# Characterisation of the heptameric pore-forming complex of the *Aeromonas* toxin aerolysin using MALDI-TOF mass spectrometry

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**Abstract** **Aerolysin, a virulence factor secreted by *Aeromonas hydrophila*, is representative of a group of β-sheet toxins that must form stable homooligomers in order to be able to insert into biological membranes and generate channels. Electron microscopy and image analysis of two-dimensional membrane crystals had previously revealed a structure with 7-fold symmetry suggesting that aerolysin forms heptameric oligomers [Wilmsen et al. (1992) EMBO J. 11, 2457–2463]. However, this unusual molecularity of the channel remained to be confirmed by an independent method since low-resolution electron crystallography had led to artefactual data for other pore-forming toxins. In this study, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was used to measure the mass of the aerolysin oligomer preparation. A mass of 333 850 Da was measured, fitting very well with a heptameric complex (expected mass: 332 300 Da). These results confirm the earlier evidence that the aerolysin oligomer is a heptamer and also show that MALDI-TOF mass spectrometry could be a valuable tool to study non-covalent association of proteins.**

*Key words:* Matrix-assisted laser desorption/ionization; Mass spectrometry; Aerolysin; Non-covalent interaction; Oligomerization; Pore-forming toxin

## 1. Introduction

Aerolysin is representative of a group of toxins that form stable homooligomers which are capable of inserting into biological membranes to generate channels. Other members of the group include the alpha toxins from *Staphylococcus aureus* [2] and *Clostridium septicum* [3], protective antigen of anthrax toxin and the oxygen-labile toxins [4] produced by a great many Gram-positive bacteria. All of these proteins have been shown to contain high proportions of β-sheet structure and may form channels of the porin type (for a review see [5]). Aerolysin is secreted by *Aeromonas* spp. as an inactive dimeric precursor that is activated by proteolytic removal of about 40 amino acids from the C-terminus [6,7]. The toxin is concentrated on the surface of target cells by binding to a specific receptor [8]. Activation and concentration lead to oligomerization, and this is a prerequisite for channel formation [9]. The structure of proaerolysin has been solved at 2.8 Å resolution [10] and low-resolution images of 2-dimensional membrane crystals revealed an aerolysin oligomer structure with 7-fold symmetry [1] suggesting that the pore is built up by seven monomers. This unusual molecularity of the channel remained, however, to be confirmed by an independent method since low-resolution electron crystallography had led to artefactual data for other pore-forming toxins [11–13]. Analyses by SDS-PAGE as well as by STEM were not accurate enough to determine unambiguously the mass [1] and analytical centrifugation or cross-linking experiments gave conflicting results (Ausio and Buckley, personal communication).

Even though the aerolysin oligomeric complex is not covalent, it may be suited to analysis by mass spectrometry due to its unusual stability. It is not disrupted by guanidine hydrochloride, pH extremes, SDS, reducing agents or heat and it is resistant to many proteases [14]. Electrospray ionization mass spectrometry (ESMS) [15] has previously been used to characterise non-covalent receptor-ligand complexes [16], oligonucleotide associations [17,18], non-covalent protein-peptide complexes [19] and more recently to study the tetramers formed by avidin [20], streptavidin [21], hemoglobin [20] as well as yeast alcohol dehydrogenase and rabbit muscle pyruvate kinase [22]. However, the use of ESMS is usually limited to mass measurements below 150 kDa due to the difficulty of resolving multiply charged ions at higher mass. Thus complexes as large as the aerolysin oligomer cannot be studied by this method, at least with the instrumentation available now.

In contrast, MALDI-TOF mass spectrometry has been successfully used to measure masses of monomeric proteins greater than 200 kDa [23]. Thanks to its high sensitivity, this technique has been shown to be especially well suited for analysis of low amounts of biological samples. However, it was initially anticipated that it would not yield masses for non-covalent protein complexes, since initial dilution of samples in the matrix solution was thought to lead to the disruption of quaternary assemblies. Thus, although successful, MALDI-TOF mass measurements on small non-covalent complexes have been published [24], very few examples of specific oligomeric complexes have been studied with this technique [25]. In this paper we present the successful mass determination of the high molecular weight aerolysin oligomer by MALDI-TOF mass spectrometry. It confirms our earlier evidence that the oligomer is heptameric.

## 2. Materials and methods

### 2.1. Protein purification

Proaerolysin was purified as previously described [26]. Protein con-

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*Abbreviations:* MALDI-TOF, matrix-assisted laser desorption/ionization; ESMS, electrospray mass spectrometry.

centrations were calculated using an absorbance of 2.5 at 280 nm for a solution of pure aerolysin at 1 mg/ml [14].

*2.2. Activation of proaerolysin and preparation of the aerolysin oligomer*

Proaerolysin (0.4 mg/ml) was activated by treatment with trypsin attached to cross-linked beaded agarose (Sigma, 0.5 units of insoluble trypsin per ml of solution) for 1 h at room temperature in a buffer containing 150 mM NaCl, 100 mM Tris-HCl, pH 8.4. At this pH, aerolysin is unable to oligomerize and therefore does not attach to the agarose beads. The sample was then centrifuged in order to pellet the trypsin. Complete conversion to the aerolysin form of the toxin was confirmed by SDS-PAGE. In order to enable the toxin to oligomerize, the aerolysin sample was dialysed against a buffer containing 30 mM NaCl, 20 mM HEPES, pH 7.4 for 48 h at room temperature. This procedure results in almost complete conversion of the aerolysin dimer [27] to the oligomer as demonstrated by SDS-PAGE.

*2.3. Preparation of the oligomer for mass spectrometry*

The sample of aerolysin oligomer was dialysed overnight against 3 changes of distilled water at 4°C. The sample was then lyophilised using a Speed-Vac. Samples of aerolysin oligomer were prepared for analysis by redissolving 40 μg in 10 μl of 0.1% aqueous trifluoroacetic acid and diluting to a solution concentration of ~2 mM. A 13 mg/ml sinapinic acid (Fluka) solution in $H_2O$, 0.1% TFA/acetonitrile (1:1) was freshly prepared prior to experimentation. 1 μl of a 1:1 mixture of sinapinic acid solution with aerolysin solution was applied on the stainless-steel probe tip. Samples were then dried under moderate vacuum before insertion.

*2.4. Time-of-flight mass spectrometry*

Mass spectrometry was performed using a Bruker (Bremen, Germany) BIFLEX matrix-assisted laser desorption time-of-flight mass spectrometer equipped with the single stage ion source SCOUT with an X-Y multisample probe, a gridless reflector and the HIMAS linear detector. Ionization is accomplished with the 337 nm beam from a nitrogen laser with a repetition rate of 3 Hz. All data were acquired at 28 kV of acceleration potential in the positive ion mode and with the linear detector set to 8–8.5 kV post-acceleration.

## 3. Results

As illustrated in Fig. 1, activation of proaerolysin using insoluble trypsin followed by extensive dialysis of the sample against a low ionic strength buffer led to a preparation of aerolysin oligomers essentially free of dimers. However, we cannot preclude that undetectable amounts on SDS-PAGE of nicked proaerolysin dimers which did not undergo the oligomerization reaction and are detectable after the first dialysis step (lane 2, Fig. 1) remain in solution at the final stage.

Measurement of the mass of these oligomers obtained by spontaneous oligomerization above a certain protein concentration threshold in the absence of receptors and membranes is relevant only if they are identical to those which are formed at the surface of the target cell membranes. There is strong experimental evidence which supports this hypothesis. These oligomers formed identical channels in lipid planar bilayers as upon injection of proaerolysin and trypsin whether or not the receptor protein is present [8,28]. There is a complete parallelism between the loss of biological activity by mutation or by the presence of zinc ions and the loss of capacity to form oligomers in solution. Finally, we checked that these oligomers migrated at the same position as those found in the erythrocytes membranes after inducing lysis with proaerolysin and trypsin (data not shown).

The MALDI-TOF spectrum of this aerolysin oligomer preparation is shown Fig. 2. Two series of multiply charged species are observed. The charge in the first series, corresponding to the smaller species, labelled M ranges from 1 to

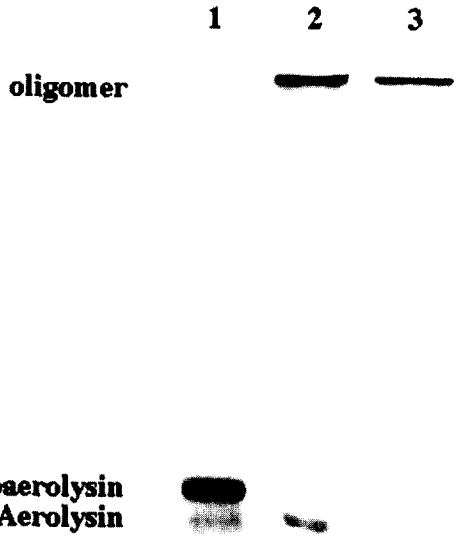


Fig. 1. Preparation of oligomeric aerolysin. The aerolysin oligomer was prepared as described in section 2. Lane: 1, proaerolysin marker showing a slight presence of activated aerolysin; 2, sample after dialysis against 30 mm NaCl, 20 mM HEPES, pH 7.4; 3, sample after dialysis against distilled water, just prior to lyophilization for mass spectrometry. The monomers seen on lane 2 indicate that a small portion of nicked proaerolysin remains as a dimer in solution. This dimer is dissociated under the SDS-PAGE experimental conditions [34].

3. The measured mass from the singly charged ion (47480 Da) can be assigned to the aerolysin monomer (expected mass: 47471 Da based on the amino acid sequence, in agreement with our previous mass determinations [7]). A small peak corresponding to the aerolysin dimer is also observed. This dimer is always present even when measuring the mass of very diluted solutions of protein indicating that this is a specific complex originating probably from cleaved proaerolysin which remains as a dimer in the preparation.The charge in the second series of peaks, labelled 7M, ranges from 1 to 4. The molecular mass of the singly charged ion in this series is 333850 Da, in good agreement with the predicted mass of the aerolysin heptamer (expected mass: 332300 Da). The sharpness of the peak at 334 kDa (resolution $m/\Delta m$ = 55 where $m$ is the mass) allows a rather accurate measurement of the mass. This resolution would allow the separation at 50% valley of two peaks with a mass difference of 5000 Da. The mass values calculated from the three other charge states of 7M (336490, 335060, 335080 Da for $7M^{4+}$, $7M^{3+}$ and $7M^{2+}$, respectively) are slightly higher, probably because of initial kinetic energy inhomogeneities [29].

In the MALDI-TOF spectra we obtained, only ions corresponding to the monomer, traces of dimers and a heptamer were observed. No ions corresponding to alternative degrees of association of aerolysin monomers were detectable (tri-, tetra-, penta-, hexa-or octameric complexes with calculated masses of 143.2, 190.9, 238.6, 286.4 or 381.8 kDa, respectively). The absence of these species is clearly not due to a limitation of the method as 5 kDa differences in molecular masses could be resolved. Moreover, the absence of this species strongly suggests that non-specific clusters, which can easily be observed in MALDI provided that the concentration of the analyte in the matrix and the fluence are high enough [30], are not formed under our experimental conditions.

It is interesting to note that aerolysin MALDI-TOF spectra could be obtained only at threshold irradiance [25] and within a defined concentration window of 1.5–0.5 pmol/μl which corresponds to an analyte/matrix ratio range from 1/20 000 to 1/50 000. This range is critical because at higher or lower analyte/matrix ratio only the monomer and traces of dimer were detected. This observation is in agreement with the results of Nelson et al. [31] who noted that the higher the analyte molecular weight, the smaller the concentration window where one can obtain satisfactory results. Nevertheless, within this concentration window spectra are remarkably reproducible from one experiment to another.

## 4. Discussion

Our results show convincingly that the aerolysin oligomer is heptameric, confirming the prediction made earlier using electron microscopy. Until recently this stoichiometry was considered rather unusual. However, studies on other pore-forming toxins indicate that association as heptamers may be characteristic of a whole class of channel-forming toxins which have substantial β-sheet structure. The same stoichiometry has been found with alpha toxin from *S. aureus*. This toxin, which has little sequence homology to aerolysin but shares a similar mode of action, was thought previously to form hexamers or tetramers based on electron microscopy [11], analytical centrifugation, cross-linking and SDS-PAGE analysis, [12,13]. However, X-ray analysis of crystals and an elegant charge shift migration assay have recently provided convincing evidence that alpha toxin forms a heptamer [32]. It has also been recently reported that the oligomeric forms of the protective antigen of anthrax toxin [33] as well as that of Vac A from *Helicabacter pylori* (R. Rappuoli, personal communication) are both heptamers.

We have demonstrated that it is possible to observe oligomeric structures formed by specific non-covalent interactions using MALDI-TOF mass spectrometry. The fact that the heptamer was not disrupted by the conditions involved in the sample preparation and production of the spectra is further evidence of the remarkable stability of this complex and may explain the success of the MALDI-TOF method with aerolysin. It is important to emphasize that the monomer-monomer interactions in the aerolysin oligomer are non-covalent since the complex can be dissociated when incubated with 70% formic acid for 30 min at room temperature. Experiments with less stable oligomers will show how generally applicable this method may be for the characterisation of non-covalent protein subunit associations. As oligomers with very high masses such as the aerolysin oligomer can only be observed in a well defined concentration window, we recommend that several analyte/matrix ratio be tested by preparing analyte solutions ranging from 10 pmol/μl to 0.1 pmol/μl.

How a dimeric protoxin assembles into a heptameric oligomer remains a puzzling question that is difficult to address. In

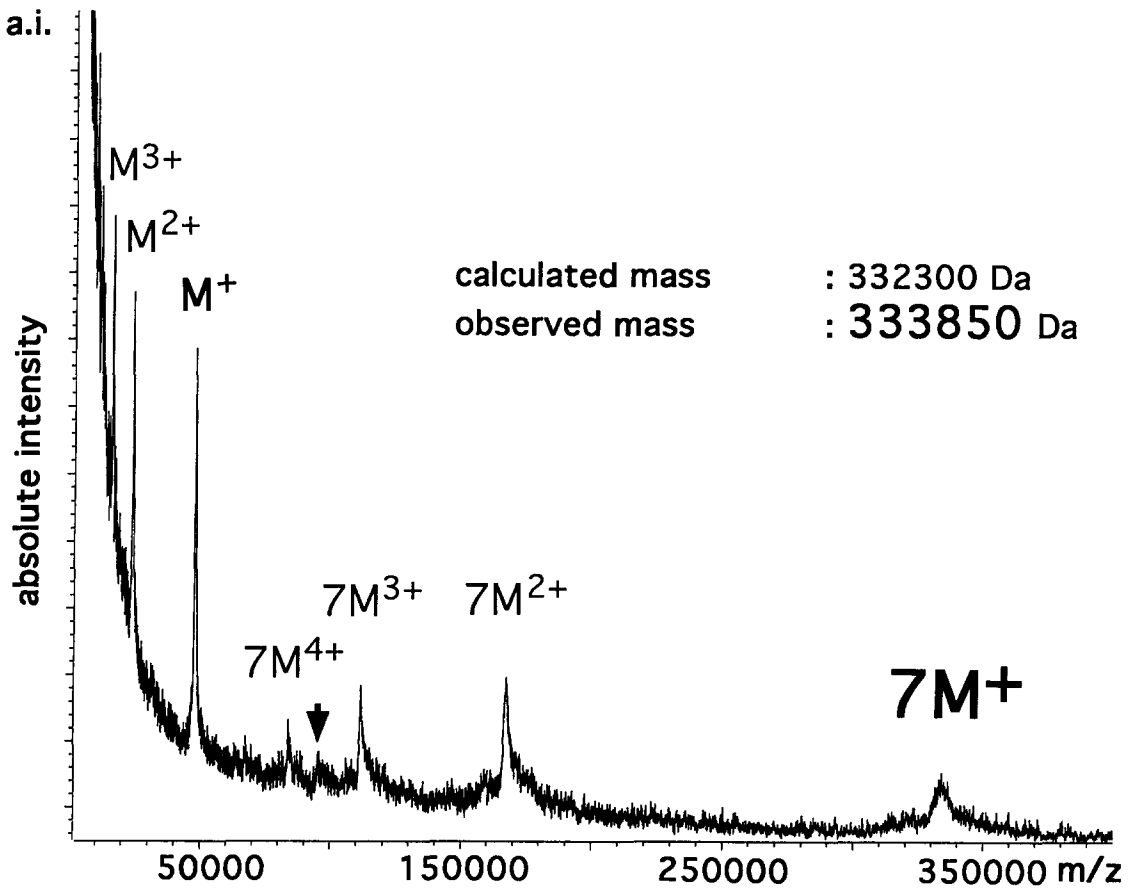


Fig. 2. MALDI-TOF mass spectrum of aerolysin heptamer. Measured molecular mass 333 850 Da. Less than 500 fmol loaded onto the target in sinapinic acid. M, monomer; 7M, heptamer. The arrow indicates a small peak corresponding to traces of aerolysin dimers.

this study, pure preformed aerolysin oligomers were analysed with success by MALDI-TOF mass spectrometry. This method may also prove to be a useful tool to study the assembly process of aerolysin into oligomers after activation of the protoxin by proteases and to detect intermediates in the association reaction.

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