

Antigenic and Allergenic Determinants of Ovalbumin

I. Peptide Mapping, Cleavage at the Methionyl Peptide Bonds and Enzymic Hydrolysis of Native and Carboxymethyl OA

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Abstract. The effects of enzymic cleavage and perturbing the conformation of the allergenic and antigenic determinants of hens egg white albumin (OA) were examined. Hens egg white extract of a total protein concentration 8.43 g/l was prepared. Isoelectric focusing in sodium dodecyl sulfate and polyacrylamide gel peptide maps for the crude egg white extract showed 26 spots visualized by staining with Coomassie blue. The OA was purified using a TSK-2000 gel filtration chromatography column. The specific allergenic reactivity of the purified OA as measured by RAST inhibition and direct RAST was relatively high: 3 µg gave an inhibition of approximately 10%.

The cleavage of OA with cyanogen bromide resulted in 4 fractions, all capable of binding specific IgE with the first peak showing the highest inhibition. Thermal denaturation of OA had no direct effect on the antigenic reactivity. RAST inhibition values for the denatured protein were similar to those of the native protein. Carboxymethylation of OA gave a product with only 20% of the inhibition reactivity. Further treatment with trypsin did not abolish the allergenic and antigenic reactivities as shown by RAST inhibition and by deflection of OA line in rocket line immunoelectrophoresis. On the other hand, limited pepsin hydrolysis destroyed the antigenic structure of the molecule. The reactivity of OA is thus relatively stable and could easily be retained making it possible to identify the allergenic determinants of enzymic hydrolysates used for elucidating the antigenic structure of the molecule.

Introduction

Hypersensitivity to different foods is a common cause of a variety of symptoms including asthma, rhinitis, atopic dermatitis and others [7]. Egg white is one of the important food items that frequently induce hypersensitivity, particularly in atopic children [21]. Hens egg has been used as a nutrient throughout the evolution of man. It is the most 'complete' animal food naturally processed for the growth of the embryo [26]. Egg is today an inherent ingredient in human and animal food and it is most difficult to be totally excluded from meals.

Egg allergy has been a subject of study for many decades [22, 25]. It has been clearly shown that among 40 different egg white proteins only 3 are major allergens, namely ovalbumin (OA), ovomucoid and ovomotransferrin [19-22]. OA is the most abundant of

the proteins, forming 58% w/w of the whole egg white. A crossed immunoelectrophoresis (CIE) reference precipitation pattern for hens egg white and a corresponding crossed radioimmunoelectrophoresis (CRIE) have recently been presented [19-20].

The function of OA is not known; it has recently been suggested that it is a good substrate for protease, and its role may involve its limited proteolysis by elastase or elastase-like enzymes [28]. The data on the complete primary structure of hens OA (385 residues, Mr 42,699) are available [24] and the antigenic structures of several proteins have been determined [1-4, 9, 10, 23]. Furthermore, OA is a model protein widely used in protein characterization and as a model immunogen particularly in studies on regulating reaginic IgE response in rodents [5, 16-18]. It is of importance that the antigenic structure of a protein as used widely as OA is studied. The present paper is the

first in a series exclusively directed towards this purpose. The effect of perturbing the native conformation and enzymic cleavage on the allergenic and antigenic reactivities are the main points examined in this paper. Furthermore, a finger print of OA and the hydrolysis products were performed and the experimental basis for the isolation and immunochemical examination of the purified peptides settled.

Material and Methods

Crude Hens Egg White

Freshly laid middle size eggs were collected from healthy white 'Gallus domesticus' hens from one of the provincial farms. Extract was prepared without delay by separating the crude white of 20 eggs [19]. The egg white was added to an equal volume of sterile physiological saline and the mixture was gently shaken at 45°C for 4 h. Then it was centrifuged at 6,000 g for 30 min. The thin layer (supernatant) was collected and divided into 4-ml portions in polystyrene tubes and stored at -20°C.

OA was purchased from Sigma Chemical Co., USA, grade VII, salt-free and free of S-OA, A 7641. OA was further purified on ACA 54 GFC-column (LKB, Bromma, Sweden). The column dimensions were 100 × 1.4 cm. The eluent buffer was 50 mmol ammonium bicarbonate containing 0.01% sodium azide. Two peaks were obtained, a major large one and a small salt/contaminant peak. The first peak was pooled, lyophilized and stored at -20°C.

Sera

Rabbit antibodies against egg white extract and OA were prepared as described [14]. Antisera were obtained from 4 animals which were intradermally injected with 2 mg of crude egg white extract or OA in Freund's complete adjuvant. The injections were repeated 3 times with 2 weeks interval in Freund's incomplete adjuvant. The animals were bled on day 10 after the last injection. IgE antibodies against egg white were obtained from 7 patients with clinical histories of food allergy including egg (RAST 2-4). The patients had not received desensitization therapy. The specific and total serum IgE were analyzed either by paper discs coated with OA or Pharmacia discs (Uppsala, Sweden).

RAST and Inhibition Experiments

OA allergosorbents were prepared by cyanogen bromide (CNBr)-activated paper discs by a previously described procedure [29]. 10 CNBr-activated paper discs were incubated with the allergen at a concentration of 1 mg/ml. For RAST inhibition, 50- μ l aliquots (1 + 1) diluted of egg allergic sera were incubated for 4 h at room temperature with serial dilutions of the allergen in question prior to coupling to the allergosorbents. These were used both for direct test or inhibition of ¹²⁵I-anti IgE binding to immobilized antibodies.

CNBr Cleavage

This was performed as described previously [12]. Samples of 1 μ mol OA and 1.7 mmol CNBr (art. 820193, Merck, FRG) in 70% v/v formic acid were taken. The fragments were fractionated on a P-6 column (d. 50 × 1.4 cm; Biorad, Calif., USA).

Denaturation

OA at a concentration of 25 nmol/ml was prepared in 4 M guanidine HCl or 8 M urea, respectively. The ultraviolet spectra were registered in the wavelength range of 240-325 nm.

Carboxymethylation and Reaction of Iodoacetate with Sulfhydryl Groups

Carboxymethylation of 2 μ mol OA was performed at pH 8.5 at 25°C under nitrogen. The pH was kept constant by triethylamine. 100 μ l mercaptoethanol was added and the pH adjusted to 8 by 2 M NaOH and kept under nitrogen for 4 h. The reaction was then proceeded in dark after the addition of 270 mg iodoacetic acid in 1 ml 1 M NaOH. The pH was kept at 8 for 15 min, then lowered to 4 by addition of 10% acetic acid. The reaction product was dialyzed (membrane cut-off 8,000 D) and finally lyophilized [13].

Enzymic Hydrolysis

Trypsin, dicyclohexylcarbodiimide-treated type XI, T-1005 (Sigma) was utilized. Extensive hydrolysis was performed using 0.5 μ mol carboxymethylated OA in ammonium bicarbonate, pH 8.0 (E/S 1:100) for 16 h at 37°C under gentle stirring. Pepsin hydrolysis was performed using pepsin from porcine stomach mucosa (lot No. 53F-8110; Sigma). The pH was adjusted to 3.5 and the reaction proceeded at an E/S ratio of 1/25 at 37°C with gentle shaking in pyridine acetate buffer. The reaction was stopped by dropwise addition of pyridine until pH 5.0 and the hydrolysate was lyophilized. Protease hydrolysis by subtilopeptidase A, type VIII (Sigma) was performed at the same E/S ratio of 1/100 at 37°C for 6 or 16 h in 100 mmol ammonium bicarbonate buffer. The reaction was terminated by lowering the pH to 3.6 and the hydrolysate mixture was further lyophilized.

High-Performance Liquid Chromatography (HPLC)

HPLC (Spectra-Physics, Santa Clara, USA) was used for the purification of OA. Ultrapac TSK G 2000, 30 cm, and an Ultrapac precolumn TSK GSWP were used for gel filtration chromatography. Supelcosil, LC-18-DB, semipreparative 25 cm × 10 mm column and a precolumn, Supelguard kit LC-18-DB, were used for the isolation of the enzymic hydrolysis peptides. The mobile phase was A = 10 mmol ammonium acetate, pH 5.6, B = acetonitrile. A linear gradient from 0 to 20% in 37 min, 20-33% in 10 min, flow rate 3 ml/min, detection = 220/280 nm and 20 μ l of peptic or tryptic digest were injected.

Immuno-electrophoretic Techniques

CIE was used for qualitative control of the rabbits' response to OA and egg white antigens [15]. CRIE was performed for the control of the specific reaginic reactivity of patients' sera [27].

Rocket line immuno-electrophoresis was performed on the enzymic digests for qualitative measurement of the lower levels of antigenic reactivities [8].

Two-Dimensional Electrophoresis

Fingerprints of hens egg white extract were obtained by isoelectric focusing (IEF) at pH gradient 3.5-9.5 in the first direction. Gel electrophoresis in the second direction was done by a gradient 5-12.5% polyacrylamide gel (PAGE) in sodium dodecyl sulfate (SDS). Aliquots of 15 μ l (1 μ g/ μ l) were used, the combined outfit of LKB and Biorad was utilized [11].

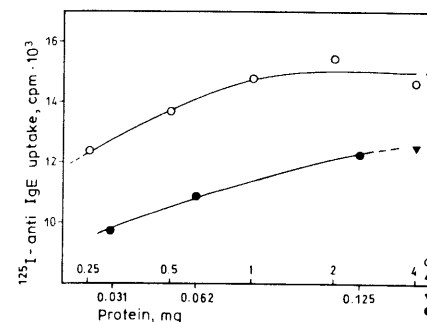


Fig. 1. The results of ¹²⁵I-anti IgE (Fc specific D 2) uptake by hens egg white extract prepared at our laboratory. An uptake of approximately 10% was obtained at an antigen concentration as low as 30 μ g per 10 discs used for each coupling. The upper and lower curves represent the uptakes at the concentrations depicted on the corresponding scales of the abscissa.

Results and Discussion

Crude Egg White and OA

The volume of the egg white was approximately 750 ml. Total protein concentration of the extract was 8.43 g/l as measured by Coomassie binding method [6]. The OA used was further purified on ACA-54 column and the fractionation resulted in 2 peaks, a large void volume peak which was lyophilized and used for further analysis and a small one which was discarded. Figure 1 demonstrates the allergenic reactivity of OA as measured by ¹²⁵I-anti IgE. The lower limit of binding serum IgE was below 30 μ g OA. This amount was used for the coupling of 10 discs 3 μ g/disc (fig. 1).

CNBr Cleavage

CNBr has the unique specificity of peptide bond cleavage on the carboxyl side of methionine residues. 16 methionine residues are included in the OA polypeptide chain; 11 out of the 17 cleavage products are relatively large peptides [24]. The reaction was performed under mild conditions. Amino acid analysis of the reaction mixture showed that methionine was lactonized to homoserine lactone. The elution diagram (fig. 2) of the gel chromatography of the CNBr cleavage reaction product shows four major fractions. Table I is deduced from the primary structure of OA [24]; it shows the theoretical order of elution of these

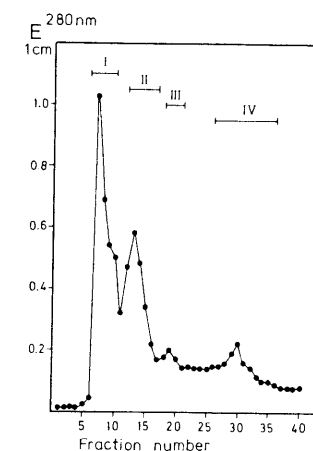


Fig. 2. Gel filtration chromatography of CNBr cleavage products of OA on P-6 column. 25% formic acid was used for the equilibrium and elution of the column. The peaks I-IV shown on the figure were pooled and directly lyophilized.

Table I. Composition of each of the CNBr fractions as deduced from OA primary structure

Peak (recovery)	Locations of peptides	Residues No.
I (65.9%)	41-172 301-385	132 85
II (7.6%)	9-35 173-196 242-273	27 24 32
III (17.6%)	1-8 197-210 212-222 228-239 274-285 288-298	8 14 11 12 12 11
IV (9.1%)	36-40 223-227 240-241 286-287 299-300	5 5 2 2 2

Figures in parentheses are the percentages of protein concentrations recovered of each fraction relative to the total protein concentration of the mixture.

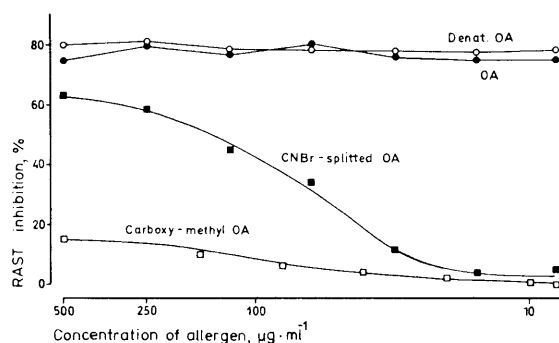


Fig. 3. RAST inhibition results using thermally denatured OA. Denat. OA showed a similar RAST-inhibition to the native OA. CNBr cleavage reaction products and carboxymethyl OA was totally inactivated at a concentration below 100 µg/ml. Amounts of 10 µl of the allergens were used in each sample and determinations were performed in triplicates.

Table II. Specific IgE inhibition percentage of the four fractions obtained by gel filtration chromatography of CNBr-OA

CNBr-OA concentration mg/ml	IgE inhibition, % (RAST), peak			
	I	II	III	IV
1.00	59	23	3	9
0.50	68	12	-	-
0.25	57	-	-	-
0.125	56	12	-	-
0.062	47	3	-	-

Fraction I showed the highest inhibition value while fractions III and IV showed the lowest. Only the highest concentration samples of these two fractions could inhibit IgE binding.

peptides according to their molecular size. Gel filtration was used for the separation of all methionine peptides depicted in table I. The pooled and lyophilized proteins were easily soluble. Their activities were tested by RAST and inhibition techniques. All the four fractions of the CNBr cleavage were capable of inhibiting the binding of reaginic IgE to the paper discs (table II). The first peak was the one with the largest inhibition value. Whether this peak contained contaminants of the intact OA was examined by reversed-phase HPLC. Results indicated that the first peak has a retention volume different from that of OA. The concentrations of the different CNBr fractions were calculated by integrating the areas of these peaks from the elution chromatogram as indicated in brackets in table I. Further purification of the N-terminal methionine peptides and examination of their reactivities are in progress.

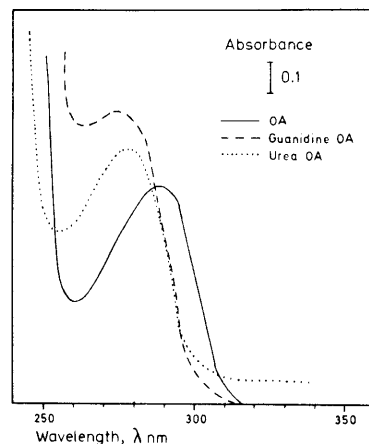


Fig. 4. Ultraviolet spectra scanning of the native OA, 8 M urea and 4 M guanidine HCl-treated OA. A shift of 5 nm towards the short ultraviolet region as compared to the native protein was observed.

Denaturation

The effects of the denaturation of OA on its reactivities are essential for further investigation of the immunological characterization of the cleavage products. Thermal denaturation by heating to 80°C for 10 min, treatment with 8 M urea or 4 M guanidine HCl were performed (fig. 3). The ultraviolet spectra of the denatured proteins were altered and showed a minimal of 5 nm shift of the peaks towards shorter ultra-

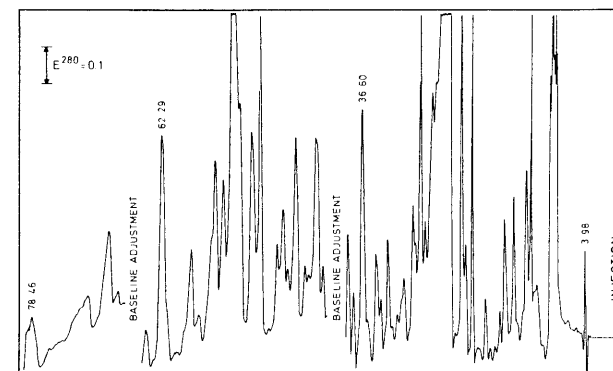


Fig. 5. HPLC elution diagram of OA hydrolyzed by pepsin in HCl for 2 h. 100 µg were injected, 10 mV, 0.05 AUFS, flow rate 3 ml/min and speed of paper 0.5 cm/min.

violet region (fig. 4). Denaturation of OA had no direct effects on the antigenic reactivity. The antigenic activity for the thermally denatured OA was similar to the untreated protein.

Carboxymethylation

The modification of the cysteinyl residues of OA to carboxymethyl cysteine had a strong effect on the allergenic activity. Carboxymethylation was assigned by amino acid analysis of the carboxymethyl OA. Analysis showed an S-carboxymethyl cysteine peak earlier than the methionine sulfoxide. The inhibition of IgE binding by carboxymethyl OA was about 20% of the original activity (fig. 3).

Trypsin Hydrolysis

Partial hydrolysis with dicyclohexylcarbodiimide trypsin (a preparation which is devoid of chymotryptic activity) yielded many peptides as was demonstrated by HPLC. The limited tryptic hydrolysis peptide product is illustrated in figure 5. Trypsin hydrolysis reduced the inhibition reactivity. Only samples with highest concentration gave RAST inhibition. At a concentration below 10 µg/ml no inhibition was found. OA gave 40% inhibition at a concentration as low as 1 µm/ml (fig. 6).

RAST inhibitions with different fractions of the limited tryptic hydrolysis from the p-6 column are indicated on the chromatogram (fig. 7).

Pepsin Hydrolysis

Pepsin is an endopeptidase with a wide specificity

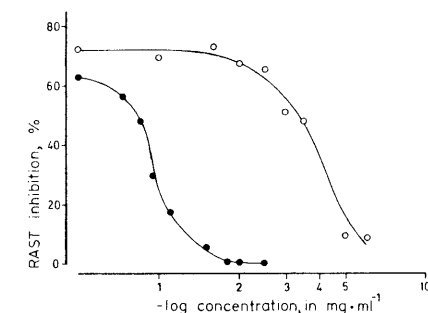


Fig. 6. RAST inhibition of OA (○—○) and 4-hour trypsin-hydrolyzed OA (●—●) at the concentrations denoted on the abscissa. The initial concentrations used were 1 mg for OA and 2.5 mg for the limited tryptic hydrolysate.

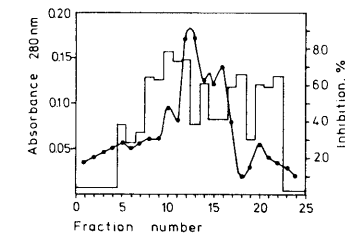


Fig. 7. Elution pattern of the limited tryptic hydrolysis of OA (●—●). The inhibition percentages of RAST values are shown for each fraction.

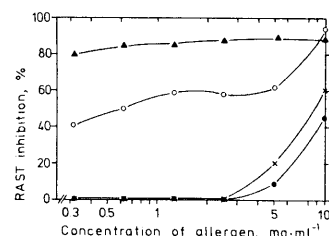


Fig. 8. RAST inhibition percentages of the hydrolysates of OA using pepsin in HCOOH (▲) or HCl (○) for 2 h. (x) Subtilisin hydrolysate for 6 h and (●) 16 h.

that catalyzes the hydrolysis of most of the peptide bonds. Particularly susceptible are those formed by aromatic amino acids. A limited pepsin hydrolysis produces therefore a large number of overlapping peptides. The effect of pepsin and subtilisin hydrolyses on the allergenic reactivity was studied and illustrated in figure 8. Limited hydrolysis with pepsin in HCl decreased the allergenic activity by approximately 50% as shown from the RAST inhibition diagram.

Rocket Line Immuno-electrophoresis

The results of rocket line deflection using OA line at a concentration of 10 µg in the gel and rabbit anti-serum pool (300 µg) against OA shown in figure 9 could be summarized as follows: carboxymethyl-treated OA deflected OA line precipitate showing a direct rocket formation. In addition, pepsin hydrolysis peptides showed no antigenic reactivity in this system; the 4-hour trypsin-hydrolyzed OA deflected OA line. The antigenic reactivity was retained after carboxymethylation and trypsin hydrolysis.

Two-Dimensional IEF/SDS-PAGE

The peptide map of the crude egg white extract (fig. 10) showed at least 26 proteins made visible by binding Coomassie stain. The figure shows the peptide map of the crude egg white as well as three IEF strips. Strip A shows the crude egg extract, strip B is a protein calibration marker, and strip C shows OA. Only the crude was run in the second-dimension SDS-PAGE.

The data obtained indicate that modification by CNBr or carboxymethylation did not totally abolish the antigenicity of OA. This suggests that the isolated peptides are also active and that isolation and charac-

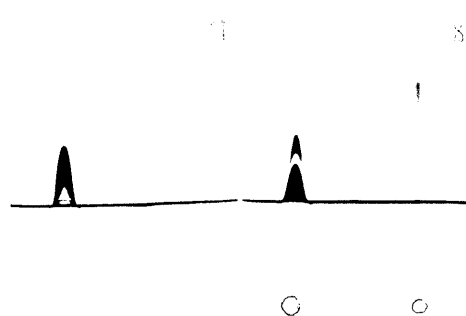


Fig. 9. Rocket line immuno-electrophoresis. The wells contained the following samples: (1) 25 µg carboxymethylated OA, (2) buffer for negative control, (3) 20 µg CNBr cleavage product of OA, (4) 25 µg pepsin-hydrolyzed OA for 2 h. OA line: 10 µg OA mixed with the primary gel. Antibodies: rabbit anti OA (300 µl) were used in the antibody gel.

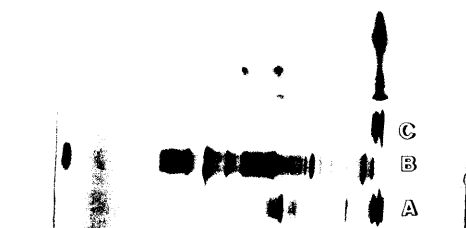


Fig. 10. Upper: two-dimensional electrophoresis. Lower: IEF. OA and crude hens egg white extract were separated according to their isoelectric points in the first dimension and according to their molecular weights in the second dimension. The high resolution capacity of this system allowed to investigate the heterogeneity of OA and the complexity of the proteins of the crude extract. 26 proteins were counted for the crude extract. C = OA; B = protein pI-marker and A = egg white extract.

terization of the active peptides should be pursued in order to assign the allergenic and antigenic determinants to specific location on the molecule.

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References

- Apold, J.; Elsayed, S.: Characterization of the immunological cross-reactivity of fragments TM1 and TM2 of Allergen M from cod. *Mol. Immunol.* 16: 205-211 (1979).
- Apold, J.; Elsayed, S.: The immunochemical reactivity of regions encompassing Tyr-30 and Arg-75 of Allergen M from cod. *Mol. Immunol.* 17: 291-296 (1980).
- Atassi, M.Z.: Antigenic structure of myoglobin: the complete immunochemical anatomy of a protein and conclusions relating to antigenic structures of proteins. *Immunochemistry* 12: 423 (1975).
- Atassi, M.Z.; Lee, C.L.: The precise and entire antigenic structure of native lysozyme. *Biochem. J.* 171: 429 (1978).
- Bazin, H.; Bernadette, P.: Production of circulating reaginic (IgE) antibodies by oral administration of OA to rats. *Immunology* 30: 679 (1976).
- Bradford, M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* 72: 248-254 (1976).
- Brostoff, J.; Carini, C.; Derek, G.: Food allergy. An IgE immune complex disorder; in Bostrom, Ljungstedt, Theoretical and clinical aspects of allergic diseases, pp. 104-122 (Almqvist & Wiksell, Stockholm 1982).
- Elsayed, S.; Titlestad, K.; Apold, J.; Aas, K.: A synthetic hexadecapeptide derived from Allergen M imposing allergenic and antigenic reactivity. *Scand. J. Immunol.* 12: 171 (1980).
- Elsayed, S.: The native and synthetic peptides of cod fish Allergen M. A short review; in Bostrom, Ljungstedt, Theoretical and clinical aspects of allergic diseases, pp. 237-253 (Almqvist & Wiksell, Stockholm 1982).
- Elsayed, S.; Apold, J.: Immunochemical analysis of cod fish Allergen M. Locations of the immunoglobulin binding sites as demonstrated by the native and synthetic peptides. *Allergy* 38: 449-459 (1983).
- Florvaag, E.; Elsayed, S.; Hammer, A.S.E.: Comparative studies on tree pollen allergens. XII. Partial characterization of the alder (*Alnus incana*) pollen extract by horizontal IEF and two-dimensional electrophoresis combined with electric transfer and immunofluorescence. Submitted for publication (1985).
- Gross, E.: The cyanogen bromide reaction; Hirs, in *Method of enzymology*, vol. XI, pp. 238-255 (Academic Press, 1967).
- Gurd, R.N.F.: Carboxymethylation; Hirs, in *Methods of enzymology*, vol. XI, pp. 532-541 (Academic Press New York, 1967).
- Harboe, N.M.G.; Ingild, A.: Immunization, isolation of immunoglobulins and antibody titre determination. *Scand. J. Immunol.* 17: suppl. 10, pp. 345-351 (1973).
- Høiby, N.; Axelsson, N.H.: Crossed immuno-electrophoresis as modified for quantitative purposes. *Scand. J. Immunol.* 17: suppl. 10, pp. 125-134 (1983).
- Ishizaka, K.: Regulation of IgE synthesis. *A. Rev. Immunol.* 2: 159-182 (1984).
- Jarret, E.E.E.; Stewart, D.C.: Rat IgE production. I. Effect of dose of antigens on primary and secondary reaginic antibody responses. *Immunology* 27: 365 (1974).
- Katz, D.H.: Recent studies on the regulation of IgE antibody synthesis in experimental animals and man. *Immunology* 41: 1-24 (1980).
- Langeland, T.A.: Clinical and immunological study of allergy to hens egg white. II. Antigens in hens egg white studied by crossed immuno-electrophoresis (CIE). *Allergy* 37: 323-333 (1982).
- Langeland, T.: A clinical and immunological study of allergy to hens egg white. III. Allergens in hens egg white studied by crossed radioimmuno-electrophoresis (CRIE). *Allergy* 37: 521-530 (1982).
- Langeland, T.: A clinical and immunological study of allergy to hens egg white. I. A clinical study of egg allergy. *Clin. Allergy* 13: 371-382 (1983).
- Langeland, T.; Harbitz, O.: A clinical and immunological study of allergy to hens egg white. V. Purification and identification of a major allergen (antigen 22) in hens egg white. *Allergy* 38: 131-139 (1983).
- Marcel, Y.L.; Hogue, M.; Weech, P.K.; Milnes, R.W.: Characterization of antigenic determinants on human solubilized apolipoprotein B. Conformational requirements for lipids. *J. Biol. Chem.* 259: 6952-6957 (1984).
- Nisbet, A.D.; Saundry, R.H.; Moir, A.J.G.; Fothergill, L.A.; Fothergill, J.E.: The complete amino acid sequence of hen OA. *Eur. J. Biochem.* 115: 335-345 (1981).
- Schloss, O.M.: A case of allergy to common foods. *Am. J. Dis. Child.* 3: 341 (1912).
- Vadehra, D.V.; Nath, R.R.: Eggs as a source of protein; in Furia, Critical reviews in food technology, pp. 193-309 (CRP Press, New York, N.Y. 1973).
- Weeke, B.; Sondergaard, I.; Lind, P.; Aukrust, L.; Lowenstein, H.: Crossed radioelectrophoresis (CRIE) for identification of allergens and determination of the antigenic specificities of patients IgE. *Scand. J. Immunol.* 17: suppl. 10, pp. 265-272 (1983).
- Wright, T.T.: OA is an elastase substrate. *J. Biol. Chem.* 259: 14335-14336 (1984).
- Yman, L.; Ponterius, G.; Brandt, R.: RAST-based allergen assay methods. *Int. WHO Lab. Symp. Standardization and Control of Allergens Administered to man*, Geneva 1974. *Dev. Biol. Standard.* vol. 29, pp. 151-165 (Karger, Basel 1975).

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