PURIFICATION AND N-TERMINAL SEQUENCE OF A FIMBRIAL PROTEIN FROM *MORAXELLA NONLIQUEFACIENS*

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1. Introduction

Flagella and fimbriae (= pili) are long, slender proteinaceous appendages of microorganisms. Flagella are well known as responsible for swimming motility [1] and the amino acid sequence of flagellin, the subunit protein of the flagellar filament, is known in one case [2]. Fimbriae are straight, thinner filaments also shown to be composed of a single protein which was purified and termed pilin [3]. Fimbriae have been found to occur in several genera of bacteria [4] and in fungi [5]. Two groups may be distinguished, one, the sex pili, found in low numbers per cell, associated with conjugating ability and DNA donor function [3,4]. The other group, common fimbriae, is of more general occurrence. It is unrelated to the donor function in bacterial mating, but has been associated with many other bacterial properties, e.g., haemagglutination [6], particular growth characteristics [3,4,7], increased competence in DNA transformation and pathogenicity [4]. Common [3,8] and sex fimbriae [9,10] have been purified and amino acid compositions reported [3,8,9] but no sequences published. Molecular weights of common fimbriae are reported in the range 17–19 000 and of sex fimbriae 11 800–12 500. Fimbriae of *Pseudomonas echinoides* which are responsible for star formation and conjugation in this species were reported to be of 8000 molecular weight [11].

We have purified common fimbriae from *Moraxella nonliquefaciens* [12] which show one band in sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis indicating an apparent molecular weight of about 17 000 and have detected one N-terminal amino acid which is uncommon. Part of the amino acid sequence has been determined.

2. Experimental

2.1. *Bacteria and cultivation*

The *M. nonliquefaciens* NCTC 7784 SC-c strain is the same as used in previous investigations [12,13]. It was cultivated on Mueller Hinton (Difco) plates with 1.7% agar, supplemented with 0.5% yeast extract (Difco), 20 ml per nine cm plate; growth for 13 h at 33°C in a humid atmosphere.

2.2. *Isolation and purification of fimbriae*

Cells from 100 agar plates were harvested into 40 ml Dulbecco phosphate buffered saline (D-PBS) pH 7.0 [12], sedimented by centrifugation at 4°C and 3600 × *g* for 10 min and washed once in D-PBS. The cellular pellet was suspended in 20 ml PBS, homogenized under ice-cooling in a Dounce homogenizer and centrifuged 3600 × *g* (supernate S 1). The pellet was rehomogenized and recentrifuged (supernate 2). The combined supernates 1 and 2 were precipitated with methanol at −65°C [14]. The sediment was suspended in 20 ml 0.01 M Tris, 0.01 M NaN_3 buffer,

pH 7.5 [15] and centrifuged at 12 000 × *g* for three min. The pellet was treated once more with 4 ml buffer. The combined supernates of these two 12 000 × *g* centrifugations were dialyzed first against 2 litres distilled water for 4 h with one change and next against 2 litres 0.1 M $MgCl_2$, 0.001 M NaN_3 for 16 h. Fimbriae were sedimented at 80 000 × *g* for 60 min [12]. Pooled material from several preparations was used.

Because the fimbrial pool material was suspected to contain bacterial lipopolysaccharides and lipid material an exhaustive organic wash procedure was utilized, for a 6 mg sample: twice with a mixture of chloroform (2 ml) and methanol (1 ml), 30 min each time by shaking at room temperature, followed by brief washes with 3 ml portions of methanol and acetone, three times each, and finally with 3 ml ether.

2.3. *SDS–polyacrylamide gel electrophoresis*

A slab-gel apparatus (Bio Rad Model 220), the Laemmli system [16], 17.5% acrylamide plus 0.5% *N,N'*-methylene bisacrylamide (both Bio Rad), a 3% stacking gel and a 1.5 mm thick slab was used. The electrophoresis buffer was 0.025 M Tris, 0.19 M glycine, 0.1% SDS (Bio Rad), pH 8.3. Samples were applied in 0.0625 M Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue and preheated 2 min at 100°C. For molecular weight estimation the following proteins were used: lysozyme, egg white grade 1, 3 × cryst. (Sigma), hemoglobin, bovine (Sigma) myoglobin, sperm whale, cryst. (Koch-Light), chymotrypsinogen A, bovine pancreas, 6 × cryst. (Koch-Light) and albumin, chicken egg, 5 × cryst. (Koch-Light).

2.4. *Protein determination*

The Schaffner and Weissman method [17] was used for all samples with bovine serum albumin (fraction V, Armour Pharmaceutical Company) as a standard.

2.5. *Amino acid analysis and sequence determination*

Protein samples of 1 mg were hydrolyzed under reduced pressure in 6 M HCl for 24, 48 and 96 h, and the amino acid analyses carried out as described [18]. A JEOL-JAS-47K Sequence Analyzer was used for automated Edman degradation with samples of 250 and 40 nmol. Reagents and solvents used were obtained from Pierce Chemical Company. The phenylthiohydantoin (PTH) amino acids were analysed by thin-layer chromatography (TLC), by gas chromatography and amino acid analysis of hydrolysates of the PTH-amino acids [18,19]. In addition to these indentification methods a thin-layer chromatography system utilizing Merck DC-Alufolien Kieselgel 60 F 254 Art. 5544 in the solvent chloroform/ethanol/methanol (88.2:1.8:10, v/v/v) was used.

3. Results and discussion

The fimbriae were prepared by a method to be reported in detail separately [12] giving a rather low yield (0.3–1.0% on the basis of protein) of pure material. In SDS–polyacrylamide gel electrophoresis, patterns given by bacteria dissolved in sample buffer, compared to the purified fimbrial protein are shown in fig.1. The bacteria display a large number of protein bands: a few are quite strong but no one dominating. Fimbriae show one strong band of slightly higher mobility than sperm whale myoglobin. The result of gel electrophoresis indicated that the fimbrial protein material was pure for further chemical characterization.

The amino acid composition of the fimbrial protein was calculated from the average or extrapolated amino acid analyses values and on the basis of a molecular weight of about 17 000, table 1. The protein has a rather high content of aspartic acid, threonine, alanine, isoleucine and leucine. Only 0.2 residues of histidine are found, for which there is no explanation. The content of tryptophan was not determined. The amino acid composition differs from that reported by others for fimbrial proteins [3,8,9]. N-terminal amino acid analyses revealed clearly only one amino acid residue, which indicates a high purity of the protein and is in agreement with the result of SDS–polyacrylamide gel electrophoresis. The PTH-amino acid derivative in step 1 of the sequencing came out as a single peak between PTH-methionine and PTH-phenylalanine on the gas chromatograph [19]. By TLC the PTH-derivative showed up as a single spot with a R_F-value slightly higher than PTH-leucine, in solvent system D [18], as well as in the chloroform/ethanol [18] solvent system. Amino acid analysis of an hydrolysate of the PTH-derivative, using the

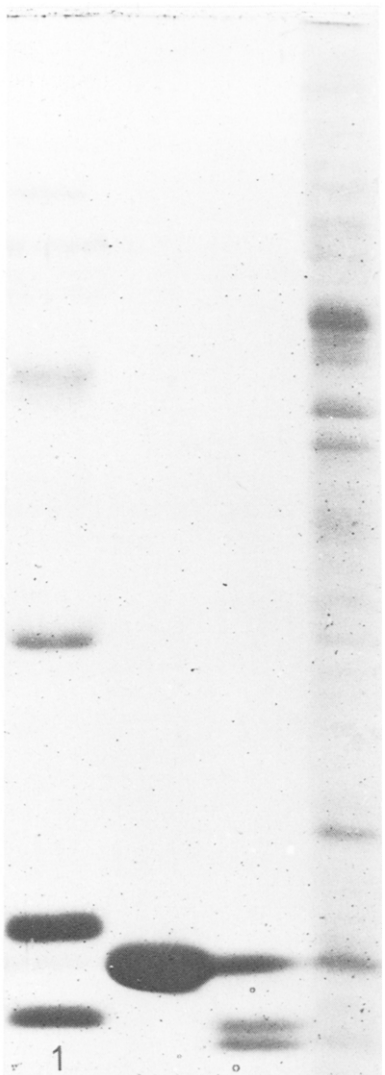

Fig.1. SDS–polyacrylamide gel electrophoresis of the fimbrial fraction and whole cells of *M. nonliquefaciens* NCTC 7784 SC-c compared to reference proteins. Only the separating gel is shown, and it is cut at the bromophenol blue position which was 85 mm from the top. Lane 1 from left (marked 1): lysozyme, myoglobin, chymotrypsinogen A and chicken egg albumin in mixture, about 1 μg of each (impurities in the upper fourth). Lane 2: fimbrial protein, 6 μg; lane 3: hemoglobin, 1 μg (double band) in mixture with some fimbrial protein from 3 Lane 4: cells, about 8 μg.

Table 1
Amino acid composition of the fimbrial protein

Amino acid	Number of residues per molecule	
Aspartic acid	20.2	(20)
Threonine	15.1	(15)
Serine	7.08	(8)
Glutamic acid	13.2	(13)
Proline	5.1	(5)
Glycine	13.9	(14)
Alanine	20.1	(20)
$\frac{1}{2}$ Cystine	1.17	(1-2)
Valine	7.16	(7)
Methionine	0.70	(1)
Isoleucine	15.7	(16)
Leucine	14.2	(14)
Tyrosine	7.63	(8)
Phenylalanine	5.84	(6)
Histidine	0.19	-
Lysine	13.7	(14)
Arginine	3.85	(4)
Total number of residues		166

Average of hydrolysates for 24, 48 and 96 h. Tryptophan was not determined.

standard 3-buffer system, revealed a peak at the position of allo-isoleucine. However, the peak width was broader than allo-isoleucine. The same peak showed also up in the chromatogram when total amino acid composition was determined from 24 h hydrolysate, but as the protein has a high content of isoleucine, it was thought to be allo-isoleucine. The size of the peak would correspond to 1 residue per molecule. This uncommon amino acid, which according to high resolution mass spectrometry is probably either *o*-(*m*- or *p*-)methyl-L-phenylalanine or L-α-amino-γ-phenyl butyric acid, is under further study to elucidate its structure [20]. The yield of this PTH-amino acid derivative in step 1 is about 100%, when using PTH-leucine as a standard for the calculation.

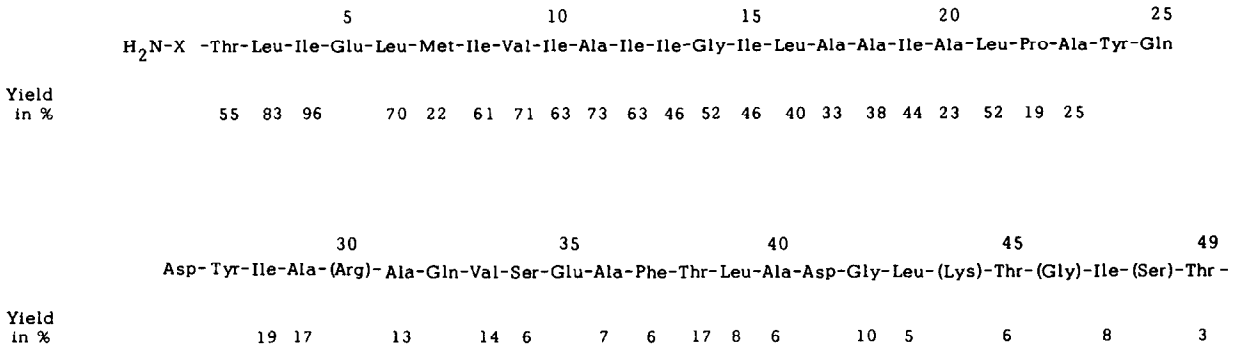

Fig.2. N-terminal amino acid sequence analysis of the fimbrial protein. Percent yield is based on gas chromatography data. The X indicates an uncommon amino acid found in the first position.

This yield is in good agreement with that obtained for the other residues (fig.2). The N-terminal sequence shows that the protein has an extremely hydrophobic region from residue 1 to about 40. According to the amino acid composition this would mean that other parts of the protein have to be very hydrophilic.

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