Insect immunity. The primary structure of the antibacterial protein attacin F and its relation to two native attacins from *Hyalophora cecropia*

Å Engström, P. Engström, Z.-j. Tao, A. Carlsson and H. Bennich

The Biomedical Center, Uppsala University, Box 575, S-751 23 Uppsala, Sweden

Communicated by H.G. Boman

The attacins are antibacterial proteins present in the hemolymph of the pupae of the silk moth *Hyalophora cecropia* after bacterial infection. We present the primary structure of one attacin, the F form. We show that this protein is derived by proteolysis from the native protein, attacin E. Using a method for rapid purification from the hemolymph of immunized pupae of the neutral attacin E and a basic attacin, both proteins were found in freshly collected immune hemolymph. We conclude that they are the native products of two attacin genes, the existence of which was inferred from the isolation of two cDNA clones as described in the accompanying paper. The two proteins, which differed in their pIs (7 and 9), were found to have similar mol. wts. (20 000) and closely related primary structures, displaying a total of 40 amino acid substitutions, 12 of which were of a non-conservative nature. **Key words:** attacin/immune protein P5/insect immunity/primary structure/antibacterial protein

Introduction

The attacins, previously referred to as immune protein P5, constitute one of the two major protein fractions to appear in the hemolymph of pupae of the giant silk moth, *Hyalophora cecropia*, in response to a bacterial infection (reviewed in Boman and Steiner, 1981). By isoelectric focusing, the attacins were shown to be a heterogeneous family of proteins, consisting of four basic (attacins A, B, C and D) and two neutral-acidic (attacin E and F) forms, with mol. wts. of 20 000–23 000. All six forms inhibited the growth of *Escherichia coli* and a few other Gram-negative bacteria (Hultmark *et al.*, 1983). Within each of the two main groups (A–C and E–F) the proteins were found to have identical amino-terminal sequences.

We now present the complete amino acid sequence of one of the forms, the attacin F, and show that this protein is likely to be derived by proteolytic digestion during preparation, from attacin E. To avoid degradation we developed a method for the rapid purification of the attacins. This method allows the purification of attacin E and one basic attacin, which have related structures and activities. These two attacins are concluded to be the only major native attacins present in the immune hemolymph. In an accompanying paper Kockum *et al.* report the isolation and sequence of two cDNA clones, corresponding to attacin E and to a basic attacin, respectively.

Results

The primary structure of attacin F

Figure 1 shows the complete primary structure of attacin F.

By automated Edman degradations of the intact protein, the sequence of 31 amino acid residues from the amino-terminal end could be determined (Hultmark *et al.*, 1983). For determination of the complete amino acid sequence, attacin F was subjected to both chemical and enzymic cleavages. The various peptides used for alignment of the complete sequence are shown in Figure 1.

Cyanogen bromide fragments (CN). From the amino acid composition of attacin F, which indicated the presence of 3–4 methionyl residues, cyanogen bromide cleavage was expected to yield a maximum of five fragments. However, the separation of the reaction mixture by gel filtration (TSK)

Fig. 1. Amino acid sequence of neutral attacin. Abbreviations: CN: cyanogen bromide, C: clostripain, V8: *S. aureus* V8 protease, TSK: gel filtration, RP: reversed-phase chromatography, N-term: sequence obtained by sequencing intact attacin F. Amino acids within brackets are the carboxy-terminal residues of attacin E, determined as described in the text. The order of the carboxy-terminal Tyr-Phe was deduced from the sequence of the cDNA clone corresponding to attacin E as determined by Kockum *et al.* (accompanying paper).

Table I. Amino acid composition of peptide CN:TSK-VI:RP-V

Amino acid	Residues/mol peptide	
	Amino acid analysis	Found in sequence
Lys	1.0	1
His	0.1	
Arg	0.2	
Asp	1.1	1
Thr	0.2	
Ser	3.1	3
H-Ser	0.0	
Glu	1.0	1
Pro	0.8	1
Gly	1.5	1
Ala	0.4	
Val	0.1	
Met	0.0	
Ile	0.2	
Leu	0.9	1
Tyr	0.0	
Phe	1.9	2
Trp	N.D.	1

H-Ser denotes homoserine, N.D. denotes not determined.

followed by reversed-phase chromatography (RP) resulted in the isolation, in sufficient quantities of two fragments (CN:TSK-VI:RP-V and CN:TSK-V:RP-VII) with acceptable purity. Since the peptide CN:TSK-VI:RP-V contained no homoserine (Table I) its sequence was assumed to represent the carboxy-terminal end of attacin F. One peptide, isolated in lower amounts, had an amino acid composition similar to CN:TSK-VI:RP-V, but lacked one amino acid residue each of leucine, serine and phenylalanine. As the remaining fragments were only poorly recovered from the reversed-phase column, one fraction from the gel filtration step (CN:TSK-IV) was used for sequencing despite its heterogeneity (see further below and Figure 1).

Clostripain fragments (C). Seven fractions were obtained by gel filtration from clostripain-digested attacin F. Sequence analysis revealed that none of the three Arg-X bonds were completely cleaved during digestion for 60 min at 33°C using an enzyme to substrate ratio as high as 1 to 10. Thus, the sequence of the peptide C:TSK-II, which comprised the amino-terminal end of attacin F, established the location of peptide C:TSK-III. The sequence of the latter established the sequence including the first methionyl residue (at position 75), while the sequence of the peptide C:TSK-V, which contained two methionyl residues, firmly established the location of fragment CN:TSK-V:RP-VII.

Staphylococcus aureus V8-protease fragments (V8). Of the several peptides produced by the V8-protease only two (V8:TSK-III and V8:TSK-VII) were required to establish the sequence of the still uncharacterized carboxyl-terminal half of attacin F. The sequence of the peptide V8:TSK-III overlapped that of the peptide C:TSK-V which placed a methionyl residue at position 101. To establish a sequence overlap between V8:TSK-III and C:TSK-III, and between V8:TSK-VII and C:TSK-V, respectively, and also to verify the identity of

Table II. Amino acid composition of attacin E and F as determined by amino acid analysis and found in the sequence of attacin F

Amino acid	Residue/mol protein		
	Attacin E	Attacin F	Found in sequence of attacin F
Lys	14.2	13.2	13
His	5.7	5.7	6
Arg	2.9	2.9	3
Asn			17
Asp	36.0	36.6	19
Thr	13.8	14.2	14
Ser	13.4	12.7	12
Gln			1
Glu	2.4	2.8	1
Pro	6.8	7.0	7
Gly	18.4	18.8	19
Ala	19.9	20.2	20
Val	8.6	8.8	10
Met	2.0	2.8	4
Ile	9.6	10.1	10
Leu	11.0	11.4	11
Tyr	3.5	2.2	2
Phe	14.9	13.2	14
Trp	1.1	0.9	1

Values determined by amino acid analysis are from Hultmark *et al.* (1983), normalised to give ~184 residues.

the amino acid residues at position 85 and 139, the heterogeneous fraction CN:TSK-IV, consisting of three peptides, was subjected to sequence analysis.

Sequence analysis of attacin E. Analysis of the 55 amino-terminal residues and a total of 55 residues in various peptides derived by clostripain digestion gave a partial amino acid sequence of attacin E, which was identical to that of attacin F except for a peptide representing the carboxy-terminal part of attacin E. The latter peptide derived from attacin E contained two additional residues, -Ser-Lys. Since clostripain cleavage of attacin E (see Materials and methods) produced several peptides derived by the cleavage of Lys-X bonds, the attacin E might well have contained additional residues at the carboxy-terminal end. Edman degradation of intact attacin E showed a contaminating sequence constituting ~20% of the material and corresponding to a fragment derived by cleavage of neutral attacin at the carboxy-terminal side of the arginine at position 139.

The amino acid composition of attacin E and F as determined by amino acid analysis and the composition found by sequence analysis of attacin F (Table II) shows that the main difference between attacin E and F, besides that already described, is the content of tyrosine and phenylalanine, corresponding to one residue more of each amino acid in attacin E.

Proteolytic activity in the immune hemolymph

The finding, that the sequence of attacin F was identical to that of attacin E except for some missing carboxy-terminal amino acid residues, prompted us to examine whether the attacins in general were sensitive to proteolytic degradation during the preparation. As shown in Figure 2, the two forms of

Fig. 2. Degradation of attacins during incubation at room temperature (22°C). A crude fraction of attacins (see text) was suspended in water and incubated at room temperature. Samples were withdrawn at the indicated times, precipitated with trichloroacetic acid (10%) and subjected to SDS-gel electrophoresis. The amount of protein in the bands corresponding to the neutral (A) and basic (B) attacins was determined as described in Materials and methods.

Fig. 3. Ion-exchange chromatography of ammonium sulfate precipitated immune hemolymph. Material corresponding to 4 ml of immune hemolymph was precipitated with ammonium sulfate at 25% of saturation, desalted and loaded on a column of Mono Q (5 x 550 mm) eluted at a flow rate of 1 ml/min with a gradient of 0–1 M (100%) sodium chloride. For details see Materials and methods. Peaks 2 and 3 correspond to basic and neutral attacins, respectively (see Figure 4 for the protein content of fractions).

attacin, as separated by SDS-gel electrophoresis (Figure 4), were in fact rapidly degraded at room temperature. Antibacterial activity was lost at a rate similar to the rate of degradation of protein (not shown). In this experiment, the attacins were precipitated out of the hemolymph by addition of am-

Fig. 4. SDS-polyacrylamide gel electrophoresis pattern of immune hemolymph (IHL) and of ammonium sulfate precipitate (P) loaded on the ion-exchange column. 40 μ l samples of the pooled fractions from ion-exchange chromatography appear in 1–6 (numbers correspond to peak numbers in Figure 3). As shown by the figure the neutral (3) and the basic (2) attacins differ in migration in the SDS gel, by a distance corresponding to ~3000 in apparent mol. wt. The material in the bands below the major band in lane 3 migrates in isoelectric focusing close to a pI of 7 and is related to the neutral attacin.

monium sulfate to 25% of saturation (Pye and Boman, 1977), resuspended in water and incubated at room temperature (22°C) for 24 h. Degradation of the proteins in the intact immune hemolymph, incubated under the same conditions, was slower, but still significant; after 20 h of incubation, ~80% of the protein remained intact, as judged by SDS-gel electrophoresis (not shown).

Rapid purification of attacins

To minimize degradation of the attacins during preparation, a fast purification method was developed (Figure 3 and Materials and methods). This method, which is based on ammonium sulfate precipitation of immune hemolymph followed by ion-exchange chromatography, allowed the preparation of two forms of attacin, one having a pI of ~7 and an apparent mol. wt. of 24 000, and the other having a pI of ~9 and an apparent mol. wt. of 21 000. By this procedure, purified attacins could be obtained within 3 h of collection of the hemolymph. The concentration of the basic and neutral attacin in the immune hemolymph was determined to be ~1.0 mg and 1.2 mg per ml, respectively. The average recovery was 25% and 30% for the basic and neutral attacins, respectively. These two proteins had pIs and apparent mol. wts., as analysed by two-dimensional gel electrophoresis (not shown), that were identical to those of the two attacins

Fig. 5. Two-dimensional gel pattern of hemolymph, collected from a pupa of *H. cecropia*, 7 days after infection by live *E. cloacae*. The hemolymph was processed for isoelectric focusing directly after collection. Isoelectric focusing (horizontal dimension) was in the pH range 5–10, acidic side left in the figure. SDS-gel electrophoresis (vertical dimension) was on a 12% polyacrylamide gel. The neutral attacin (attacin E) focused at an approximate pI of 7.0, the basic at an approximate pI of 9.0. The positions of the two proteins are indicated in the figures by arrows labeled N and B, respectively. The small arrow indicates a protein present, as a contaminant, in the purified basic attacin (see text).

observed on gels obtained from freshly collected hemolymph from immunized pupae (Figure 5) and which constituted >90% of the protein of the apparent mol. wt. of attacin (e.g., 20 000–25 000) in the immune hemolymph.

The neutral protein (pI 7) (fraction 3, Figure 3) was found to have a homogenous amino-terminal sequence identical to that of attacin E/F (59 amino acids sequenced). The purified carboxy-terminal cyanogen bromide fragment from the neutral protein, had an amino acid composition similar to the carboxy-terminal fragment of attacin F but, in addition, contained one residue each of serine, lysine, tyrosine and phenylalanine. The neutral protein co-migrated with attacin E (isolated as described by Hultmark *et al.*, 1983) in isoelectric focusing (not shown). We thus conclude that the neutral protein (pI 7) in fraction 3 (Figure 3) is identical to attacin E and

that the protein is present in the hemolymph of immunized pupae. No protein with the properties of attacin F was found either in freshly collected immune hemolymph or in our preparations. Attacin F is thus likely to be a degradation product of attacin E, generated during the preparation of attacins. The basic attacin (pI 9) was contained in fraction 2 (Figure 3). This fraction, in most preparations, was contaminated with small amounts of material from fraction 1, a protein with an apparent mol. wt. of ~10 000–15 000. This contaminant did not have antibacterial activity by itself, neither did it affect the antibacterial activity of attacins. The major portion of the basic attacin fraction migrated in isoelectric focusing as a single band with a pI of ~9, thus close to the upper pH limit of the system used. Minor species of protein with the same apparent mol. wt. as the major form, but having slightly different pIs and constituting <10% of the basic attacin, were reproducibly found. In three different experiments, using protein from three different preparations, the basic attacin fraction gave no recovery of amino-terminal amino acids upon Edman degradation, although the amount of protein, according to amino acid analysis, should have been sufficient. However, when the basic attacin had been treated with pyroglutamate aminopeptidase to remove a possible blocking pyroglutamyl residue, an amino-terminal sequence was obtained that was identical to the sequence of attacin A–C as previously reported (Hultmark *et al.*, 1983). In a parallel experiment where basic attacin was subjected to the same incubation procedure, but where the enzyme was omitted, the yield in the sequencing was ~20% of the yield obtained after treatment with pyroglutamate aminopeptidase. The lack of success in our previous attempts to determine the amino-terminal sequence of attacin D (Hultmark *et al.*, 1983) is likely to have been caused by the presence of a blocking pyroglutamyl residue. This conclusion is also supported by the amino acid analysis of attacin D (Hultmark *et al.*, 1983), which indicated the presence of one additional glutamic acid residue as compared with the attacins A–C.

The hydropathic character of attacins

Figure 6 shows the profiles of hydropathy (Kyte and Doolittle, 1982) for basic and neutral attacin together with the 40 positions of amino acid substitutions. Both attacins contain three distinct hydrophobic regions, which are located within the amino-terminal one third of the molecules.

Twelve amino acid differences represent non-conservative substitutions (Dayhoff, 1972) and these are all located outside of the three major hydrophobic regions except for the leucine/tyrosine substitution at position 53. A particular concentration of seven substitutions is observed within the small segment (residue 22–30) located between the first and second hydrophobic regions. The difference in pI between the two attacins is explained by the presence of four aspartic acids in attacin E in positions where the basic attacin contains lysine, asparagine, glycine and threonine, respectively. All four substitutions are located within the carboxy-terminal two-thirds of attacin E.

Discussion

The results of the structural analysis of the acidic attacins E and F, defined and purified as described (Hultmark *et al.*, 1983), indicated that they had an identical amino acid sequence, except for a carboxy-terminal tetrapeptide sequence Ser-Lys-Tyr-Phe, which was missing in attacin F. Since this

Fig. 6. Hydropathy profile of attacin E and basic attacin. Hydropathic values are according to Kyte and Doolittle (1982) and values presented are mean values of nine residual segments. Positions for amino acid substitutions are indicated with bars. The sequence of the basic attacin (Kockum *et al.*, accompanying paper) is displaced to give a maximum homology, and the amino-terminal pyroglutamyl residue is not incorporated.

kind of structure was consistent with the sequence of a cDNA clone as reported by Kockum *et al.* (1984), we conclude that attacin F is derived from attacin E, as a result of proteolytic cleavage. Since the protease(s) acting on attacin E seemed to have exopeptidase activity (carboxypeptidase type), the preparation of attacin F may have consisted of a mixture of partially degraded attacins, all having the same net charge and the same amino-terminal sequence but differing in their carboxy-terminal sequence. An indication of this kind of restricted heterogeneity was provided by the finding of two, partially identical, carboxy-terminal cyanogen bromide fragments from attacin F, which differed only in size.

These results, taken together with the findings by two-dimensional gel electrophoresis of freshly collected immune hemolymph of only two major forms of attacins, prompted us to develop a simplified procedure for the rapid isolation of purified attacin. One of the attacins, which had a pI of 7, was found to be structurally identical to attacin E. The other attacin had a basic pI of 9 and a blocking pyroglutamyl amino terminus followed by a sequence, which was identical to that previously reported for the basic attacins A–C (Hultmark *et al.*, 1983). This sequence, including the amino-terminal glutamine, was identical to the amino-terminal sequence deduced from the sequence of a cDNA clone corresponding to a basic attacin as reported by Kockum *et al.* (1984). Since, by using our fast purification procedure, we could isolate only one major form of basic attacin which, by the two criteria, pI and molecular size, was indistinguishable from the basic attacin present in the immune hemolymph, we conclude that this protein represents the native product of the gene for basic attacin.

The possibility that other minor basic forms, present in fraction 2, Figure 3, also represent native attacins cannot be excluded. However, we find it more plausible that they represent proteolytically derived modifications of the native basic attacin, in the same way as attacin F was found to represent a proteolytically derived product of the neutral attacin E. The assumption that the heterogeneity of attacins, as described by

Hultmark *et al.* (1983), is a result of proteolytic degradation of the two native forms, was further supported by the finding of proteolytic activity in the immune hemolymph. The origin of this activity is not known, but at present we cannot exclude the possibility that it is derived from the injected bacteria.

The activity of the two different attacins against *E. coli* is very similar in nature, the prime target most likely being the bacterial cell envelope. Likewise, the species specificity appears to be similar for the two antibacterial proteins. However, the specific activity of the basic attacin is more than twice as high as that of the neutral protein (Hultmark *et al.*, 1983 and our unpublished observations). The function of the attacins at a molecular level is not known. However, the establishment of the primary structures of the two different attacins provides us with an opportunity to determine what structures are necessary for the antibacterial activity, and what features are responsible for the difference in specific activity between the two proteins.

Materials and methods

Insects, preparation of immune hemolymph

Diapausing pupae of *H. cecropia* were purchased from W. Shelton, Hilton, NY, and stored at 8°C. The procedure for injection of living *Enterobacter cloacae* strain β 12, and collection of hemolymph after 7 days at 25°C was as described (Hultmark *et al.*, 1980).

Preparation of attacins

Preparation of attacin E and F was performed as described (Hultmark *et al.*, 1983) and the batches used for amino acid sequence analysis were kindly provided by D. Hultmark. For the rapid purification of attacins from freshly collected immune hemolymph, the following procedure was used: the starting material for each batch of attacins consisted of 4 ml of haemolymph from three immunized pupae, collected in chilled polypropylene tubes (1.5 ml) containing 1–2 mg of phenylthiourea (Merck), and centrifuged for 2 min in a Beckman microfuge B. The supernatant was diluted 7.5 times with ice-cold distilled water, and centrifuged for 10 min at 12 000 g at 4°C in a Sorvall RC-2B centrifuge. Saturated ammonium sulfate (pH 6.5) was added to the supernatant to a final saturation of 25%. After centrifugation for 10 min at 12 000 g, 4°C, the precipitate was dissolved in 2 ml distilled water and immediately desalted on Sephadex G25, HPD 10 column (Pharmacia, Sweden), equilibrated with the starting buffer used in the ion-exchange experiment. The

eluate (3.5 ml) was subjected to ion-exchange chromatography in an FPLC-system (Pharmacia, Sweden) equipped with a mono Q anion-exchange column, at room temperature (22°C). The column was equilibrated with 20 mM diaminopropane, adjusted to pH 10.1 with hydrochloric acid. The column was eluted with a gradient of 1 M sodium chloride in starting buffer (Figure 3). The effluent was continuously monitored at 280 nm, and pooled fractions were desalted on a PD 10 column equilibrated with 0.1 M ammonium bicarbonate and subsequently freeze-dried.

Antibacterial activity

Determination of antibacterial activity was done by using an agar diffusion method (Hultmark et al., 1983). *Escherichia coli* strain D22 was used as the test organism.

Electrophoresis

For SDS-gel electrophoresis, the system of Laemmli (1970), with the modifications described by Randall and Hardy (1977), was used. The concentration of acrylamide (BDH, UK) was 12–14%. The same SDS-gel system was used for the second dimension in the two-dimensional gel system (O'Farrell, 1975). The isoelectrofocusing gel used in the first dimension was a 0.25 mm thick slab gel, polymerized onto GelBond PAG film (LKB, Sweden). The gels were prepared and run according to the manufacturer (LKB, Sweden), except that the gels contained 1.25% ampholine pH 5–7, 1.25% ampholine pH 7–10 and 7 M urea. The gels were prerun (1000 Vh) prior to application of the samples. After staining with Coomassie blue, relevant sample strips (10 mm) were cut out from the gel, washed for 10 min in water and soaked for 2 min in SDS-sample buffer and applied on top of a 1 mm thick SDS gel, identical to the SDS gel described above. The pIs of proteins were estimated by measuring the pH in the isoelectrofocusing gel with a surface pH electrode.

Determination of protein concentration

The Bio-Rad Protein Assay (Bio-Rad Laboratories, FRG) was used for determinations of protein concentrations in the preparations of purified attacin. Bovine serum albumin was used as a reference, and concentration values were calibrated by use of amino acid analysis. For an estimation of the concentration of attacins in immune hemolymph, the following procedure was used. Samples of immune hemolymph were run on an SDS-gel together with reference samples containing known amounts of basic and neutral attacin, respectively. The gel was stained, dried onto cellophane and scanned in a Quick Scan Jr-TLC (Helena Laboratories, USA). The areas of immune hemolymph were measured and the concentration for each of the two attacins was calculated by use of the reference samples. The amount of protein in each band of attacin was kept within the range of linearity for the relation between the amount of protein and the peak area.

Chemical and enzymatic cleavages

Cyanogen bromide (Eastman, USA) dissolved in acetonitrile (LiChrosolv, Merck) to give a concentration of 1 g/ml was kept at –20°C until use. For chemical cleavage, 75 nmol of attacin F was mixed with a 2-fold excess by weight of cyanogen bromide over protein in 70% formic acid. The reaction mixture was blown with nitrogen and left at room temperature (22°C) in the dark for 24 h and then diluted 10 times with distilled water and finally freeze dried.

Digestion with clostripain from *Clostridium histolyticum* (Boehringer, FRG) was done on 30 nmol of attacin F in 300 µl of a solution of 1% ammonium bicarbonate, 2.5 mM dithioerythritol (DTE, Aldrich Chem. Co., USA), 0.02 M calcium chloride and 2 M urea. The mixture was incubated at 33°C for 60 min using an enzyme to substrate ratio of 1:10 (w/w). For the clostripain digestion of attacin E, 25 nmol of protein were dissolved in the same buffer as above but exclusive of urea, and incubated at 22°C for 13 h using an enzyme to substrate ratio of 1:25 (w/w).

Digestion with *S. aureus* V8 protease (Miles Laboratories, UK) was done on 45 nmol of attacin F in 160 µl of 0.1 M ammonium bicarbonate at 37°C for 2 h, using an enzyme to substrate ratio of 1:50 (w/w).

Removal of amino-terminal pyroglutamic acid was done by treatment with pyroglutamate aminopeptidase from calf liver (Boehringer, FRG). The protocol of Podell and Abraham (1978) was followed with the following modifications. The deblocking buffer was prepared at the double concentration and mixed with an equal volume of the protein, dissolved in distilled water. After digestion the reaction mixture was desalted on a PD-10 column.

Isolation of peptides

Separation of the mixtures of peptides produced by chemical and enzymic cleavage was done by gel filtration on a Ultropac TSK-SW 200 column, 7.5 x 600 mm (LKB, Sweden) in 0.2 M ammonium acetate, pH 6.0 at a flow rate of 0.5 ml/min and monitoring the eluate at 230 nm; or by reversed-phase chromatography on a Radial Pac C18 column, 10 µm, 8 x 100 mm (Waters, USA) eluted with a linear gradient of acetonitrile (LiChrosolv, Merck, FRG) in 0.1% trifluoroacetic acid (Merck), at a flow rate of 1 ml/min, and

monitoring the eluate at 220 nm. Aliquots corresponding to 10–20% of the material in pooled fractions were subjected to amino acid analysis.

Amino acid analysis

Samples of peptides or purified protein were freeze-dried and subjected to hydrolysis *in vacuo* in sealed Pyrex tubes with 6 N hydrochloric acid containing 1% phenol at 110°C for 24 h. After rotary evaporation the hydrolysates were dissolved in 300–500 ml of Beckman sample dilution buffer, pH 2.2. The analyses were performed in a Beckman 121 M Amino Acid Analyzer equipped with a Hewlett-Packard 3388A Integrator.

Amino acid sequence analysis

Automated Edman degradations (Edman and Begg, 1967) were performed in a Beckman 890C spinning cup sequencer updated with a high vacuum valve kit and a cold trap (Beckman, USA). All chemicals for the sequencing were purchased from Rathburn Chemicals Ltd. (UK) except for the Quadrol which was purchased from Beckman, USA. The sequencing program used was essentially that of Thomsen et al. (1976) including a reversed delivery of 0.2 M Quadrol and phenylisothiocyanate and shortened cleavage times. Prior to the application of sample, 3 mg of Polybrene (Aldrich Chemical Co., USA) dissolved in distilled water was added to the cup and subsequently subjected to three sequenator cycles (Tarr et al., 1978; Klapper et al., 1978). To minimize the destruction of thiazolinone derivatives of serine residues, the butyl chloride extracts were collected in tubes containing 0.3 ml of 1 M hydrochloric acid and 0.1% ethanethiol. The butyl chloride was evaporated in the fraction collector. The concentrated derivatives dissolved in the residual volume of hydrochloric acid were then heated at 80°C for 10 min for conversion to phenylthiohydantoin (PTH) derivatives, and subsequently dried by rotary-evaporation.

The PTH amino acids were identified by h.p.l.c. using a Waters dual 6000 A pump and a Waters 660 Solvent Programmer. The analyses were performed on a Nucleosil C-18, 5 µm column (4 x 300 mm) packed by Skandinaviska GeneTec, Sweden, equilibrated with 5 mM sodium acetate pH 5.0 adjusted to pH 4.0 with trifluoroacetic acid. The column was eluted with a convex gradient (program 2) of acetonitrile (LiChrosolv, Merck, FRG) for 5 min at a flow rate of 0.9 ml/min at 37°C. The total running time was 22 min. The effluent was monitored at 254 nm using a Waters 440 absorbance detector, and the peaks were automatically integrated for quantitation using a Spectra Physics 4000 data system.

Acknowledgements

We are grateful to Hans G. Boman and Kerstin Kockum and their collaborators for many fruitful discussions and for providing us with information about the results of their work on the cDNA clone for a basic attacin. We also thank Ludmilla Nirk and Ulf Hellman for performing the amino acid analyses. This work was financially supported by the Swedish Medical Research Council (grants 13X-03556 and 13P-05037). T.Z.-j. was supported by a grant from Sarec to H.G. Boman.

References

- Boman, H.G. and Steiner, H. (1981) *Curr. Top. Microbiol. Immunol.*, **94/95**, 75–91.
- Dayhoff, M.O. (1972) *Atlas of Protein Sequence and Structure*, published by National Biomedical Research Foundation, Washington, DC.
- Edman, P. and Begg, G. (1967) *Eur. J. Biochem.*, **1**, 80–91.
- Hultmark, D., Steiner, H., Rasmuson, T. and Boman, H.G. (1980) *Eur. J. Biochem.*, **106**, 7–16.
- Hultmark, D., Engström, A., Andersson, K., Steiner, H., Bennich, H. and Boman, H. (1983) *EMBO J.*, **2**, 571–576.
- Klapper, D.G., Wilde, C.E. and Capra, J.D. (1978) *Anal. Biochem.*, **85**, 126–131.
- Kyte, J. and Doolittle, R. (1982) *J. Mol. Biol.*, **157**, 105–132.
- Laemmli, U.K. (1970) *Nature*, **227**, 680–685.
- O'Farrell, P. (1975) *J. Biol. Chem.*, **250**, 4007–4021.
- Podell, D. and Abraham, G. (1978) *Biochem. Biophys. Res. Commun.*, **81**, 176–185.
- Pye, A.E. and Boman, H.G. (1977) *Infect. Immun.*, **17**, 408–414.
- Randall, L.L. and Hardy, S.J.S. (1977) *Eur. J. Biochem.*, **75**, 43–53.
- Tarr, G.E., Beecher, J.F., Bell, M. and McKean, D.J. (1978) *Anal. Biochem.*, **84**, 622–627.
- Thomsen, J., Bucher, D., Brunfeldt, K., Nexoe, E. and Olesen, H. (1976) *Eur. J. Biochem.*, **69**, 87–96.

Received on 18 June 1984