

SHORT PAPER

A Synthetic Hexadecapeptide Derived from Allergen M Imposing Allergenic and Antigenic Reactivity

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Elsayed, S., Titlestad, K., Apold, J. & Aas, K. A Synthetic Hexadecapeptide Derived from Allergen M Imposing Allergenic and Antigenic Reactivity. *Scand. J. Immunol.* 12, 171–175, 1980.

A solid phase synthesis (SPPS) hexadecapeptide corresponding to residues 49–64 of allergen M from cod was found to bind specifically IgE antibodies from sera of cod-allergic individuals. The SPPS peptide was similarly reactive in IgG-mediated reactions; it could interfere with Allergen M line precipitate in rocket-line immunoelectrophoresis, giving a distinct deflection. It is the only reported synthetic polypeptide imposing allergenic and antigenic reactivity. The hexadecapeptide is composed of two homologous tetrapeptides located at its termini, tentatively concluded to be mutually critical for the immunological specificity. The data obtained confirmed the predicted reactivity of the Ca^{2+} -binding CD loop of the intact Allergen M.

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Cod Allergen M is composed of 114 residues in a single polypeptide chain with a molecular formula weight of 12,328 [12]. It is a member of the Ca^{2+} -binding parvalbumin group, present in abundance in most vertebrate skeletal muscles. Several known primary structures and one tertiary structure among the group showed that parvalbumins have preserved an analogous three-domain conformation [16]. The strong interaction of two of these domains with two moles of calcium per mole protein is well documented. The Ca^{2+} coordination groups are located in the CD and EF loops, residues 51–62 and 90–101, respectively [6, 14, 16].

The immunochemical structure of Allergen M has been studied, using selective and limited enzymic hydrolysis peptides in the native, polymerized, and modified states [1–4, 9, 10]. Studies on sequences encompassing Ca^{2+} -coordination residues of the EF loop (peptide 88–108 and peptide 88–113) suggested that the nonapeptide 88–96 is incorporating the active site of this region [9]. On the basis of this,

interest was directed towards the isolation of the analogous CD loop Ca^{2+} chelating sequence. It has proved difficult to obtain a selective enzymic peptide located in the CD loop of Allergen M in the size and yield adequate for studying the reactivity of the region.

A solid phase peptide synthesis (SPPS) of the hexadecapeptide corresponding to residues 49–64 of Allergen M, covering the whole CD loop, was used. The allergenic and antigenic reactivity of this peptide and the synthesis procedures will be reported below.

MATERIAL AND METHODS

Solid phase peptide synthesis. The SPPS hexadecapeptide corresponding to residues 49–64 of Allergen M from cod (Fig. 1) was synthesized by use of chloromethylated polystyrene-1% divinylbenzene resin [17]. The α -amino position of the amino acids was protected by a *t*-butoxycarbonyl (Boc) group, the side chains of aspartic acid and glutamic acid were protected by benzyl groups, and the ϵ -amino position of lysine was blocked by a benzyloxycarbonyl group. Boc-Lys(Z)-OH was

esterified to the resin and the appropriate Boc-amino acids added stepwise in a 2.5 molar excess [13]. All coupling reactions were mediated by dicyclohexylcarbodiimide with methylene chloride being used as solvent. The Boc groups were removed with 40% trifluoroacetic acid in methylene chloride, and the resin was neutralized with 10% triethylamine in methylene chloride.

The synthesized peptide was cleaved from the resin by hydrogen bromide in trifluoroacetic acid, which also removed the protecting groups. The resulting crude peptide was purified for contaminants from the resin and then used in tests at concentrations of 1.79 or 2.03 nmol ml⁻¹.

The synthesis of H-Phe-Ile-Glu-Glu-Asp-Glu-Leu-Lys-OH. The synthesis of the octapeptide corresponding to residues 57–64 of Allergen M from cod was carried out in solution by stepwise addition of the individual Boc-amino acids to H-Lys(Z)OBzl as the starting unit. The side chains of aspartic acid and glutamic acid were protected by a benzyl group.

The Boc-protecting group was removed with trifluoroacetic acid, and the coupling reactions were carried out in methylene chloride with dicyclohexylcarbodiimide as the coupling agent. The protected intermediates were washed with dilute acid and base, and their identity and purity were confirmed by NMR spectroscopy and thin-layer chromatography.

The final octapeptide was deblocked by hydrogenolysis, and the resulting crude peptide submitted for tests at a concentration of $1.69 \text{ nmol ml}^{-1}$.

Primary structure analysis. Amino acid analyses, N-terminal analyses, and partial stepwise Edman-DNS degradation of the hexadecapeptides and octapeptides were performed according to techniques previously described [2, 3, 11, 12].

Allergens and antigens. Allergen M and birch pollen extract (BV extract) were prepared as outlined elsewhere [5, 8].

Unrelated antigens from human serum containing standard titres of albumin, haptoglobin, transferrin and μ -chain were purchased from Behring Institute, Mannheim, West Germany.

Human sera. Sera were obtained from patients with clinical history of hypersensitivity and positive radio-allergosorbent test (RAST) (class 2-4) to the respective allergens. The serum pool was prepared from equal parts of ten individual sera.

Rabbit antisera. Anti-Allergen M was produced as described earlier [2]. Anti-human sera against albumin,

haptoglobin, transferrin and μ -chain were purchased from Behring.

RAST inhibition. RAST inhibition experiments were performed as previously described, with only minor modifications [3, 18]. Human sera were diluted 1:1 with phosphate buffer, and 50 μ l aliquots were preincubated with an equal volume of allergen dilutions. Allergen-coupled paper discs and radio-labelled anti-IgE were purchased from Pharmacia, Uppsala, Sweden.

Passive transfer (PK) tests. Details of the performance of the PK tests were described in a previous communication [9]. The recipient skin sites were sensitized with undiluted serum and challenged by prick tests. Results were given in square millimetres, with the reaction in the non-sensitized control sites subtracted.

Rocket-line immunoelectrophesis. Immunoelectrophoresis was run essentially as described elsewhere [4].

RESULTS AND DISCUSSION

The covalent structure of the hexadecapeptides and octapeptides derived from the known primary structure of cod Allergen M and corresponding to residues 49–64 and 57–64, respectively, are shown in Fig. 1.

These peptides were only partially purified; the ultimate purification and a detailed study of the immunological reactivity of the peptides are in progress.

The mean values of the amino acid composition of four samples of each of the peptides are presented in Table. I. The values were calculated on the basis of peptide formula weights of 2879.89 and 763.85, respectively, and shown to be in close proximity to the amino acid sequence values.

The N-terminal amino acid analyses of the hexadecapeptides and octapeptides showed distinct Ile and Phe residues, respectively, in addition to minor contaminants. Dansyl-Edman N-terminal sequence indicated Ile-Ala-Asp- for the hexadecapeptide and Phe-Ile-Glu- for the

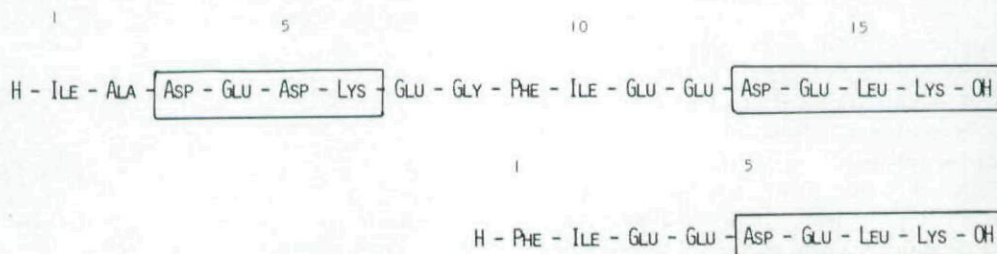


FIG. 1. The amino acid sequence of the synthetic peptides derived from the known primary structure of Allergen M from cod. The homologous tetrapeptides located at the termini of the hexadecapeptide and octapeptide are shown in boxes.

TABLE I. The amino acid composition of the hexadecapeptide and the octapeptide. The samples were hydrolysed in 6 M HCl (containing norleucine as internal standard) in vacuo at 110°C for 24 h.

Amino acid	Hexadecapeptide		Octapeptide	
	Sequence values, residues	Obtained values, nmol/nmol	Sequence values, residues	Obtained values, nmol/nmol
Lysine	2	2.13	1	1.07
Aspartic acid	3	2.37	1	1.12
Glutamic acid	5	5.49	3	3.00
Glycine	1	1.11	—	—
Alanine	1	0.78	—	—
Isoleucine	2	1.71	1	0.40
Leucine	1	1.23	1	1.03
Phenylalanine	1	1.00	1	0.35

octapeptide. No trial was made on the C-terminal sequence; it was found unnecessary since a hydrolysate of the Lys-resin ester was analysed prior to further synthesis and gave an excellent yield of the C-terminal Lys.

The 16-amino-acid residue molecule is so far the only allergenic synthetic polypeptide reported. It has the capacity of inhibiting the binding of specific IgE antibodies to cod allergens in a serum from individuals allergic to cod. The results of RAST inhibition showed a relatively high inhibition percentage (38.6) for the hexadecapeptide as compared with Allergen M (68.3) (Table II). Preliminary experiments have tentatively showed that the SPSS hexadecapeptide could also inhibit, although to a much lower extent, the binding of specific IgE antibodies to birch pollen allergosorbent. No RAST inhibition was obtained in identical experiments with sera from individuals allergic to dog epithelium and the respective allergosorbent.

Peptide 49–64 gave a PK test wheal area of 12 mm². Rechallenge of these sites with Allergen

M produced a wheal area of 45 mm², whereas the reaction to Allergen M in previously non-reacted sites was 65 mm². The ability to elicit a direct in vivo reaction so far precludes the possible use of this peptide for blocking clinical allergy reactions.

The SPSS hexadecapeptide distinctly deflected the Allergen M line precipitate in rocket-line immunoelectrophoresis. When line precipitates from BV extract were used as control, one particular line out of four was deflected. The antigenic specificity was further examined with four unrelated human serum antigens (albumin, haptoglobin, transferrin and IgM μ -chain). None of their line precipitates were deflected under identical experimental conditions (Fig. 2). Studies on the capacity of the hexadecapeptide to inhibit RAST to birch pollen or other unrelated allergens and to deflect one particular antigenic line are currently in progress.

In conclusion, the data obtained for the CD loop hexadecapeptide are indeed in concert with the reactivity predicted on the basis of the

TABLE II. The results of radioallergosorbent test inhibition of the solid phase synthesis hexadecapeptide, using sera pooled from cod- or birch-pollen-allergic patients

Serum	Inhibition percentage				
	Mean			Relative	
	Hexadecapeptide	Allergen M	BV extract	Peptide/All.M	Peptide/BV
Cod-allergic individuals	38.6	68.3	—	53.5	—
Birch-pollen-allergic individuals	17.5	—	77.8	—	22.3

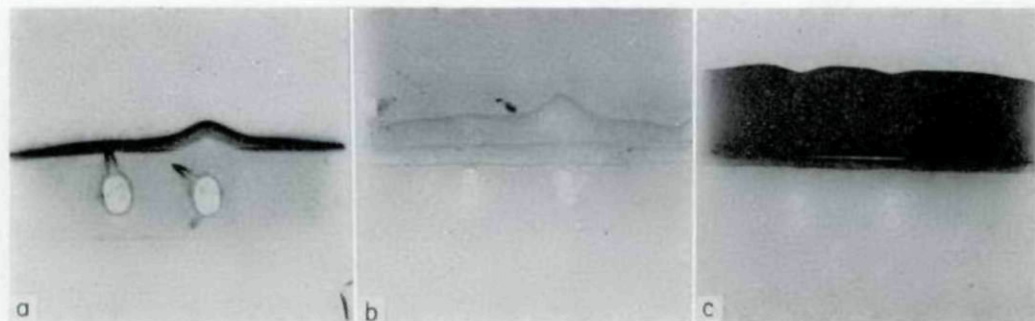


FIG. 2. Rocket-line immunoelectrophoresis patterns. The octapeptide was applied in the first well and the hexadecapeptide in the second (100 pmol of each peptide was used). (a) A distinct deflection of Allergen M line precipitate by the hexadecapeptide. 10 μ g of Allergen M were applied in the primary gel and 250 μ l of rabbit anti-Allergen M in the antibody gel. (b) Line precipitate of BV extract, in which the hexadecapeptide interfered with only one line precipitate of the four lines shown. 10 μ g of BV extract were incorporated in the primary gel, and 200 μ l of rabbit anti-BV crude extract in the antibody gel. (c) Rocket line-precipitate, using an unrelated antigen (haptoglobin). Neither the hexadecapeptide nor the octapeptide caused deflection in the haptoglobin line precipitate. Identical patterns were obtained with albumin, transferrin, or IgM heavy chain. The amount of haptoglobin was 22.5 μ g, and the amount of anti-human haptoglobin used was 200 μ l serum.

sequence homology and the analogous tertiary structure with the EF loop of Allergen M.

On the other hand, the octapeptide corresponding to residues 57–64 of Allergen M did not show any capacity in RAST to inhibit cod or any other allergen. Similar results were previously encountered with synthetic tetrapeptides and octapeptides derived from Allergen M [7].

The hexadecapeptide encompassed two repetitive sequences (Asp-Glu-Asp-Lys- and Asp-Glu-Leu-Lys), whereas the octapeptide included only the C-terminal one, as presented in boxes in Fig. 1. The lack of reactivity of the octapeptide overlapping the C-terminal half of the hexadecapeptide does not imply that it is not involved in the immunological specificity of the region. It is rather believed that the two homologous tetrapeptides, located at each of the termini, are mutually critical for the specificity and enhancement of antibody-binding.

Further synthesis of peptides with variable length of ligands connecting the two tentative sites could reveal whether an optimum number of residues is crucial for the steric fit between the peptide and antibody molecule.

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Received 15 February 1980

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