl of stopping solution (1 M NaOH) the absorbance was measured at 405 nm. All EAST-inhibition experiments were performed in duplicate and data were given in mean values.

3. Results and discussion

3.1. Quantification of proteins

Tables 2a and b show the averaged relative protein contents per gram hen's egg of the six different extracts and the control as well as the protein content as the percentage of the control including the individual results of extractions 1 and extractions 2 (see Section 2.3) measured with the method of Bradford (1976). Since the extractions 2 resulted in protein contents of only 5–10% of that of extractions 1, respectively, a third extraction was not performed. The determined mean value of the control is 40.42 mg/g. The protein content of hen's egg given in the literature (Souci, Fachmann, & Kraut, 1991) is 106–124 mg/g. Hence the protein content of the control measured with the method according to Bradford (1976) is approximately one third of the protein content given in the literature (Souci et al., 1991). The reason for this loss of proteins is certainly due to the method of protein detection according to Bradford (1976). As a consequence of different response factors of various proteins, this method only provides a relative but not an absolute quantification of the proteins. According

Table 2a
Protein contents of extraction 1, 2, and total for different extraction solutions in mg/g

Nr.	Extraction solution	Protein content extraction 1 (mg/g)	Protein content extraction 2 (mg/g)	Total protein content (mg/g)
1	Physicological saline (0.15 M)	27.85 (±2.15)	1.56 (±8.33%)	29.41 (±1.76%)
2	PBS	28.88 (±1.83%)	1.80 (±5.96%)	30.68 (±1.31%)
3	Water	23.87 (±4.01%)	2.58 (±2.22%)	26.45 (±3.74%)
4	Urea (8 M)	57.59 (±1.55%)	3.37 (±4.03%)	60.96 (±1.67%)
5	Acetate buffer (pH 3.8, 0.1 M)	23.18 (±1.58%)	2.47 (±1.65%)	25.65 (±1.35%)
6	Tween 20 (0.2%)	27.99 (±0.70%)	1.77 (±1.92%)	29.76 (±0.55%)
7	Control	–	–	40.42 (±2.12%)

Table 2b
Protein contents of extraction 1, 2, and total for different extraction solutions in %

Nr.	Extraction solution	Averaged protein content extraction 1 (%)	Averaged protein content extraction 2 (%)	Averaged total protein content (%)
1	Physicological saline (0.15 M)	68.9	3.9	72.8
2	PBS (pH 7.4)	71.5	4.5	75.9
3	Water (pH 8)	59.1	6.4	65.4
4	Urea (8 M)	142.5	8.3	150.8
5	Acetate buffer (pH 3.8, 0.1 M)	57.4	6.1	63.5
6	Tween 20 (0.2%)	69.3	4.4	73.6
7	Control	–	–	100

to a study by Tal et al. (1980), the calibration of the Bradford (1976) method with the commonly used standard bovine serum albumin gives underestimated values of mass for most proteins. This assumption is supported by the presented results. Since the Bradford (1976) method gives reproducible and rapid results for a relative quantification of the same kind of proteins, it is well applicable for these investigations. In order to assure these results of relative protein contents, the total protein content of the control was measured with the method of DIN EN 25663 – H 11 (Kjeldahl) (Meyer, 2006). The averaged absolute protein content of the control was 110.71 mg/g (±1.92%) and consequently within the range of 106–124 mg/g given in the literature (Souci et al., 1991). Thus, the relative protein content of the control measured with the method of Bradford (1976) was designated as 100% and the relative protein contents of the six extracts measured by Bradford were referred hereto. Fig. 1 shows the absolute protein contents of the six different extraction solutions based on the protein content of the control. The variation coefficients of the triple determinations are between 0.55% and 3.74% (see Table 2a). Consequently the standard deviation of the particular mean value is in an adequate range for every extraction method.

At 150.8% in relation to the control, the protein content of the urea extract is unrealistically high (see Fig. 1, No. 4). All other extracts besides the urea extract provide protein contents of less than 100% in relation to the control. The significantly highest protein content – with a mean value of 75.9% – was determined in the extraction with PBS and the second highest with Tween 20 extraction, whereas the lowest protein content could be measured in the acetate buffer extract at averaged 63.5%.

The method for the determination of the relative protein content according to Bradford (1976) is based on the protein binding properties of the dye Coomassie Brilliant Blue G-250. The binding of the dye to the proteins causes a shift in the absorption maximum of the dye from 465 to 595 nm, and it is the increase in the absorption at 595 nm which is monitored (Bradford, 1976). Dye binding requires a macromolecular form with certain reactive functional groups (Compton & Jones, 1985). Assay interferences by bases, detergents, and other components are explained (Bearden, 1977; Compton & Jones, 1985; Merck, 2006; Pierce & Suelter, 1977; Sedmak & Grossberg, 1977; Spector, 1978). Table 1 shows the concentrations of the reagents that were used to establish the extraction solutions within the scope of this study. In comparison to that, Table 3 shows concentrations of these solutions that caused assay interferences in different studies. This comparison clearly indicates that in this study extraction solutions containing NaCl, Tween 20 or sodium acetate were used in much lower concentrations than those causing assay interferences in other studies. Thus, it could be assumed that no assay interferences were caused by these extraction solutions within the scope

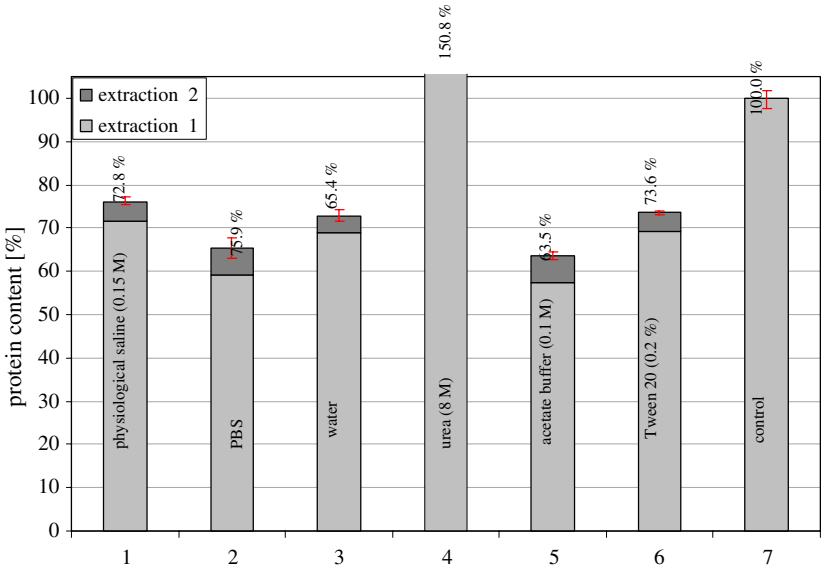

Fig. 1. Protein contents of extraction solutions in comparison to the control.

Table 3
Concentrations of reagents that cause assay interferences in comparison to the concentrations used in this study (Bearden, 1977; Compton & Jones, 1985; Merck, 2006; Pierce & Suelter, 1977; Sedmak & Grossberg, 1977; Spector, 1978)

Reagent	Concentration that causes assay interferences	Concentrations used in this study
NaCl	>5 M	0.15 M
Tween 20	>0.5%	0.2%
Sodium acetate	>0.5 M	0.1 M
Urea	>6 M	8 M

Table 4
C_{50}-values of EAST-inhibitions of the extraction solutions in comparison to the control

Reagent	C_{50}-value (μg/ml)
Control	0.4
Water	0.4
NaCl	0.4
Tween 20	0.4
Urea	26.3
PBS	0.4
Acetate buffer	0.4

of this study. By contrast, in this study an extraction solution suggested for allergen extraction by Berkelmann and Stenstedt (1998) was used with a urea solution in a concentration higher than 6 M that is known to cause assay interferences according to Merck (2006). In order to exclude the possibility of higher measured values caused by interactions of urea with other components besides proteins during the dye-reaction, a blank value of urea with the Bradford (1976) method was measured and subtracted from the measured protein value of urea extraction. This urea solution blank value amounts less than 0.3% of the urea protein extract solution. Fig. 1 demonstrates the protein content of the urea extraction after the subtraction of the urea blank. In conclusion, these results of protein contents of more than 100% must be caused by interactions between urea and proteins in the presence of Coomassie Brilliant Blue G250. Detailed determinations could not be performed in this study.

3.2. Quantification of allergenicity

The EAST-inhibition was accomplished for the quantification of the relative allergenic potential of the six protein extracts in relation to the control. Fig. 2 shows the inhibition curves of the extracts and the control. Table 4 shows the corresponding C_{50}-values. The higher the C_{50}-values, the lower the allergenic potential of the protein extract is. The C_{50}-value of the inhibition curve of the urea extract is 26.3 μg/ml. C_{50}-values of all other inhibition curves – including that of the control – are 0.4 μg/ml. Consequently the allergenic potential of the urea extract is reduced more than 65-fold compared to that of the control, whereas the allergenic potentials of the other extracts correspond to that of the control. Thus, it becomes apparent that the allergenic potential of the urea extract significantly decreased during the extraction procedure. Urea (>6 M) has the ability to convert egg white proteins to a molten globule state, which is partially denatured but retains its' native-like structure (Tal et al., 1980). While urea treatment showed no consequences to IgE binding of ovalbumin and ovomucoid, conalbumin and lysozyme showed significantly higher binding activities to human IgE in a study by Mine and Zhang (2002). These results can not be supported, as the urea (>6 M) treatment of egg proteins in this study resulted in a strong decrease of binding activities to human IgE in relation to the untreated control.

4. Conclusion

On the basis of these results, PBS is the most qualified for the extraction of hen's egg allergens amongst the six tested extraction solutions. With PBS proteins could be

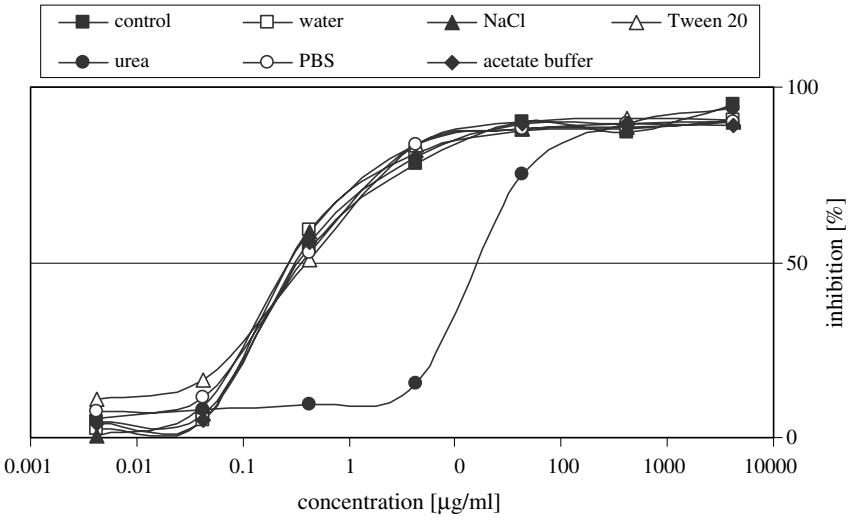

Fig. 2. EAST-inhibition of IgE-binding to hen's egg proteins with extraction solutions as inhibitors.

extracted out of the matrix quantitatively and reproducibly without an alteration in their allergenic potential during the extraction process. Physiological saline (0.15 M), water, acetate buffer (0.1 M) and Tween 20 solution are also applicable but less quantitative than PBS. Urea (8 M) has the ability to reduce the allergenicity of hen's egg allergens and is therefore not qualified as an extraction solution for the analysis of hen's egg allergens. In addition to that, urea (8 M) leads to overestimations of proteins in the Bradford assay.

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References

Bearden, J. C. Jr. (1977). Quantitation of submicrogram quantities of protein by an improved protein–dye binding assay. *Biochimica and Biophysica Acta, 533*(2), 525–529.

Berkelmann, T., & Stenstedt, T. (1998). *2-D Electrophoresis using immobilized pH gradients, principles & methods*. Uppsala, Sweden: Amersham Pharmacia Biotech.

Bernhisel-Broadbent, J., Dintzis, H. M., Dintzis, R. Z., & Sampson, H. A. (1994). Allergenicity and antigenicity of chicken egg ovomucoid (Gal d III) compared with ovalbumin (Gal d I) in children with egg allergy and in mice. *Journal of Allergy and Clinical Immunology, 93*, 1047–1059.

Besler, M., Steinhart, H., & Paschke, A. (2000). Allergens of animal origin. Stability and allergenicity of processed foods. *Internet Symposium on Food Allergens, 2*(4), 171–184.

Bradford, M. M. (1976). Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein–dye binding. *Analytical Biochemistry, 72*, 248–254.

Ceska, M., & Lundkvist, U. (1972). A new and simple radioimmunoassay method for the determination of IgE. *Immunochemistry, 9*, 1021–1030.

Compton, S. J., & Jones, C. J. (1985). Mechanism of dye response and interference in the Bradford protein assay. *Analytical Biochemistry, 151*, 369–374.

Crespo, J. P., Pascual, C., Burks, A. W., Helm, R. M., & Esteban, M. M. (1995). Frequency of food allergy in a pediatric population from Spain. *Pediatric Allergy and Immunology, 6*, 39–43.

Désormeaux, A., Blochet, J. E., Pézolet, M., & Marion, D. (1992). Amino acid sequence of a non-specific wheat phospholipid transfer protein and its conformation as revealed by infrared and Raman spectroscopy. Role of disulfide bridges and phospholipids in the stabilization of the alpha-helix structure. *Biochimica and Biophysica Acta, 1121*, 137–152.

Fernández, S. S., Padilla, A. P., & Mucciarelli, S. (1999). Protein extraction from Atriplex lampa leaves: potential use as forage for animals used for human diets. *Plant Foods for Human Nutrition, 54*(3), 251–259.

Hirose, J., Kitabatake, N., Kimura, A., & Narita, H. (2004). Recognition of native and/or thermally induced denatured forms of the major food allergen, ovomucoid, by human IgE and mouse monoclonal IgG antibodies. *Bioscience, Biotechnology, and Biochemistry, 68*(12), 2490–2497.

Langeland, T. (1982a). A clinical and immunological study of allergy to hen's egg white. II. Antigens in hen's egg white studied by crossed immunoelectrophoresis (CIE). *Allergy, 37*(5), 323–333.

Langeland, T. (1982b). A clinical and immunological study of allergy to hen's egg white. III. Allergens in hen's egg white studied by crossed radio-immunoelectrophoresis (CRIE). *Allergy, 37*(5), 521–530.

Martinez, A., Fernandez-Rivas, M., Martinez, J., & Palacios, R. (1997). Improvement of fruit allergenic extracts for immunoblotting experiments. *Allergy, 52*(2), 155–161.

Merck. (2006). *Methodenbeschreibung, 1.10306. Bioquant® Protein (nach Bradford-Methode)*. Merck KGaA, 64271 Darmstadt, Germany.

Meyer, A. H. (2006). Lebensmittelrecht, Verlag C.H. Beck München, Stand: 1. February 2006, § 64, Lebensmittel- und Futtermittelgesetzbuch, Amtliche Sammlung von Untersuchungsmethoden, Nr. L 06.00-7.

Mine, Y., & Zhang, J. W. (2002). Comparative studies on antigenicity and allergenicity of native and denatured egg white proteins. *Journal of Agricultural and Food Chemistry, 50*, 2679–2683.

Nørgaard, A., & Bindslev-Jensen, C. (1992). Egg and milk allergy in adults. *Allergy, 47*, 503–509.

Pastorello, E. A., & Trambaioli, C. (2001). Isolation of food allergens. *Journal of Chromatography B: Biomedical Sciences and Applications, 756*(1), 71–84.

Pierce, J., & Suelter, C. H. (1977). An evaluation of the Coomassie Brilliant Blue G-250 dye-binding method for quantitative protein determination. *Analytical Biochemistry, 81*(2), 478–480.

Sedmak, J. J., & Grossberg, S. E. (1977). A rapid, sensitive, and versatile assay for protein using Coomassie Brilliant Blue G250. *Analytical Biochemistry, 79*, 544–552.

Souci, S. W., Fachmann, W., & Kraut, H. (1991). *Lebensmitteltabellen für die Praxis*. Stuttgart: Wissenschaftliche Verlagsgesellschaft mbH.

Spector, T. (1978). Refinement of the coomassie blue method of protein quantitation A simple and linear spectrophotometric assay for less than or equal to 0.5 to 50 microgram of protein. *Analytical Biochemistry, 786*(1), 142–146.

Tal, M., Silberstein, A., & Nusser, E. (1980). Why does Coomassie Brilliant Blue® interact differently with different proteins? *Journal of Biological Chemistry, 260*, 9976–9980.

Vieths, S., Schöning, B., & Petersen, A. (1994). Characterization of the 18-kDa apple allergen by two-dimensional immunoblotting and microsequencing. *International Archives of Allergy and Immunology, 104*, 399–404.

Wüthrich, B. (1993). Zur Nahrungsmittelallergie – Häufigkeit der Symptome und der allergieauslösenden Nahrungsmittel bei 402 Patienten. *Allergologie, 16*, 280–287.

Wittemann, A. M., Akkerdas, J. A., van Leeuwen, J., van der Zee, J. S., & Aalbersee, R. C. (1994). Identification of a cross-reactive allergen (presumably tropomyosin) in shrimp, mite and insects. *International Archives of Allergy and Immunology, 105*, 56–61.