

Antigenic and Allergenic Determinants of Ovalbumin

II. The Reactivity of the NH₂ Terminal Decapeptide

S. ELSAYED, E. HOLEN & M. B. HAUGSTAD

Allergy Research Group, Laboratory of Clinical Biochemistry, University Hospital, Bergen, Norway

Elsayed, S., Holen, E. & Haugstad, M.B. Antigenic and Allergenic Determinants of Ovalbumin. II. The Reactivity of the NH₂ Terminal Decapeptide. *Scand. J. Immunol.* 27, 587–591, 1988

The decapeptide 1-10 of hen's egg ovalbumin (OA), deduced from the known amino acid sequence (Gly-Ser-Ile-Gly-Ala-Ala-Ser-Met-Glu-Phe), was synthesized by Merrifield solid phase peptide synthesis with a yield of >70%. The completeness of the insertion of amino acids during synthesis was monitored by the amino acid compositions of the peptide-resin, prior to coupling of the preceding residue. The linearity of the synthesis was supported by dansyl Edman degradation and detection of three NH₂ terminal residues 5-dimethylaminonaphthalene-1-sulphonyl (DNS)-Gly, -Ser, -Ile, respectively. The peptide was purified by gel filtration chromatography and analytical reversed-phase high-performance liquid chromatography (HPLC). The homogeneity of the preparation was calculated both from the amino acid analysis and by integrating the peaks of HPLC to be >83%. The antigenicity of the purified (P2) peptide could be detected by precipitation inhibition with the nephelometric technique. The decapeptide could also specifically react with functional structures on reagenic IgE molecule from the sera of individuals allergic to eggs, inhibiting its further binding to ovalbumin. An in-vivo experiment using direct skin test on two patients allergic to eggs showed no activity, rendering further testing unnecessary. The results suggest that the decapeptide of the NH₂ terminal segment of OA encompasses an Ig-binding haptenic epitope.

S. Elsayed, Allergy Research Group, Laboratory of Clinical Biochemistry, Haukeland Sykehus, University Hospital, N-5021 Bergen, Norway

Previous studies from this laboratory on cod fish allergen M provided evidence of the antigenic (IgG-binding sites) and allergenic (IgE-binding sites) activity of the molecule. Four peptides located in the calcium- or non-calcium-binding domain and the NH₂ terminal segment are able to bind specific antibodies against the intact molecule. The reactivity of these peptides was studied by inhibiting the binding of human reagenic IgE and rabbit IgG against the intact allergen. The peptide's capacity to bind the Ig molecule was found to associated with an optimal chain length [6, 7].

Hen's egg and fish muscle proteins are common causes of atopic hypersensitivity symptoms particularly in children and young adults [7, 18–20]. Egg is an essential ingredient in human and animal food and it is very difficult to totally

eliminate it [8]. Ovalbumin (OA) was found to be the most dominant among the three major allergens of egg white [20]. The complete amino acid (AA) sequence of OA has been elucidated [23]. OA is widely used as a model protein in structural as well as immunological studies [4, 13, 16, 17]. So far, very few studies on the antigenic structure of OA have been reported [8]. The antigenic structures of several proteins have been elucidated and were extremely useful for studying their interactions with antibodies [1, 7–12, 21]. The immunological reactivity of OA was shown to be relatively stable and could easily be retained in derivatives, or after major cleavages of the molecule, allowing possible identification of the fragments. The effect of enzymic cleavage and perturbation of the conformation of allergenic and antigenic determi-

nants of OA have been examined previously. The cleavage of OA with cyanogen bromide resulted in four fractions, all capable of binding specific IgE. Thermal denaturation of OA apparently had no direct effect on the antigenic reactivity as shown by crossed immunoelectrophoresis (CIE). Carboxymethylation of OA reduced its allergenicity by 80%. Furthermore, trypsin hydrolysis of the carboxymethyl OA did not abolish the activity, while limited pepsin hydrolysis could destroy the allergenic activity of the molecule [8].

The peptide 1-10 of OA was predicted from its AA sequence to be mostly helical in structure [5] and was selected for investigating the immunochemical reactivity of the NH₂ terminal region.

MATERIALS AND METHODS

Materials. Crude hen's egg white and rabbit antibodies against egg white extract were prepared as previously described [8-14]. OA, type VII No. A-7641, was purchased from Sigma (St Louis, Mo., USA) and further purified by gel filtration chromatography (GFC) on an ACA-54 column (100×1.5 cm) (LKB, Bromma, Sweden). Two peaks were obtained and the larger one (void volume peak) was used for further experiments.

Synthesis of the peptide. Solid phase synthesis (SPS) of the decapeptide (shown in Fig. 1) was performed manually by Merrifield SPS, with chloromethylated 2% divinylbenzene cross-linked polystyrene resin [2, 3, 9-12, 22]. Approximately 2.5 molar excess of the appropriate tert-butyl oxycarbonyl amino acid (Boc-AA) was coupled twice to the resin. The synthesized peptide was cleaved from the resin by hydrogen bromide (HBr) gas in trifluoroacetic acid (TFA), which also removed all protecting groups.

Purification of the peptide. The crude peptide was purified by GFC, on a P2 column (300×10 mm) (Biorad Laboratories, Richmond, Calif., USA). Two peaks were obtained, and the larger one, eluted in the void volume of the column, was used for further purification. High-performance liquid chromatography (HPLC), using Lich. 5RP 8 reversed phase column (150×4.6 mm), supplied by Chrompack (Middelburg, The Netherlands), was used for the purification of the P2 preparation. A Spectra-Physics delivery system connected to a Spectro Monitor D, LCD/Milton Roy (Fla., USA) was used. HPLC-grade reagents were used for the preparation of the solvents. A 10 min linear gradient of 70% of 0.1% TFA:30% acetonitrile (MeCN) was used; the absorbance was monitored at 210 nm.

Both the homogeneous and the crude products were used at initial concentrations of 1.85 µmol. A dilution series of the following concentrations: 1.39, 0.93, 0.46, 0.23, 0.12 µmol, was used for testing both the allergenic and antigenic activity.

NH₂ terminal determination and AA analysis. To

check the sequence linearity, NH₂ terminal analysis was performed by stepwise degradation of the first three AA by dansyl Edman degradation and the DNS-AA were detected as previously described [8-11, 15]. AA analysis was performed using 50-µl aliquots of the peptide resin removed from the reactor at the end of each coupling to screen the completeness of the insertion of AA. The samples were hydrolysed in a vacuum in 6 M HCl at 110°C, for 24 h. A biotronic amino acid analyser model LC 7000 (Wissenschaftliche Geräte, Munich, FRG), connected to a Spectra-Physics SP 4200 Computing Integrator (Calif., USA) was used. The results were given in µmol/l and used for the calculation of the homogeneity of the peptide on the basis of an *M_r* value of 1097.2 daltons. The AA concentrations were converted to nmol/nmol Lys (the Lys esterified resin particle).

The allergenic and antigenic reactivity. Specific IgE antibodies against egg were obtained from 14 patients with clinical histories of food allergy including egg and sometimes fish, with radioallergosorbent test (RAST) class 2-4. Informed consent was obtained from the patients before blood samples were taken. None of them has been treated with systemic corticosteroids or desensitization therapy.

Total and egg white-specific IgE from the sera were analysed by RAST and by inhibiting the binding of specific IgE to OA discs, respectively. A serum pool or individual sera from egg-allergic individuals were diluted one-fold, 100-µl aliquots were incubated for 4 h at room temperature with equal volumes of serial dilutions of the peptide. The inhibited sera were allowed to react with OA bound to paper discs [8, 24]. For all the peptide batches four different runs including triplicate samples of the series of dilutions and controls were performed. Control samples with no peptide (non-inhibited), with crude or purified unrelated peptide, and with the intact antigen (total inhibition), were always included under identical experimental conditions.

The peptide was also preliminarily tested *in vivo* by a direct skin prick test (SPT) on two cod-allergic volunteers (SPT was kindly done by Dr Høgne Vik from our group). No reactivity could be found to justify a more elaborate performance of SPT.

The antigenicity of the peptide was detected by immunoprecipitation inhibition test using rabbit anti-OA at a 10-fold dilution and a series of peptide dilutions. One hundred-microlitre aliquots of P2 peptide at the following concentrations: 1, 0.5, 0.25, 0.125, 0.0625, 0.03125 µmol, were incubated with the rabbit antibodies for 1 h at room temperature. This was followed by the addition of equal aliquots from 1.25 µg/ml OA solution (equivalence concentration pre-estimated by the quantitative immunoprecipitation curve for the intact antigen and rabbit antibodies). The second incubation was done for 1 h at room temperature. The light scatter was read in a Laser Nephelometer (Behring Institute, Marburg, FRG).

RESULTS AND DISCUSSION

Studies on the allergenic and antigenic activity can be approached either by using purified



FIG. 1. The primary structure of peptide 1-10 as deduced from the amino acid sequence of hen's egg ovalbumin [23]. Lysine was esterified to the resin and replaced Cys-11 of the original sequence.

enzymic fragments from the intact native molecule or by using synthetic peptides of particular sites of the molecule [1, 6–12].

The manual SPS introduced by Merrifield was successfully used for the synthesis of the amino terminal peptide of OA, resulting in an overall yield of >70%. The covalent structure of this synthetic decapeptide is illustrated in Fig. 1.

Because of the toxicity and corrosiveness of hydrogen fluoride (HF) gas, anhydrous HBr/TFA was used for cleavage. Since this peptide has no arginines, the less noxious HBr/TFA cleavage should likewise allow optimal cleavage. The first 10 residues of the synthetic peptide were identical to those of the NH₂ terminal segment of OA. Lys-11 esterified to the solid support replaced Cys-11 of the OA sequence [23]. This replacement of Cys by Lys in this peptide remains to be examined in connection with preceding ones.

The crude peptide was easily soluble in 0.01 M ammonium bicarbonate or 0.1% TFA buffers, which makes its purification convenient. No racemization was experienced during the coupling stages. The peptide was first purified with P2-GFC. The void volume peak was applied on a reversed-phase HPLC column (Fig. 2), which generated a satisfactory degree of purity. The calculated mean area of peak II from seven semi-preparative HPLC runs was >83%.

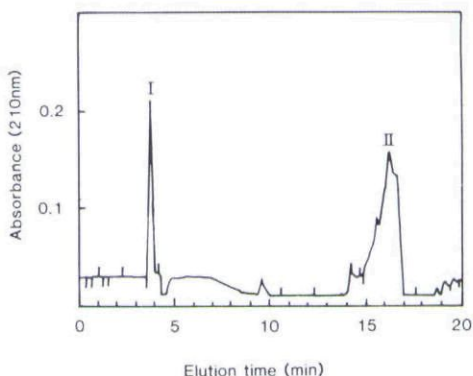


Fig. 2. HPLC diagram of the partially purified peptide. The mobile phase gradient: 0.1% aq. TFA 70% and 30% MeCN; time 10 min; flow rate 0.5 ml/min; chart speed 0.5 cm/min; absorbance at 210 nm. The composition of the two peaks were assigned from the AA analysis. Peak I was small deletion fragments while peak II contained the major portion of the peptide in question and also the biological activity.

To check that the residues were incorporated in a linear fashion, the NH₂ terminal sequence as well as the quantitative AA composition after each coupling was noted. Three stages of NH₂ terminal degradation resulted in 5-dimethylaminonaphthelene-1-sulphonyl (DNS)-Gly, -Ser and -Ile with satisfactory yields. The mean values of the AA composition were calculated on the basis of M_r 1097.2 daltons. The single lysine value was used for the calculation of the total number of residues. The results of the AA analysis are given in Table I. The table presents the peptide resin composition status at the end of each coupling stage. In addition it shows the composition of the crude and HPLC-purified peptide and the theoretical number of residues.

TABLE I. The amino acid analysis of the peptide resin after the end of each stage of SPPS. Lys-P (stage XI) was used to calculate the number of residues/mol of the growing peptide. The amino acid monitoring was used to indicate the status of the peptide P and for the estimation of its completeness.

[illegible]

TABLE II. Specific IgE binding inhibition percentage of the crude peptide 1-10 of OA before and after GFC purification. Two controls, OA I and crude egg white extract II, were used at a concentration of 100 μ g each. OA and crude egg white extract were able to inhibit the serum pool at mean values of 61.8% and 67.75% respectively.

Peptide concentration (μ mol used)	IgE-binding inhibition percentage (mean value)	
	Crude peptide	After P2 purification
1.85	34.9	16
0.93	20.7	12
0.46	18.4	12
0.23	18.0	8
0.12	10.2	3

The AA composition of the peptide resin prior to each insertion was generally in good agreement with the expected values. The methionine residue was reduced in all the analyses, probably because of incomplete coupling of this residue or 6 M HCl hydrolysis. The low values for methionine from the crude analyses were improved by HPLC purification of the peptide. Generally, the AA composition after each step, for the crude preparation, and the HPLC-purified peptide confirmed that the synthesis had been successful.

The allergenic activity of the peptide was tested by the inhibition of specific IgE from a serum pool and individual sera from patients allergic to egg. The data shown in Table II were representative of four different runs. The peptide was tested with 16 individual sera. Two sera

gave no inhibition, one showed a very weak inhibition (<5%), and another serum gave moderate inhibition (>10%). The other 12 sera could be inhibited by the decapeptide as shown by the results of the serum pool illustrated in Table II. Assuming an M_r of 42,699 daltons for OA [23], the peptide inhibited IgE binding at approximately 800, 400, 200, or 50 times higher concentrations than the concentrations used to obtain maximum inhibition by OA control. This was previously experienced with other synthetic peptides when they were allowed to inhibit large native protein molecules [9]

Higher IgE binding inhibition for the crude rather than for the purified peptide was found with the serum pool. Some of the individual sera from the pool could be inhibited at a higher ratio by the purified than by the crude form and vice versa. The reason for these individual reactions may be the different affinities of the various specific IgE to this epitope. Furthermore, the presence of shorter contaminating peptides with higher affinity for some specific IgE, contributing to the higher activity of the crude preparation, could not be excluded.

The antigenicity of the peptide was examined with rabbit precipitating antibodies against OA. The purified P2 peptide was able to inhibit the complex precipitation. The precipitation inhibition diagram presented in Fig. 3 seemed to follow the general pattern of quantitative precipitation inhibition curves. The high percentage of inhibition obtained at extremely high concentrations of the peptide obviously does not imply that rabbit antibodies are exclusively directed against this epitope. No inhibition was obtained at peptide concentrations of 1.95, 3.90, 7.80, 15.65, 31.50, or 62.50 nmol. At 125 nmol, the inhibition was 24%, while at extremely high concentrations (1000 nmol), it was 90%. The later high value could be due to the concentration differences between the inhibiting epitope and the inhibited OA molecule or to the accumulation of interfering contaminants.

The above tentative results with both reaginic and rabbit antibodies suggested that the NH_2 terminal segment of OA encompassed an Ig-binding haptenic epitope.

This peptide will be used in studies on the regulation of IgE antibody response in mononuclear cell cultures from patients with atopic dermatitis, high IgE titres, and egg allergy (work in progress).

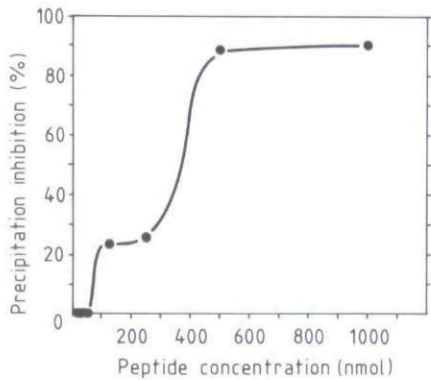


Fig. 3. Precipitation inhibition diagram using P2 preparation at the concentrations indicated. Control system including non-inhibited, inhibited by an unrelated crude or purified peptide, and by the whole antigen under identical experimental conditions.

ACKNOWLEDGMENTS

The present study was supported in part by the Research Fund of the Norwegian Asthma and Allergy Federation and by the Norwegian Research Council for Science and the Humanities.

REFERENCES

- Atassi, H. & Atassi, M.Z. Antibody recognition of ragweed allergen R3: localization of the full profile of the continuous antigenic sites by synthetic overlapping peptides representing the entire protein chain. *Eur. J. Immunol.* **16**, 229, 1986.
- Auffret, A.D., Hirst, W., Meade, L.G. & Thacker, W. Advances in fully automated peptide synthesis. P. 15 in Peeters, P. (ed.) *Protides of the Biological Fluids*. Pergamon Press, Oxford, 1986.
- Barany, G. & Merrifield, R.B. Special Methods in Peptide Synthesis. Vol. 2 of Gross, E. & Meienhofer, J. (eds) *The Peptides. Analysis, Synthesis, Biology*. Academic Press, New York, 1980.
- Buus, S., Sette, A., Colon, S.M., Miles, C. & Grey, H.M. The relation between major histocompatibility complex (MHC) restriction and the capacity of Ia to bind immunogenic peptides. *Science* **235**, 1353, 1987.
- Chou, P.Y. & Fasmar, G.D. Prediction of the secondary structure of proteins from their amino acid sequence. *Adv. Enzymol.* **47**, 45, 1978.
- Elsayed, S. The native and synthetic peptides of cod fish Allergen M. Pp. 237–253 in Boström, H. & Ljungstedt, N. (eds) *Theoretical and Clinical Aspects of Allergic Diseases*. Almqvist & Wiksell, Stockholm, 1982.
- Elsayed, S. & Apold, J. Immunochemical analysis of cod fish allergen M: Location of the immunoglobulin binding sites as demonstrated by the native and synthetic peptides. *Allergy* **38**, 449, 1983.
- Elsayed, S., Hammer, S.S.E., Kalvenes, M.B., Florvaag, E., Apold, J. & Vik, H. Antigenic and allergenic determinants of ovalbumin. I. Peptide mapping, cleavage at the methionyl peptide bonds and enzymic hydrolysis of native and carboxymethyl OA. *Int. Arch. Allergy Appl. Immunol.* **79**, 101, 1986.
- Elsayed, S., Ragnarsson, U., Apold, J., Florvaag, E. & Vik, H. Allergenic synthetic peptide corresponding to the second calcium-binding loop of cod allergen M. *Scand. J. Immunol.* **14**, 207, 1981.
- Elsayed, S., Ragnarsson, U. & Netteland, B. Solid-phase synthesis of the non-calcium binding loop of cod allergen M. Direct evidence of the reactivity of the amino-terminal segment. *Scand. J. Immunol.* **17**, 291, 1983.
- Elsayed, S., Sørnes, S., Apold, J., Vik, H. & Florvaag, E. The immunological reactivity of three homologous repetitive tetrapeptides in the region 41–64 of allergen M from cod. *Scand. J. Immunol.* **16**, 77, 1982.
- Elsayed, S., Titlestad, J., Apold, J. & Aas, K. A synthetic hexadecapeptide derived from allergen M imposing allergenic and antigenic reactivity. *Scand. J. Immunol.* **12**, 171, 1980.
- Guillet, J.G., Lai, M.Z., Briner, T.J., Buus, S., Sette, A., Grey, H.M., Smith, J.A. & Geeter, M.L. Immunological self, non-self discrimination. *Science* **235**, 865, 1987.
- Harboe, N.M.G. & Ingild, A. Immunization, isolation of immunoglobulins and antibody determination. *Scand. J. Immunol.* **17**, suppl. 10, 345–351, 1973.
- Hartley, B.S. Strategy and tactics in protein chemistry. The first BDH lecture. *Biochem. J.* **119**, 805, 1970.
- Ishizaka, K. Twenty years with IgE: From the identification of IgE to regulatory factors for the IgE response. *J. Immunol.* **135**, 1, 1985.
- Iwata, M., Fukutomi, Y., Hashimoto, T., Sato, Y., Sato, H. & Ishizaka, K. Augmentation of the antibody response by antigen-specific glycosylation-enhancing factor. *J. Immunol.* **138**, 2561, 1987.
- Langeland, T. *A clinical and immunological study of egg allergy*. Dissertation, University of Oslo, Representanten, Oslo, Norway, 1983.
- Langeland, T. A clinical and immunological study of allergy to hen's egg white. I. A clinical study of egg allergy. *Clin. Allergy* **13**, 371, 1983.
- Langeland, T. & Harbitz, O. A clinical and immunological study of allergy to hen's egg white. V. Purification and identification of a major allergen (antigen 22) in hen's 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, 1984.
- Merrifield, R.B. Solid phase synthesis. I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* **85**, 2149, 1963.
- 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, 1981.
- Yman, L., Ponterius, B. & Brandt, R. RAST-based allergen assay method. *Int. WHO Labs Symposium on Standardization and Control of Allergens Administered to Man*, Geneva, 1974. *Dev. Biol. Standard* **29**, 151.

Received 8 September 1987

Accepted in revised form 11 January 1988

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.