

## THE PRIMARY STRUCTURE OF A MAJOR ALLERGEN (COD): NH<sub>2</sub>-TERMINAL AMINO ACID SEQUENCE OF FRAGMENT TM 2

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**Abstract**—The NH<sub>2</sub>-terminal amino acid sequence of a major allergenic fragment (TM 2) from cod was elucidated and shown to have the following structure:

Ala—Leu—Thr—Asp—Ala—Glu—Thr—Lys—Ala—Phe—Leu—Lys—Ala—Gly—Asx—Ser—Asx . . .  
1    2    3    4    5    6    7    8    9    10   11   12   13   14   15   16   17

Tryptic hydrolysis of the molecule yielded 6–8 oligopeptides. No allergenicity was found for either of these peptides or for fragment TM 2 after the removal of the 17 NH<sub>2</sub>-terminal residues.

### INTRODUCTION

An allergenic protein was prepared by ion exchange chromatography and gel filtration of the low ionic strength extracts of the white muscle of cod (Aas and Jebsen, 1967). Isoelectric focusing of the resultant major allergen yielded an acidic protein (pI 4.75) which was designated Allergen M and has been shown to represent the major allergenic determinant(s) of cod (Elsayed and Aas, 1971a). No heterogeneity could be detected for Allergen M by polyacrylamide gel electrophoresis, ultra centrifugation analysis and high d.c. potential electrophoretic migration (Elsayed *et al.*, 1971). Later it was shown that the allergenicity of the major allergen of cod was sequentially rather than conformationally dependent (Elsayed and Aas, 1971b). Recent investigations have shown that the amino terminal residue of Allergen M was N-acetylated. By masking of the  $\epsilon$ -NH<sub>2</sub> groups of lysine residues of Allergen M, the tryptic cleavage of the molecule at its single arginine site, yielded two polypeptide fragments (TM 1, mol. wt 8500 and TM 2, mol. wt 6500). Both were allergenically active as assessed by direct skin tests, passive transfer, passive transfer cross neutralization tests and the radio-allergosorbent technique (R.A.S.T.).

On acid hydrolysis fragment TM 2 yielded 50–53 amino acid residues in the following proportion: Asp 7–8, Thr 2, Ser 2, Glu 4, Gly 6, Ala 10,  $\frac{1}{2}$  Cys 0–1, Val 4, Ile 2, Leu 4–5, Phe 3, Lys 5 and Trp 1. This fragment contained no carbohydrate and alanine was found as the NH<sub>2</sub>-terminal residue (Elsayed *et al.*, 1972).

In the present investigation the sequence of 17 amino acid residues from the NH<sub>2</sub>-terminal of fragment TM 2, will be reported as well as investigations on the allergenicity of the tryptic peptides from this fragment.

### MATERIAL AND METHODS

Reagents used in the present studies were of the highest, commercially available, purity grade.

#### Fragment TM 2

The allergenic fragment was prepared from cod Allergen M by the methods described previously (Elsayed *et al.*, 1972).

#### NH<sub>2</sub>-terminal sequence analysis

The NH<sub>2</sub>-terminal sequence was determined by Edman degradation of fragment TM 2. A manual degradation was performed on three different aliquots of 1  $\mu$ mole each. The procedure of Edman (1960) was followed as described by Doolittle (1965) and Sletten *et al.* (1968). The first aliquot involved 12 cycles, the second and the third 17 cycles. The phenyl thiohydantoin amino acids (PTH-AA) were identified by several methods including u.v. spectrophotometry, thin layer chromatography and conversion of the PTH-AA to free amino acids followed by amino acid analysis. Ultra violet absorption of the PTH-AA derivatives was recorded in a Bekman DU spectrophotometer. The ratio of E<sub>245</sub>/E<sub>260</sub> served as a purity index of the PTH-AA investigated (Sj  quist, 1957). PTH-Ser and PTH-Thr were also identified as their PTH-dehydro derivatives. One dimensional thin layer chromatography was performed on a small proportion of the PTH-AA which were identified according to Edman and Sj  quist (1956) and as outlined in a previous communication (Elsayed *et al.*, 1972). Standard PTH-amino acids (Sigma) and silica gel chromatogram sheets 6060 (Eastman) were used.

#### Gas-liquid chromatography of the PTH-AA

The first 10 residues were co-identified on a Varian 2100 gas chromatograph fitted with a flame ionization detector

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according to the method described by Pisano and Bonzert (1969). A U-shaped glass column, 4 mm I.D.  $\times$  183 cm, was packed with 10 per cent DC 560, on 100–120 mesh Gas Chrom P prepared by the method of Horning *et al.* (1963). The column was conditioned for 24 hr at 300°C. Highly purified and dried nitrogen gas, at a flow rate of 40 ml/min, was used. The column temperature was programmed at 180–320°C with a heating rate of 8°C/min. The  $H_2$  and air flow rates were 25 and 300 ml/min respectively. The temperature of the detector and injector was adjusted to 240°C and 260°C respectively. 0.05–0.1  $\mu$ mole of each of the PTH-AA derivatives including also the volatile ones were silylated using an equal volume of  $N_{10}$ -Bis (trimethyl silyl) acetamide (BSA) in a 1 ml capacity reacti-Vial (Pierce Chem. Comp.) under nitrogen. Silylation was carried out at 50°C for 10–15 min, after which 1–5  $\mu$ l of the trimethyl silyl phenyl thiohydantoin amino acid (TMSi-PTH-AA) derivatives were directly injected into the gas chromatograph.

#### Amino acid analysis of the PTH-AA

An aliquot of the PTH-AA was hydrolysed in 6 *N* HCl in vacuum for 24 hr at 150°C (Van Orden and Carpenter 1964). The analysis was performed in a Biocal BC 200 automatic amino acid analyzer as described by Hannestad and Sletten (1971).

#### Tryptic hydrolysis

Hydrolysis of fragment TM 2 was carried out with 1:25 (w/w) of 2 $\times$  crystalline salt-free lyophilized bovine trypsin (Sigma) at 37°C in 0.05 *M* ammonium bicarbonate at pH 8.2 for 5 hr as described earlier (Elsayed *et al.*, 1972). Ascending paper chromatography of the tryptic digest was run at 24°C on 3 MM Whatman paper for 18 hr using the solvent acetic acid:butanol in the ratio 5:7 at pH 2.4. The peptides were designated PC 1, PC 2, PC 3, PC 4, PC 5 and PC 6 according to their chromatographic migration. The locations of the peptides were marked by using a ninhydrin-stained guide-strip and eluted by 0.1% ammonia. The fractions were lyophilized and gel filtered on a 15 $\times$ 0.9 cm column of Sephadex G-25. The paper strips in between the spots were similarly treated for detecting peptides with weak ninhydrin-staining capacity, by their spectrophotometric absorption at 235 m $\mu$ . The purity of the fractions was checked by ascending thin layer chromatography on silica gel plates using the solvent mixture chloroform-methanol-25% ammonia, 2:2:1 v/v (Sletten *et al.*, 1968).

#### Preparation of the material for the biological activity tests

The material of fragment TM 2 after 1 and 17 cycle(s) of Edman degradation were dialysed for 48 hr against six changes of 21 (0.05 *M*) phosphate buffer at pH 7.3. The dialysis bags were pretreated to decrease their permeability as outlined by Kupke (1961). Aliquots of 0.2 mg per ml were Millipore filtered as described previously (Aas, 1967), and submitted to toxicity tests which were performed (Nygaard & Co. A/S, Oslo) by direct injection into 10 mice. A second aliquot of fragment TM 2 after 17 cycles Edman degradation was identically prepared for biological activity except that desalting was performed by gel filtration instead of dialysis. The six peptides (PC 1–PC 6) were similarly prepared at a concentration of 0.2 mg/ml and Millipore filtered as described for fragment TM 2.

#### Allergenic activity tests

The allergenic activity of TM 2 and of the peptides ma-

terial, with buffer blank samples used as controls, was studied by means of direct scratch tests in six cod-allergic individuals and in six control individuals tolerating fish, and by passive transfer tests (PK-tests) using one cod allergic reference serum (D.E.) in a non-allergic recipient as described before (Aas and Elsayed, 1969). Due to the content of irritants in some of the samples, only minor quantities sufficient to leave a bleb of approximately 1 mm diameter were injected into the test sites. All reactions were read and registered after 20 min. Allergenic activity was also assessed by means of a passive transfer cross neutralization technique. For this, the sensitized site was first challenged twice with one of two substances to be compared, and then challenged with the second substance after 1, 6 and 24 hr, respectively, if the reaction to the first substance was negative, and after 24 hr if it was positive. Finally all cross-neutralized test sites were challenged with unrelated allergens (Cows dander extract, ALK 1/1000, w/v) to which the serum donor (D.E.) was allergic, to assure that the sites still possessed the ability to react.

#### RESULTS

Manual Edman degradation of the PTH-AA permitted the identification of the first 17 residues from the  $NH_2$ -terminal part of fragment TM 2 as follows:

|      |       |        |      |      |      |      |        |      |
|------|-------|--------|------|------|------|------|--------|------|
| Ala— | I.eu— | Thr—   | Asp— | Ala— | Glu— | Thr— | I.y.s— | Ala— |
| 1    | 2     | 3      | 4    | 5    | 6    | 7    | 8      | 9    |
| Phe— | I.eu— | I.y.s— | Ala— | Gly— | Asx— | Ser— | Asx—   |      |
| 10   | 11    | 12     | 13   | 14   | 15   | 16   | 17     |      |

The symbol Asx was used whenever the experimental evidence was, as yet, insufficient to allow distinction between amide and the dicarboxylic amino acid.

In gas chromatographic identification of the PTH-AA the use of thermally stable polar silicone phase and silylation with BSA gave the PTH-AA derivatives good chromatographic properties. The registration of the first ten residues were shown in Fig. 1. Trimethyl silylated diphenyl thiourea and phenyl thiourea gave sometimes small peaks which were resolved earlier than the TMSi-alanyl thiohydantoin, the most volatile derivative. Leucyl and isoleucyl derivatives were difficult to distinguish. The identification of the residues No. 2 and 11 was, however, confirmed from the amino acid analysis. Slight contaminant peaks were observed especially of alanyl derivatives before the two threonine residues No. 3 and 7. The yield of the PTH-AA at each step of degradation were in the range of 100–55 per cent for the first 10 residues and 55–38 per cent for the last ones.

From the sequence data is was observed that this region of fragment TM 2 possessed a relatively high content of alanine residues, and that the two threonine residues were both located in the  $NH_2$ -terminal octapeptide ending with lysine.

The results of the ascending paper chromatography of the tryptic digested TM 2 showed three major spots PC 3, PC 5, PC 6 and three minor ones

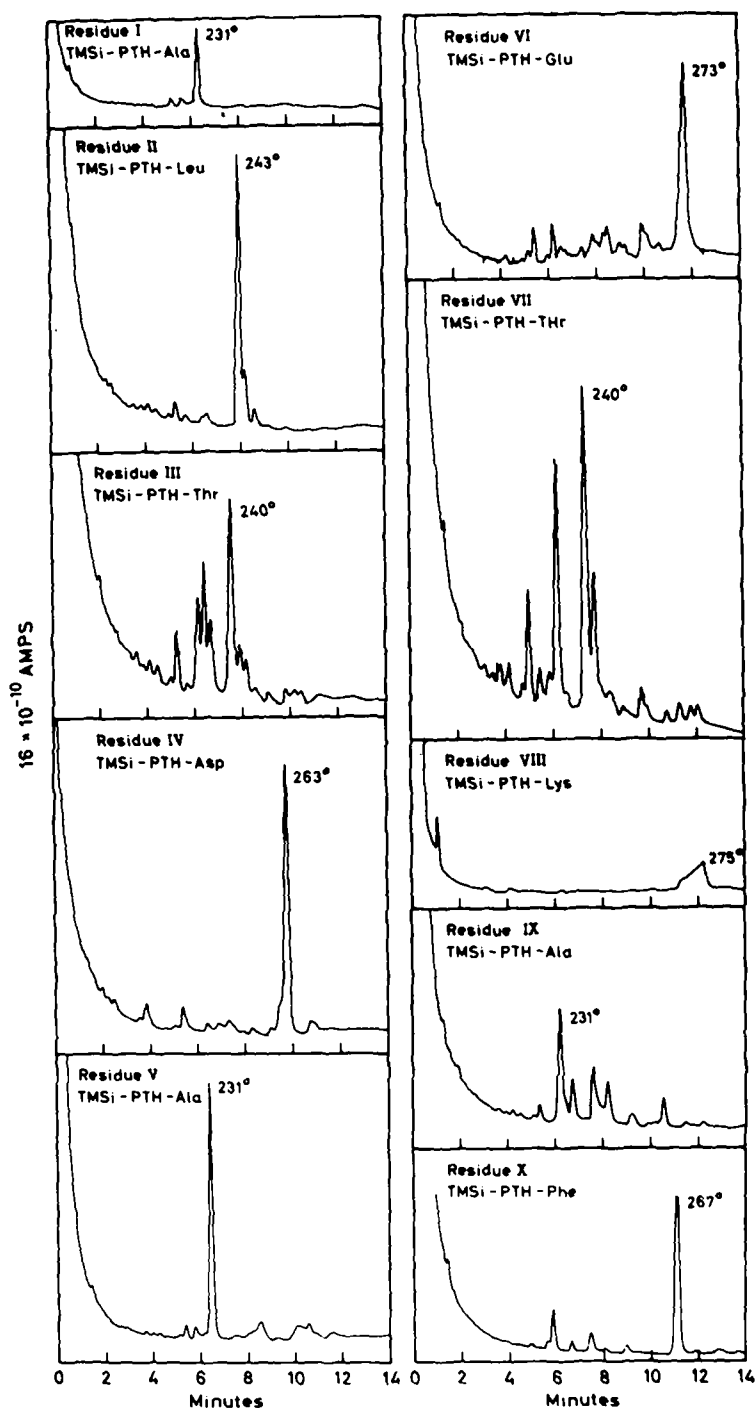


Fig. 1. Gas chromatographic registration of the first 10  $\text{NH}_2$ -terminal PTH-AA residues by using 10 per cent DC-560 on 100-120 mesh Gas Chrom P. For the conditions of operation refer to the text.

PC 1, PC 2 and PC 4. One or two additional ninhydrin shadows were also observed (Fig. 2). No peptides or amino acid residues could be detected in the elution buffer of the strips corresponding to these ninhydrin-shadows. The six peptide spots (PC 1-PC 6) were shown to be homogeneous on as-

cending silica gel thin layer chromatography after gel filtration on Sephadex G-25 column.

#### *Allergenic activity tests*

Fragment TM 2 and the same material after only one Edman degradation cycle elicited marked posi-

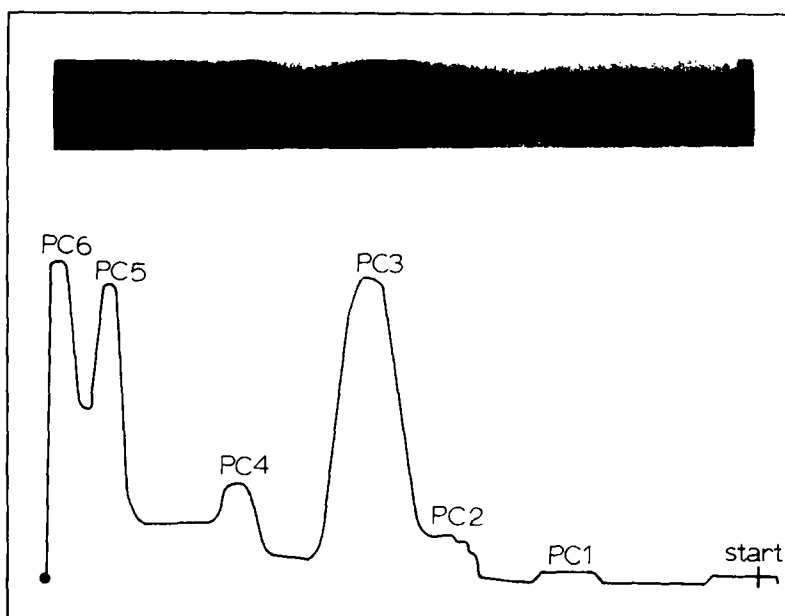


Fig. 2. Ascending paper chromatography of the tryptic digest of fragment TM 2. The solvent used was *n*-butanol: acetic acid, 5:7 (v/v) at pH 2.4. The spots were stained by 0.1 per cent ninhydrin in acetone. Three major spots PC 3, PC 5 and PC 6, and three minor ones PC 1, PC 2 and PC 4, were also shown by Chromoscan registration curve.

tive skin test reactions in all cod-allergic patients tested but not in any of the control individuals. The tryptic peptides from fragment TM 2 as well as fragment TM 2 after 17 Edman degradation cycles, were negative in all patients, eliciting only slight toxic-type erythema reactions in nine of the 12 individuals so tested.

Passive transfer tests with TM 2 were positive with a lower end point reaction at  $2 \times 10^{-10}$  g per ml concentration. The peptide material gave a local burning sensation when injected into the sensitized and non-sensitized sites but were negative in the PK-system. TM 2 neutralized the sensitized sites for a second challenge with TM 2 in equal or lower concentration. Fragment TM 2 in concentrations as

low as  $10^{-12}$  g/ml used in the first challenge modified the PK reaction in cross neutralization experiments. A subsequent challenge with 100 times higher concentrations or more of TM 2 regularly resulted in a doughnut whealing reaction with no oedema at the central site of the first challenge. When the tryptic peptides were similarly used at top concentrations in the first challenge, no neutralization occurred (Table 1).

#### DISCUSSION

The study of the mechanism of reaginic allergy on the molecular level requires detailed information about the molecular nature of the counterparts of the interaction. Fragment TM 2 was found to be a

Table 1. Example of passive transfer cross neutralization (*in vivo*) tests. Refer to the text for detailed description of the test

|      |         | Challenges*/result |            |            |            |             |                   |
|------|---------|--------------------|------------|------------|------------|-------------|-------------------|
| Site | Serum   | 24 hr              | 25 hr      | 30 hr      | 48 hr      | 54 hr       | 72 hr             |
| 1    | DE      | TM 2/+ + +         |            |            | TM 2/-     | PC 1-PC 6/- | Cows dander/+ +   |
| 2    | Control | TM 2/-             |            |            | TM 2/-     | PC 1-PC 6/- | Cows dander/-     |
| 3    | DE      | PC 1-PC 6/-        | TM 2/+ + + |            | TM 2/-     |             | Cows dander/+ +   |
| 4    | Control | PC 1-PC 6/-        | TM 2/-     |            | TM 2/-     |             | Cows dander/-     |
| 5    | DE      | PC 1-PC 6/-        |            |            | TM 2/+ + + |             | Cows dander/+ +   |
| 6    | Control | PC 1-PC 6/-        |            |            | TM 2/-     |             | Cows dander/-     |
| 7    | DE      | PC 1-PC 6/-        |            | TM 2/+ + + |            | TM 2/-      | Cows dander/+ + + |
| 8    | Control | PC 1-PC 6/-        |            | TM 2/-     |            | TM 2/-      | Cows dander/-     |

\*In this experiment TM 2 was used at a concentration of 0.002 mg per ml and the peptides PC 1, PC 2, PC 3, PC 4, PC 5 and PC 6 (PC 1-PC 6) each at a concentration of 0.2 mg per ml in intradermal challenges.

good model for a major allergen. It consists of one low mol. wt chain with no conjugates or prosthetic groups (Elsayed *et al.*, 1972). Its allergenic activity was shown to be dependent on the amino acid sequence rather than on the molecular conformation (Elsayed and Aas, 1971b). The results from the sequence data showed that the two threonine residues of fragment TM 2 were both located in the NH<sub>2</sub>-terminal part. It also showed that the four alanine residues were all interspaced with three residues, the third being a charged amino acid. If the COOH-terminal arginine residue in fragment TM 1 is taken into account, there are three regions where basic amino acids (Arg-Lys) were followed by an alanine residue in this part of Allergen M (Elsayed and Sletten, 1972).

Tryptic hydrolysis of fragment TM 2 yielded 6-8 peptides. This number was higher than the expected, five lysyl peptides plus a COOH-terminal one. The six spots PC 1-PC 6 were homogeneous on silica gel thin layer chromatography; the two other spots might represent some sort of microheterogeneity.

Fragment TM 2 was allergenically active although the PK lower end point was somewhat higher than that found for DS 22 and Allergen M (Elsayed *et al.*, 1972; Elsayed and Aas, 1971a). This was probably at least caused by reduced reactivity of the recipient's skin. The allergenic activity of the remaining part of fragment TM 2 after the 17 Edman degradation cycles was apparently lost. This might be due to loss of one or more essential allergenic determinant(s) incorporated in the deprived 17 residues. During Edman procedure all the free  $\epsilon$ -NH<sub>2</sub> groups of lysine residues were blocked by coupling the phenyl isothiocyanate reagent. These groups are frequently involved in binding sites and their masking may effect the allergenicity and/or the antigenicity of the molecule (Marsh *et al.*, 1970). The effect of masking of the free amino groups on the biological activity of the major allergen in cod is the subject of separate study. Preliminary results indicated that the allergenic activity was not affected by converting these groups to aminomethylol derivatives through prolonged treatment with formaldehyde.

The tryptic peptides obtained from TM 2 were all allergenically inactive. These peptides probably have molecular weights below the lower limit for a substance to be allergenic in the reaginic immune system even if allergenic determinants are present (Aas, 1972). From results of experiments with synthetic antigens there is reason to assume that the part of the molecule which determines the antigenicity is relatively short (Goodman, 1969). The elicitation of the immediate hypersensitivity reaction is thought to require bivalent antigens (Stan-

worth, 1971; de Weck and Schneider, 1969). Monovalent, haptenic antigens may inhibit-binding site of the complementary antibody molecule, so that bridging of IgE molecules by means of native allergens cannot take place (de Weck and Girard, 1972). The cross neutralization experiments in the present study were designed to investigate the presence of any single antigenic determinant in the peptides able to inhibit the allergic reaction to TM 2 in the skin. The demonstration that the peptides were completely inactive in this system may suggest that the structure of the allergenic determinants have been perturbed during the tryptic hydrolysis of TM 2. If for example an allergenic active site should be determined by the amino acid sequence Lys-Ala (8-9 and 12-13) within other structural characteristics, the activity would be lost by the tryptic cleavage used in the preparation of the peptides studied. Further detailed studies are required to settle the structural and immunological relationships of the allergenic and antigenic sites in different allergens.

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