The amino acid sequence of delta haemolysin purified from a canine isolate of *S. aureus*

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The amino acid sequence of a staphylococcal delta haemolysin purified from a canine isolate has been determined by a combination of chemical and mass spectroscopic sequence methods. The toxin molecule consists of 26 amino acid residues and is compared with the sequence of a delta haemolysin purified from a human isolate. The two sequences are found to be homologous although amino acid differences are observed in 9 of the 26 residues.

Delta hemolysin *Toxin* *Amino acid sequence*

1. INTRODUCTION

Delta haemolysin is one of a number of extracellular cytolytic polypeptides produced by many isolates of *Staphylococcus aureus* [1]. The toxin is surface active [2] and exhibits pronounced effects on the membranes of a wide variety of cells and organelles. The amino acid sequence of delta haemolysin purified from culture supernatants of *S. aureus* strain 186X [3] has been determined and shown to consist of 26 amino acids, contains no proline, cysteine, tyrosine, histidine or arginine, and has an N-terminal formylmethionine residue [4]. Preliminary physicochemical characterisation suggests that the toxin can undergo solvent-mediated dissociation of the multimeric form into tetramers [5].

The purification of an immunologically distinct delta haemolysin from a canine strain of *S. aureus* has been reported [6] and the studies described here were performed to elucidate the molecular basis for this observation.

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2. MATERIALS AND METHODS

Canine delta toxin was purified as in [6] using a combination of ammonium sulphate and acid precipitation.

Amino acid analyses were performed as in [4] using a single-column amino acid analyser (Locarte, London) employing sodium citrate buffers.

Tryptic digestion of delta haemolysin (10 mg/ml) was performed at 37°C in 50 mM ammonium bicarbonate (pH 8.0) with bovine trypsin (0.1 mg/ml) recrystallised in the presence of diphenylcarbamyl chloride (BDH, Poole). The digestion was terminated after 4 h by freezing and lyophilisation and the peptides purified by high-voltage paper electrophoresis at pH 2.0. Peptides were located by reaction with dilute fluorescamine and were eluted with 5% ammonia prior to lyophilisation and sequence analysis by the Dansyl-Edman procedure [7].

Digestion with staphylococcal protease (Miles, Slough) was performed in an identical manner with the exception that the digestion time was 20 h.

Canine delta haemolysin was subjected to N-terminal solid-phase sequence analysis following deblocking with HCl (1 M) in methanol for 2 h at

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20°C as in [4,7], PTH-amino acids were identified by TLC and amino acid analysis following back hydrolysis to free amino acids by HI.

Proteolytic digestion of delta haemolysin (10–100 mmol/100 μl) for subsequent mass spectrometric analyses was performed at 37°C in 100 μl of 40 mM ammonium bicarbonate (pH 8.0) using 20-μl aliquots of freshly prepared solutions containing either elastase, chymotrypsin, or *S. aureus* protease (1 μg/μl) dissolved in the same medium or with trypsin (1 μg/μl) dissolved in 1 mM HCl. Solutions were lyophilized after 1–12 h.

M_r values of peptides in the trypsin, chymotrypsin and *S. aureus* digests were determined by fast atom bombardment mass spectroscopy with a high-field magnet [8,9]. A 1 μl aliquot of lyophilized sample in 5% acetic acid (100 μl) was used for this purpose. On probe acetylation of the samples with 1 μl of 2:1 methanol–acetic anhydride was employed to determine the number of free amino groups in each peptide [8,9].

Sequence analysis of the peptides produced in the elastase and *S. aureus* digests was accomplished using the combination of fast atom bombardment and collision-activated dissociation on a Finnigan triple-stage quadrupole mass spectrometer [10]. Peptides in the lyophilized samples were dissolved in 5% acetic acid (100 μl) and fractionated by HPLC on a μBondapak C_{18} preparative column using a linear gradient elution from 10:90 (v/v) to 40:60 (v/v) propanol–5% acetic acid at a flow rate of 1 ml/min for 30 min [11]. Each of the 1-ml fractions collected was lyophilized and then treated with 100 μl of 1:1 $C^1H_3O^1H$/$C^2H_3O^2H$ containing thionyl chloride (10 μl/ml) to convert peptides to their 1:1 O-C^1H_3/O-C^2H_3 esters. After 3 h at room temperature the solutions were lyophilized and samples were allowed to stand in methanol for 5 min to back exchange deuterium bonded to peptide heteroatoms. Methanol was removed under vacuum and the peptides were then dissolved in a minimum amount of 5% acetic acid (1–5 μl) and applied directly to the glycerol matrix on the fast atom bombardment sample probe. $(M+H)^+$ ions (multiplets containing signals separated by 3 amu) from the peptides in each fraction were separated from one another using the first quadrupole of the triple quadrupole instrument and caused to fragment on collision with argon atoms in quadrupole 2. The resulting fragment ions used to deduce the sequence of amino acids in the purified peptide $(M+H)^+$ ion were then separated in quadrupole 3 and detected with a conventional conversion dynode electron multiplier.

3. RESULTS AND DISCUSSION

Canine delta toxin purified as in [6] was found to be considerably less soluble than delta toxin prepared previously from *S. aureus* 186X [4] and was less stable to heating. The amino acid composition of the toxin was in good agreement with that in [6] (table 1) although, in contrast with toxin purified from 186X, low levels of arginine, cysteine, histidine, proline and tyrosine were found suggesting that the toxin preparation was not homogeneous. Consequently the purity of the preparation was tested by silica gel TLC (*n*-butanol–acetic acid–water, 9:5:6) which indicated a major component (R_f = 0.66) and a minor component (R_f = 0.61), the minor component being estimated visually followed staining with ninhydrin to be present in a ratio of approx. 1:3.

Mass spectra of the delta haemolysin sample recorded under fast atom bombardment conditions showed pairs of $(M+H)^+/(M-H)^-$ ions in the positive and negative ion modes, respectively, at m/z 2963/2961, 2509/2507 and 2493/2491. The latter two sets of ions probably arise from the impurities in the delta haemolysin sample since all of the data presented below are consistent with a molecule having an M_r of 2962. Mass spectrometry was found to be an ideal method for sequencing the delta haemolysin peptide despite the minor impurities. These did not give interfering ions after sub-digestion.

$(M+H)^+$ ions observed in mass spectra recorded directly on trypsin, chymotrypsin and *S. aureus* protease digests of the impure delta haemolysin sample are summarised in fig.1 above the primary structure assigned to canine delta haemolysin (C-DHL). On probe acetylation of these samples caused a 42 amu mass shift for each Lys residue present in the individual peptides. The $(M+H)^+$ ions at m/z 819, 902, 1276, 407 and 663 were shown to contain 1, 1, 2, 2 and 3 Lys residues, respectively. No peptide $(M+H)^+$ ions containing residues located at the N-terminus of the delta haemolysin molecule were observed in these experiments.

Table 1

Amino acid composition of delta toxin

Amino acid	mol% [6]	mol% (here)	mol residue/ mol haemolysin	Residues found in sequence
Aspartic acid	10.2	9.6	2.5	1
Threonine	4.6	5.4	1.4	2
Serine	3.2	4.2	1.1	1
Glutamic acid	13.1	11.5	3.0	3
Proline	2.2	2.3	0.6	0
Glycine	4.1	6.5	1.7	0
Alanine	5.2	7.3	1.9	3
Cysteine	0.3	N.D.	N.D.	0
Valine	6.9	8.1	2.1	3
Methionine	0.2	3.1	0.8	1
Isoleucine	11.0	11.5	3.0	5
Leucine	8.9	8.1	2.1	1
Tyrosine	2.2	1.5	0.4	0
Phenylalanine	8.2	6.2	1.6	2
Histidine	1.7	1.5	0.4	0
Lysine	12.9	10.8	2.8	4
Tryptophan	N.D.	0.0	0.6	0
Arginine	3.3	2.3	0.6	0
Total residues			26	26

N.D., not detected

Results obtained from sequence analysis of HPLC-fractionated peptides from *S. aureus* and elastase digests using the combination of fast atom bombardment and collision-activated dissociation mass spectrometry on a triple quadrupole mass spectrometer are summarised in fig.1 above the primary structure assigned to C-DHL. Peptides produced by these two proteolytic enzymes overlap sufficiently to provide a unique sequence for the canine delta haemolysin molecule. The assigned sequence is consistent with both the mass of the parent $(M+H)^+$ ion (m/z 2962) and the $(M+H)^+$ ions observed in the digests performed with trypsin, chymotrypsin, and *S. aureus* protease.

N-terminal solid-phase sequence analysis resulted in the unambiguous determination of the first 21 amino acid residues of the canine haemolysin with the exception of residue 14 where no PTH-amino acid derivative was detected consistent with the identification of lysine at this position which would have remained bound to the solid-phase support.

Digestion with proteolytic enzymes resulted in the purification and sequence analysis of 4 peptides derived from the C-terminal region (residues 15–26) of the canine haemolysin (table 2).

The results derived from mass spectroscopic and classical sequence analysis allow the unambiguous mass and sequence of the canine delta haemolysin to be deduced (fig.1) and compared with the amino acid sequence of delta haemolysin purified from a human pathogenic strain of *S. aureus*.

Both haemolysins contain an N-terminal formylmethionine residue and a C-terminal Lys-Lys sequence. The amino acid differences are fairly evenly distributed throughout the sequence and are of a conservative nature.

Interestingly, the single tryptophan residue at position 15 in the human isolate is replaced by a leucine residue in the canine isolate, consistent with the relatively low absorbance at 280 nm (0.6 A/mg per ml canine *vs* 2.1 A/mg per ml for the human isolate).

Delta haemolysin is thought to form an am-

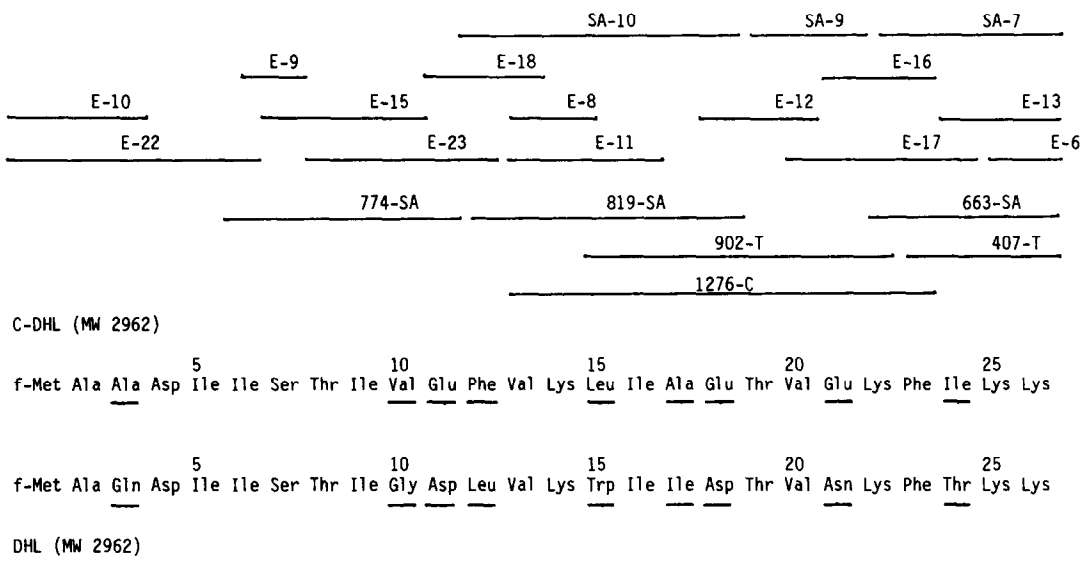

Fig.1. Primary structure of canine delta haemolysin (C-DHL) and comparison with non-canine delta haemolysin (DHL). Peptides from elastase (E) and *Staphylococcus aureus* protease (SA) digests that were sequences on the triple quadrupole mass spectrometer are shown above the primary structure of C-DHL. Numbers refer to fractions (1 ml) eluted from an HPLC column. Sequences that match *m/z* values for $(M + H)^+$ ions recorded on direct analysis of trypsin (T), chymotrypsin (C) and *S. aureus* (SA) digests using fast atom bombardment on the ZAB high-field magnet instrument are shown directly above the primary structure of C-DHL. Sequence differences are underlined.

Table 2

Peptides isolated by high-voltage paper electrophoresis following protease digestion

Peptide[a]	Composition	Sequence	Position in sequence
TB	Ile (1.0), Phe (0.9), Lys (2.3)	Phe-Ile-Lys-Lys	23–26
TC	Ile (1.0), Phe (0.9), Lys (1.3)	Phe-Ile-Lys	24–26
TD	Glx (2.0), Thr (0.9), Ala (1.0), Val (1.0), Ile (0.8), Lys (1.0), Leu (0.9)	Leu-Ile-Ala (Glu_2, Thr, Val, Lys)	15–22
SPA	Ile (1.1), Phe (1.0), Lys (3.1)	Lys-Phe-Ile-Lys (Lys)	22–26

[a] Arbitrary designations: T refers to peptides derived from tryptic digestion; peptide SPA was purified from a staphylococcal protease digest

Sequences were determined by the dansyl-Edman method

phipathic α-helix [5] and to interact with and cause lysis of biological membranes in an analogous manner to the amphipathic peptide melittin, isolated from bee venom [12–14]. Despite the amino acid differences the two microbial haemolysins must adopt similar amphipathic helical conformations and cause membrane lysis via a common mechanism although the differences in sequence are sufficient to account for the immunological dissimilarity observed in [6]. Naturally occurring amphipathic α-helices may prove to be a general theme whereby proteins interact with biological membranes and in the case of haemolysins from human and canine origins and bee venom melittin elicit a cytolytic response.

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REFERENCES

[1] Rogolsky, M. (1979) Microbiol. Rev. 43, 320–360.
[2] Colacicco, G., Basu, M.K., Buckelew, A.R. and Bernheimer, A.W. (1977) Biochim. Biophys. Acta 465, 378–390.
[3] Heatley, N.G. (1971) J. Gen. Microbiol. 69, 269–278.
[4] Fitton, J.E., Dell, A. and Shaw, W.V. (1980) FEBS Lett. 115, 209–212.
[5] Fitton, J.E. (1981) FEBS Lett. 130, 257–260.
[6] Turner, W.H. (1978) Infect. Immun. 20, 485–494.
[7] Hartley, B.S. (1970) Biochem. J. 119, 805–822.
[8] Morris, H.R. (1982) Int. J. Mass Spectrom. Ion Phys. 45, 331–342.
[9] Morris, H.R., Dell, A., Etienne, A.T., Judkins, M., McDowell, R.A., Panico, M. and Taylor, G.W. (1982) Pure Appl. Chem. 54, 267–279.
[10] Hunt, D.F., Bone, W.M., Shabanowitz, J., Rhodes, J. and Ballard, J.M. (1981) Anal. Chem. 53, 1704–1706.
[11] Morris, H.R., Etienne, A.T., Dell, A. and Albuquerque, R. (1980) J. Neurochem. 34, 574–582.
[12] Dawson, C.R., Drake, A.F., Helliwell, J. and Hider, R.C. (1978) Biochim. Biophys. Acta 510, 75–86.
[13] Terwilliger, T.C. and Eisenberg, D. (1982) J. Biol. Chem. 257, 6010–6015.
[14] Terwilliger, T.C. and Eisenberg, D. (1982) J. Biol. Chem. 257, 6016–6022.