Biochem. J. (1988) 252, 87–93 (Printed in Great Britain)

The amino acid sequence of a gonococcal growth inhibitor from *Staphylococcus haemolyticus*

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A gonococcal inhibitor produced by *Staphylococcus haemolyticus* was separated into three components by reverse-phase h.p.l.c. The amino acid composition analysis of each of the three components indicated extensive similarities. *N*-Terminal sequence analysis of all three components allowed the identification of the first 27–30 residues of each. The complete primary structure of each component was determined from the sequence analysis of trypic peptides and peptides generated by mild acid hydrolysis. Each component is composed of 44 amino acid residues, with evidence suggesting the presence of an *N*-terminal formylmethionine residue in each. The components I, II and III have respectively 33, 29 and 33 identical amino acid residues in their sequences, which represents 75%, 65.9% and 75% homology. These components contain a high proportion of hydrophobic amino acids, and their hydrophobicity profiles are closely related. Also, each of the three components contains a positively charged residue (lysine) as the third residue, followed by a core of hydrophobic residues. These results suggest that the three components are possible signal sequences of one or more secreted or membrane-associated proteins.

INTRODUCTION

Staphylococci are the aerobic micro-organisms isolated from the urogenital flora that most frequently inhibited the growth of gonococci on solid medium (Bisaillon *et al.*, 1980). The purification of a bactericidal inhibitor produced by coagulase-negative staphylococci no. 7 was described by Beaudet *et al.* (1982). The characterization of the purified substance revealed that it was a lipoprotein or a lipid-associated protein in which the protein component is the active part. The protein component was a low-M_r peptide that in the absence of a dissociating agent is present as large-M_r complexes or aggregates. Bisaillon *et al.* (1985) have identified strain no. 7 as *Staphylococcus haemolyticus*.

The chemical and biological characterization of the gonococcal growth inhibitor was pursued (Frenette *et al.*, 1984). This substance lysed cells such as erythrocytes, HeLa cells and WI 38 cells. The haemolytic spectrum of the inhibitor against erythrocytes of different animal species showed that human and horse erythrocytes were the most susceptible. The haemolytic and antigonococcal activities of the purified substance were inhibited in the presence of phosphatidylcholine. Many of the properties exhibited by the inhibitor are similar to those reported for staphylococcal δ-lysin (Kreger *et al.*, 1971; Chow *et al.*, 1983). However, the two substances are different, mainly on the basis of their physical and chemical characteristics such as M_r, isoelectric point and amino acid composition.

The present work was undertaken to determine the amino acid sequence of the gonococcal growth inhibitor produced by *S. haemolyticus*. This inhibitor is composed of three different constituent peptides presenting high degrees of homology in their amino acid sequences. On the basis of the results obtained, it is suggested that these peptides may be ideal candidates for the signal sequence of one or more secreted or membrane-associated proteins.

MATERIALS AND METHODS

Bacterial strains

Staphylococcus haemolyticus no. 7 was used for the production of the bactericidal inhibitor (Bisaillon *et al.*, 1980; Lafond *et al.*, 1981; Beaudet *et al.*, 1982). The reference target strain was *Neisseria gonorrhoeae* G-10 (Bisaillon *et al.*, 1981) and it was cultivated as previously described (Frenette *et al.*, 1984). These strains were kept either in a freeze-dried state or as frozen suspensions at −76 °C.

Production and purification of the gonococcal inhibitor

The production and purification of the gonococcal inhibitor were carried out as previously described (Beaudet *et al.*, 1982). The inhibitor was produced on brain/heart-infusion semi-solid medium and purified by methanol extraction, acetone fractionation, dialysis and chromatography on Ultrogel AcA 54. The protein component was separated from the lipids by chromatography on Ultrogel AcA 54 in the presence of 4 M-urea.

H.p.l.c. fractionation of the gonococcal inhibitor

Fractionation of the gonococcal inhibitor to homogeneity was achieved by reverse-phase h.p.l.c. on an Altex Ultrapore RPSC C3 column (Beckman Instruments, Palo Alto, CA, U.S.A.) with a Varian 5000 liquid chromatograph. The inhibitor (1.0 mg), dissolved in

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500 μl of 0.05% (v/v) trifluoroacetic acid (BDH Chemicals, Toronto, Ont., Canada), was injected on to the column, which had been previously equilibrated in 0.05% trifluoroacetic acid. Elution was with a linear acetonitrile (BDH Chemicals) gradient (1%/min) at a flow rate of 1.5 ml/min.

Removal of tentative *N*-terminal formyl groups

Removal of the tentative *N*-terminal formyl groups from the *N*-terminus of the purified components was performed in methanolic 1 M-HCl at room temperature for 3 h. Each fraction was diluted 5-fold with distilled water and freeze-dried.

Trypsin cleavage of inhibitor components

Trypsin [bovine pancreas, twice crystallized, treated with L-*N*-tosylphenylalanylchloromethane ('TPCK')] was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and was further purified by reverse-phase h.p.l.c. before use according to the method of Titani *et al.* (1982). Component peptide samples (200 μg) were dissolved in 50 mM-NH_4HCO_3, pH 8.0, and trypsin was added in a 1:100 (w/w) ratio and incubated for 3 h at 37 °C, when the addition of trypsin and incubation were repeated. The reaction mixture was then freeze-dried. The resultant tryptic-digest peptides of the individual components were fractionated by elution with an acetonitrile gradient (1%/min) at a flow rate of 1.5 ml/min from a Synchropak RP-P column (4.1 mm × 250 mm) (Synchrom, Linden, IN, U.S.A.) previously equilibrated in 0.05% trifluoroacetic acid. The effluent was monitored at 220 nm, and individual peptides were collected and freeze-dried.

Mild acid cleavage

Cleavage at aspartic acid/asparagine residues was performed by mild acid hydrolysis (Schroeder *et al.*, 1963) of approx. 200 μg of individual components by incubation in 0.25 M-acetic acid under vacuum at 110 °C for 8 h, followed by freeze-drying. The mild-acid-cleavage peptides of the individual inhibitor components were fractionated by elution from a Synchropak RP-8 column (4.1 mm × 250 mm) in the same manner as described for the tryptic-digest peptides.

Amino acid composition analysis

Amino acid composition analyses of purified peptides were performed with a Durrum D-500 analyser. Peptide samples were hydrolysed for 20 h *in vacuo* [2.6 Pa (20 mTorr)] at 110 °C in 6 M-HCl (AristaR; BDH Chemicals). Tryptophan determinations were performed after hydrolysis in 4 M-methanesulphonic acid containing 0.2% 3-(2-aminoethyl)indole (Pierce Chemical Co., Rockford, IL, U.S.A.) at 110 °C for 20 h *in vacuo* (2.6 Pa). Inhibitor components I, II and III were hydrolysed for 24 h, 48 h and 72 h respectively to correct for hydrolytic losses.

Automated gas-phase sequence analyses

Automated gas-phase sequencing was performed on an Applied Biosystems 470A sequencer (Foster City, CA, U.S.A.) with 0.1–0.5 nmol quantities of purified peptides. The samples were dissolved in 30 μl of Milli-Q water (Millipore, Bedford, MA, U.S.A.) and applied to a glass-fibre disc containing 1.5 mg of precycled Polybrene (Applied Biosystems). The phenylthiohydantoin

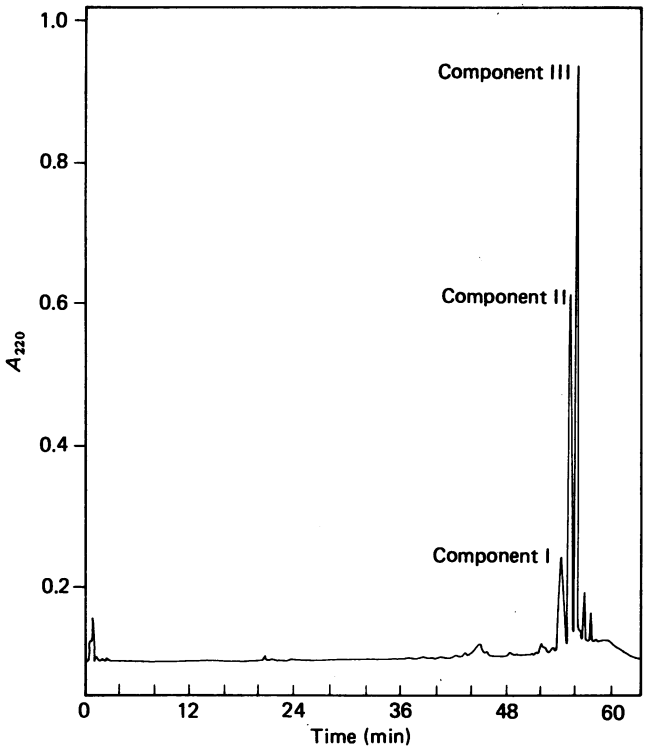

Fig. 1. Separation of the gonococcal inhibitor components by h.p.l.c.

Elution was with an acetonitrile gradient (1%/min) at 1.0 ml/min from an Altex Ultrapore RPSC column equilibrated in 0.05% trifluoroacetic acid. Amino acid compositions of components are given in Table 1.

Table 1. Amino acid compositions of inhibitor components

Values in parentheses are values obtained by sequence analysis.

Amino acid	Component...	Amino acid composition (mol of residue/mol) I	II	III
Asx		3.14 (3)	5.12 (5)	4.22 (4)
Thr		1.82 (2)	2.94 (3)	0.91 (1)
Ser		1.79 (2)	3.86 (4)	2.89 (3)
Glx		4.11 (4)	5.20 (5)	5.14 (6)
Pro		–	–	–
Gly		7.24 (7)	4.16 (4)	7.32 (6)
Ala		6.18 (6)	4.08 (4)	4.11 (4)
Cys		–	–	–
Val		3.96 (4)	2.97 (3)	4.94 (5)
Met		1.92 (2)	0.96 (1)	0.91 (1)
Ile		4.87 (5)	4.91 (5)	3.89 (4)
Leu		2.03 (2)	3.11 (3)	4.09 (4)
Tyr		–	–	–
Phe		1.98 (2)	2.04 (2)	2.08 (2)
His		–	–	–
Lys		3.89 (4)	4.06 (4)	3.12 (3)
Arg		–	–	–
Trp*		0.86 (1)	0.94 (1)	0.91 (1)
Total ...		44	44	44

* Determined by 4 M-methanesulphonic acid hydrolysis.

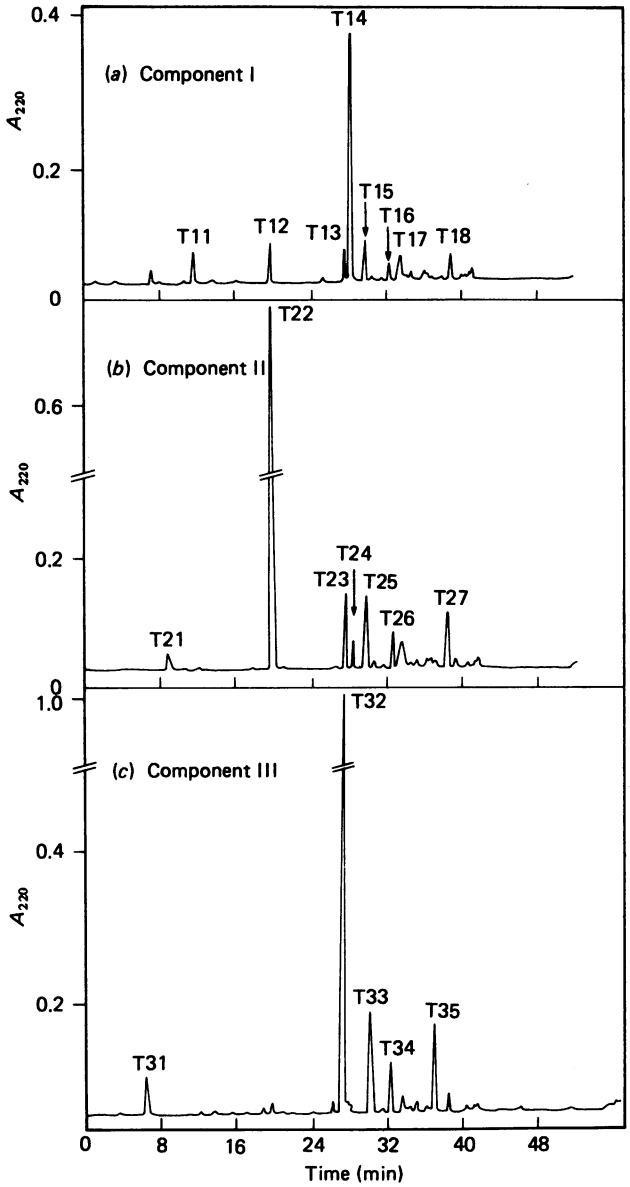

Fig. 2. Separation of peptides from tryptic cleavage of components I (*a*), II (*b*) and III (*c*)

Elution was with an acetonitrile gradient (1%/min) at 1.5 ml/min from a Synchropak RP-P column equilibrated in 0.05% trifluoroacetic acid. The letter T denotes trypsin cleavage. Amino acid compositions of these peptides are given in Table 2.

derivatives of amino acids were identified by h.p.l.c. on a Varian Vista-56 chromatograph with an IBM-Cyano column (4.5 mm × 25 cm) (IBM Instruments, Meriden, CT, U.S.A.) maintained at 37 °C in 30 mM-sodium acetate / 5% (v/v) tetrahydrofuran, pH 4.85, with gradient elution with 100% acetonitrile (BDH Chemicals).

The amino acid placed at any given position in the sequence was not necessarily that present in the greatest amount, but was taken to be that amino acid which was shown to be increasing over that amount present in the previous sequence cycle. It was also dependent on all other amino acids decreasing over the amount present in the previous step.

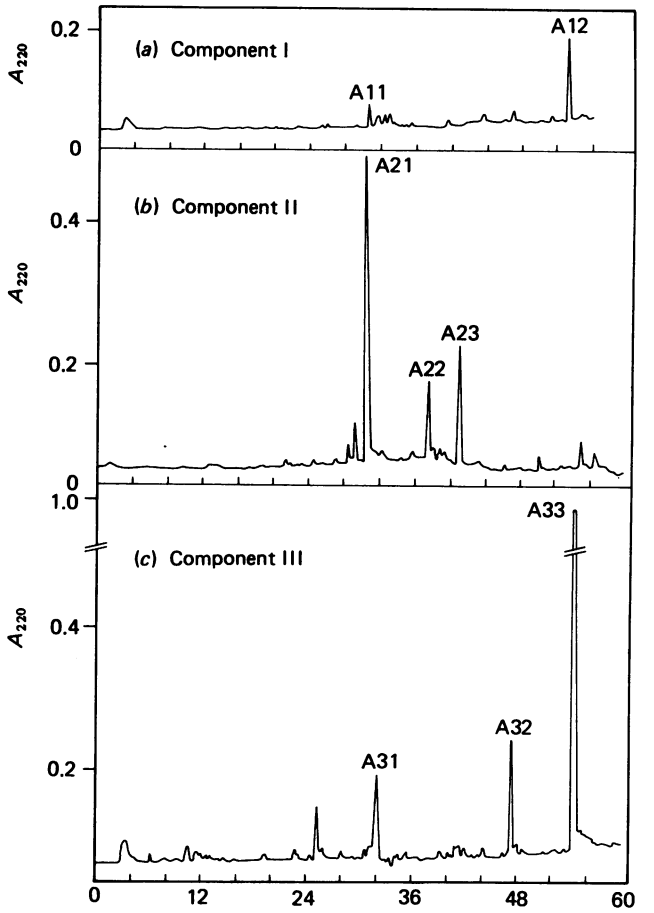

Fig. 3. Separation of peptides from mild acid cleavage of components I (*a*), II (*b*) and III (*c*)

Elution from a Synchropak RP-8 column was as described in Fig. 2 legend. The letter A denotes mild acid cleavage. Amino acid compositions of these peptides are given in Table 3.

Hydrophobicity profile

On the basis of the amino acid sequence of inhibitor components I, II and III, we determined their hydrophobicity profiles by the method of Kyte & Doolittle (1982). The window length used in calculating hydrophobic profiles was nine residues according to this method. Such determinations were also done for δ-lysin of *Staphylococcus aureus* on the basis of the amino acid sequence reported by Fitton *et al.* (1980) and for staphylokinase (Sako & Tsuchida, 1983) and β-lactamase (McLaughlin *et al.*, 1981) of *S. aureus*.

RESULTS

Fractionation by reverse-phase h.p.l.c. and subsequent *N*-terminal sequence determination

The final purification step of the gonococcal inhibitor consisted of reverse-phase h.p.l.c. on an Altex Ultrapore RPSC C3 column. Elution with a linear acetonitrile gradient resulted in the base-line resolution of three components as shown in Fig. 1. Amino acid composition analysis of each of the components indicated that, though they displayed extensive similarity, distinct

Table 2. Amino acid compositions of tryptic peptides of the three components

Nomenclature for peptides is T for trypsin cleavage; the first number represents the component and the second number the order of elution of the peak.

	Amino acid composition (mol of residue/mol)													
	Component I					Component II					Component III			
Amino acid	T11 (residues 1–3)	T12 (residues 19–22)	T14 (residues 4–18)	T15 (residues 41–44)	T18 (residues 23–40)	T21 (residues 1–3)	T22 (residues 4–9)	T23 (residues 10–22)	T24 (residues 41–44)	T27 (residues 23–40)	T31 (residues 1–3)	T32 (residues 4–22)	T33 (residues 41–44)	T35 (residues 23–40)
Asx	–	1.03 (1)	0.96 (1)	–	1.03 (1)	–	0.95 (1)	2.12 (2)	–	1.98 (2)	–	2.81 (3)	–	1.01 (1)
Thr	–	–	–	–	1.93 (2)	–	–	0.89 (1)	–	1.91 (2)	–	–	–	0.95 (1)
Ser	–	–	0.91 (1)	–	0.89 (1)	–	–	0.93 (1)	–	2.87 (3)	0.94 (1)	0.93 (1)	–	0.84 (1)
Glx	0.98 (1)	–	2.12 (2)	–	1.09 (1)	0.98 (1)	–	3.21 (3)	–	1.04 (1)	–	4.96 (5)	–	1.25 (1)
Gly	–	0.97 (1)	1.10 (1)	1.01 (1)	4.17 (4)	–	–	1.10 (1)	1.02 (1)	2.13 (2)	–	–	0.97 (1)	5.12 (5)
Ala	–	–	5.87 (6)	–	–	–	2.10 (2)	2.23 (2)	–	–	–	3.81 (4)	–	–
Val	–	–	0.92 (1)	–	2.82 (3)	–	0.91 (1)	–	–	1.88 (2)	–	1.94 (2)	–	2.86 (3)
Met	0.92 (1)	–	–	–	0.91 (1)	0.96 (1)	–	–	–	–	0.99 (1)	–	–	–
Ile	–	–	0.89 (1)	0.97 (1)	2.82 (3)	–	0.97 (1)	0.97 (2)	0.90 (1)	1.81 (2)	–	1.07 (1)	–	2.88 (3)
Leu	–	–	0.95 (1)	–	1.11 (1)	–	–	–	–	3.10 (3)	–	1.07 (1)	0.99 (1)	1.85 (2)
Phe	–	–	–	2.09 (2)	–	–	–	–	2.16 (2)	–	–	–	2.08 (2)	–
Lys	1.02 (1)	1.11 (1)	1.02 (1)	–	1.13 (1)	1.03 (1)	1.03 (1)	1.09 (1)	–	1.04 (1)	1.06 (1)	1.06 (1)	–	0.93 (1)
Trp*	–	(1)	–	–	–	–	–	(1)	–	–	–	(1)	–	–

* As determined in sequence analysis.

Table 3. Amino acid compositions of peptides generated from the three components by mild acid cleavage

Nomenclature for peptides is A for mild acid hydrolysis; the first number represents the component and the second number the order of elution of the peak.

	Amino acid composition (mol of residue/mol)							
	Component I		Component II			Component III		
Amino acid	A11 (residues 1–16)	A12 (residues 20–44)	A21 (residues 20–28)	A22 (residues 1–18)	A23 (residues 30–44)	A31 (residues 1–9)	A32 (residues 11–18)	A33 (residues 20–44)
Asx	–	0.95 (1)	–	2.18 (2)	1.09 (1)	–	1.08 (1)	1.02 (1)
Thr	–	1.86 (2)	1.89 (2)	–	0.92 (1)	–	–	0.99 (1)
Ser	0.89 (1)	0.89 (1)	0.93 (1)	0.97 (1)	1.90 (2)	1.83 (2)	–	1.04 (1)
Glx	3.12 (3)	1.09 (1)	–	4.12 (4)	1.07 (1)	1.06 (1)	4.12 (4)	1.00 (1)
Gly	1.10 (1)	6.26 (6)	1.05 (1)	1.16 (1)	2.21 (2)	–	–	6.29 (6)
Ala	6.25 (6)	–	–	3.94 (4)	–	0.99 (1)	2.08 (2)	1.18 (1)
Val	0.90 (1)	2.79 (3)	–	0.91 (1)	1.76 (2)	0.98 (1)	0.96 (1)	2.72 (3)
Met	0.87 (1)	0.86 (1)	–	0.76 (1)	–	0.84 (1)	–	–
Ile	0.89 (1)	3.79 (4)	1.10 (1)	1.95 (2)	1.77 (2)	0.99 (1)	–	2.74 (3)
Leu	0.96 (1)	1.08 (1)	1.93 (2)	–	1.10 (1)	1.06 (1)	–	3.17 (3)
Phe	–	2.06 (2)	–	–	2.01 (2)	–	–	2.07 (2)
Lys	1.12 (1)	2.10 (2)	1.13 (1)	2.21 (2)	1.20 (1)	1.19 (1)	–	2.18 (2)
Trp*	–	(1)	(1)	–	–	–	–	(1)

* As determined in sequence analysis.

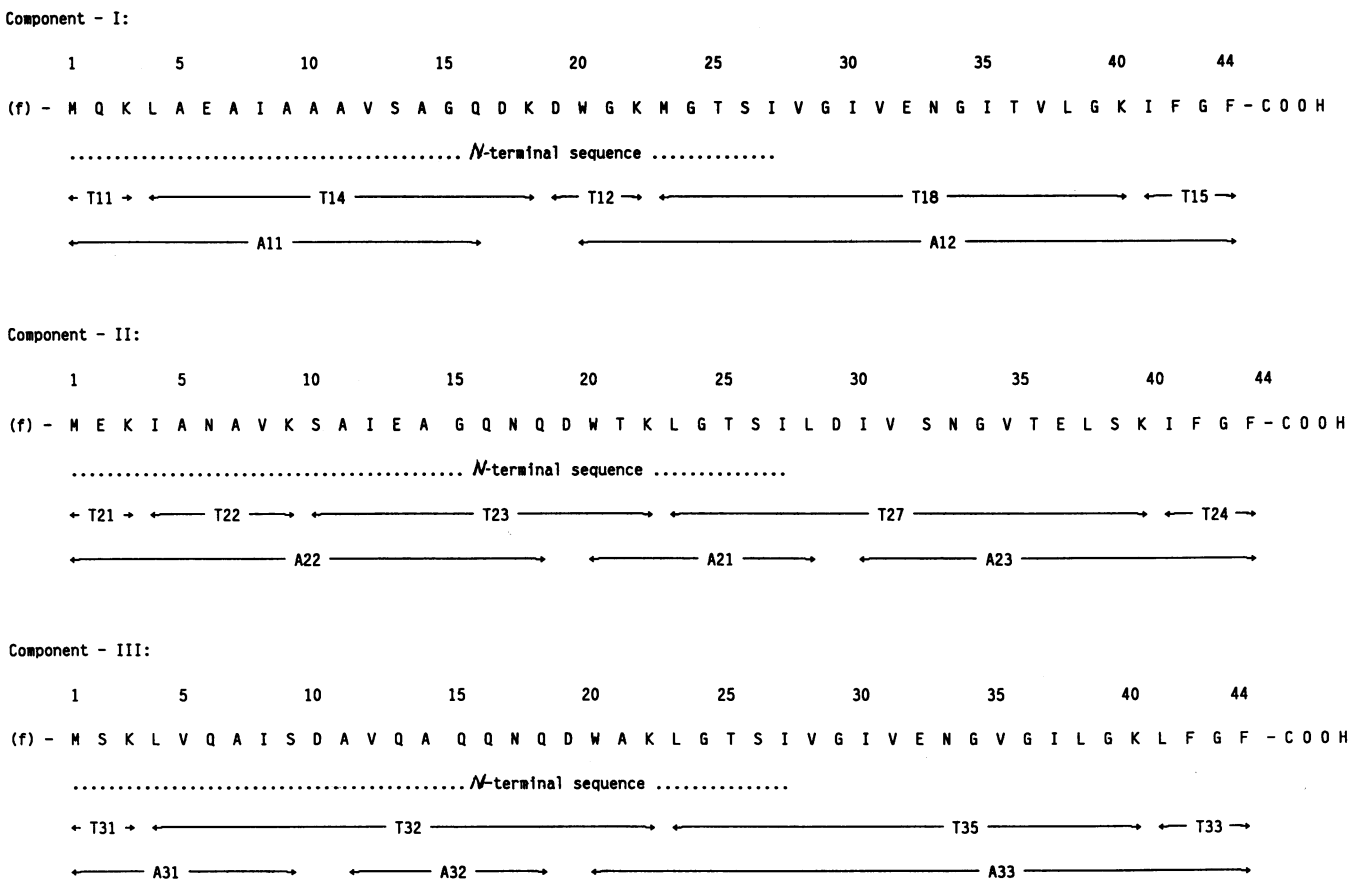

Fig. 4. Complete amino acid sequences of the gonococcal inhibitor components I, II and III

Continuous lines indicate sequences obtained from peptides after digestion with trypsin (T) and mild acid cleavage (A). Dotted lines represent *N*-terminal sequences obtained after removal of the tentatively assigned *N*-terminal formyl (f) blocking groups.

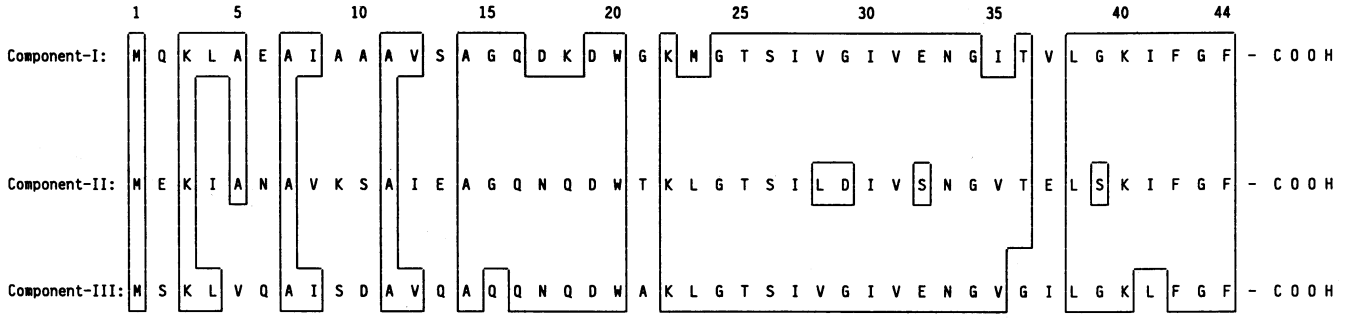

Fig. 5. Comparison of the amino acid sequences of the gonococcal inhibitor components

The boxes indicate identity between the components.

differences are apparent (Table 1). Initial attempts at *N*-terminal sequence analysis of all three components resulted in low yields, approx. 5–10% of methionine after one cycle of automated Edman degradation. After treatment with 1 M-HCl in methanol at room temperature for 3 h, subsequent *N*-terminal sequence analysis of each component allowed the unambiguous identification of the first 27, 30 and 30 residues of components I, II and III respectively.

Cleavage of components I, II and III with trypsin

Amino acid composition analysis (Table 1) indicated the presence of four lysine residues in both components I and II and three lysine residues in component III. Each of the three components was therefore subjected to trypsin cleavage and the resulting peptides were separated (Fig. 2). Samples of each peptide were removed for amino acid composition analysis (Table 2) and subsequent sequence analysis. Trypsin cleavage at the conserved Lys-3 and Lys-22 residues in addition to the Lys-18 in component I and Lys-9 in component II made possible the alignment of the peptide sequences through overlap with the *N*-terminal sequences previously determined for all three components. The tentative placement of the *C*-terminal tryptic peptide in each component was made on the basis of its lack of a *C*-terminal lysine residue.

Mild acid cleavage of components I, II and III

Conclusive evidence for the nature of the *C*-terminus and the complete primary structure was provided by overlapping sequences after sequence analysis of peptides derived by mild acid cleavage in 0.25 M-acetic acid. These peptides were separated (Fig. 3) and their amino acid compositions were determined (Table 3). Sequence analysis of the peptides generated by cleavage at the conserved Asp-19 in addition to the Asp-17 in component I, Asp-29 in component II and Asp-10 in component III provided overlapping sequences for all the tryptic-digest peptides, thereby providing additional evidence for the correct alignment of the tryptic-digest peptides.

Amino acid sequences of components I, II and III

The complete amino acid sequences of the components I, II and III based on the identification of amino acids and alignment of peptides obtained are presented in Fig. 4. Peptides I, II and III have respectively 33, 29 and 33 identical residues in their sequences, which represent 75%, 65.9% and 75% homology (Fig. 5). The highest

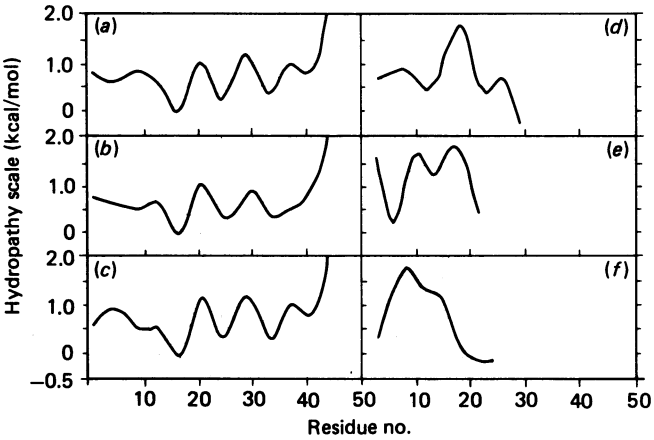

Fig. 6. Comparison of the hydrophobicity profiles of inhibitor components I (*a*), II (*b*) and III (*c*), δ-lysin (*d*), *S. aureus* staphylokinase (*e*) and β-lactamase (*f*) signal peptides

Positive values on the hydropathy scale indicate hydrophobic residues. The values expressed in kcal/mol should be multiplied by 4.184 to convert them into kJ/mol.

degree of homology was found in the area between residues 14 and 44, where it was between 81 and 84% as compared with between 31 and 54% homology for the 13 residues at the *N*-terminal extremity. The M_r values of the components are respectively 4555, 4759 and 4596.

Hydrophobicity profile

The hydrophobicity profiles presented in Fig. 6 are quite similar for the three components. Despite a slight difference in the patterns of the first 15 residues for each of the polypeptides, and the absence of a pronounced peak around the 37th residue for component II as compared with components I and III, the three hydrophobicity profiles are almost superimposable. However, these profiles are different from the ones found for δ-lysin and the signal peptides for staphylokinase and β-lactamase of *S. aureus*.

DISCUSSION

Previous work (Beaudet *et al.*, 1982) suggested that the antigonococcal substance produced by *S. haemolyticus* no. 7 was composed of one small polypeptide chain present as aggregates or complexes. The present study

has clearly shown that the inhibitor is composed of three peptides of similar length differing only slightly in their amino acid sequences. This discrepancy is due to the very similar M_r values of the peptides (4550–4750), which could not be differentiated by polyacrylamide-gel electrophoresis in the presence of SDS and urea. In the presence of 4 M-urea the antigonococcal substance was present as a complex estimated to be of M_r approx. 15000 (Beaudet *et al.*, 1982). This complex may result from the association of the three peptides either randomly or in a particular arrangement. The presence of three peptides raises many questions on the structure of the complex and on the activity of each peptide; comparison of the antigonococcal and haemolytic activity of the purified peptides alone and after their reassociation in different arrangements will be needed to resolve these problems.

To our knowledge, the amino acid sequences of the peptides I, II and III are different from those reported in the literature for other proteins of staphylococci. Moreover, they are also different from the amino acid sequence of δ-lysin of *S. aureus* reported by Fitton *et al.* (1980) even though many properties are shared by these substances. Frenette *et al.* (1984) have shown that the antigonococcal substance and δ-lysin have similar haemolytic spectra, affinity for some phospholipids, the absence of proline, tyrosine, histidine, arginine and cysteine from their amino acid compositions, a high predominance of hydrophobic residues and the ability to disrupt tissue-culture cells. However, on the basis of the limited amino acid sequence homology between these substances, the peptides I, II and III cannot be considered as another class of δ-haemolysin, as is the case for dog δ-lysin (Fitton *et al.*, 1980). Moreover, the three components can also be differentiated from δ-lysin on the basis of their hydrophobicity profiles.

On the basis of their amino acid sequences, it is suggested that the peptides I, II and III could possibly act as signal sequences for three or more secreted or membrane-associated proteins. In his compilation of published signal sequences, Watson (1984) has found that signal peptides are characterized by the presence of an *N*-terminal formylmethionine residue, 20–40 amino acid residues and a high content of hydrophobic amino acids. Also, the general format of a signal seems to include a charged residue within the first five amino acid residues, followed by a core of hydrophobic residues. These properties are all observed for the peptides I, II and III. Signal peptides similar in length to the three peptides have been observed, for example the β-lactamase signal peptide of *Bacillus cereus* contains 45 amino acid residues (Sloma & Gross, 1983). Fitton *et al.* (1980) have already suggested that δ-lysin could act as a signal sequence, although Lee & Birkbeck (1984) suggest that this is unlikely.

It has been demonstrated that signal peptides share little primary sequence homology, and the study by Kendall *et al.* (1986) demonstrates the importance of overall structural features. When compared with the signal peptides of *S. aureus* for β-lactamase and staphylokinase, which contain 24 and 19 amino acid residues respectively, the primary sequence and the hydrophobicity profiles of peptides I, II and III are different. Although there is evidence suggesting that the three peptides represent signal sequences, the final proof of their role will result from the isolation and purification of the precursor proteins and comparison of their amino acid sequences with those of the three peptides described here. Confirmation of the signal peptide function of these peptides will allow us to study the process of localization and secretion of certain proteins by the staphylococcal cell. The small differences in the amino acid sequences among peptides I, II and III could be a unique tool to study the relationship between the structure and function of signal peptides.

We are grateful to Louis Racine and Rita Alary for excellent technical assistance. This study was supported by the Medical Research Council of Canada Grant no. MA-9253. This is N.R.C.C. publication no. 28341.

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Received 17 November 1987; accepted 15 January 1988