Structure determination of the virulence factor AsaP1 from *Aeromonas salmonicida* subsp. *Achromogenes*

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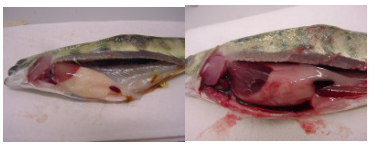
Introduction

Aeromonas commonly found in waters, wastewaters and mud are non-motile gram negative bacteria. Atypical strains[1] of *Aeromonas salmonicida* are known to be largely responsible for causing a lethal disease in Atlantic salmons and other farmed fishes[2]. The available commercial vaccines are reported to show different levels of protection[3]. Also the emergence of drug resistance are reported against the current chemotherapy.

Amongst the other virulence factors predicted from the genome of the strain A449, the *Aeromonas salmonicida* subsp. *achromogenes* peptidase 1 (AsaP1) is primarily responsible for causing the classical furunculosis. The mature active exotoxin Asap1 is a 20 kDa extracellular metallo-protease[4] and belongs to the family of aspzincins.

Aspzincins are metalloendopeptidase that needs Zn^{2+} in its active site. It contains a conserved HExxH zinc-binding motif where the two histidines are considered to serve as anchoring ligands for the Zn^{2+} and the glutamate as a catalytic residue.

Figure 1. Atlantic cod showing atypical infection by *Aeromonas salmonicida* subsp. *achromogenes* (Ref 1)



(a) (b)

Figure 2. A comparison of normal (a) and an infected cod (b). An infection leads to haemorrages in internal organs.

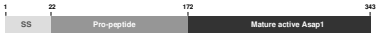


Figure 3. A schematic representation of Asap1. The aa sequence 1-22 represents the signal sequence (SS). The mature Asap1 responsible for furunculosis consists of aa from 172 to 343.

Expression, Purification and Crystallization

The native and 4 mutants (E294Q; E294A; Y309F and Y309 A) of AsaP1 were cloned into pJOE E 3075 vector and were successfully expressed as N-terminal 6xHis tagged soluble proteins in *E. coli* BL21 Pri 3715 strain. All proteins were purified using Ni-NTA, followed by gelfiltration as a monomer of 37kDa (Figure 4).

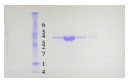


Figure 4. Purified Asap1 protein

To screening for the crystallization conditions, 900 conditions were tested for each protein using 5 mg/ml as a starting point by sitting drop method with the help of the CyBi®-CrystalCreator.

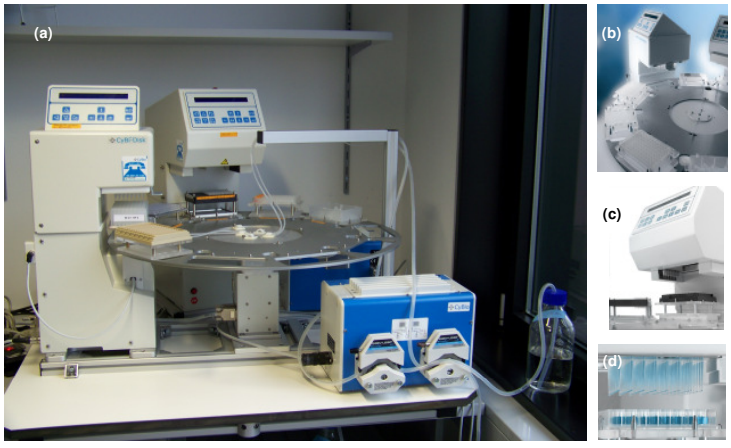


Figure 5. CyBi® -CrystalCreator: (a) A set up of the crystallization robot, (b) Rotating-disc with platforms, (c) 8-channel pipettor, (d) 96-channel pipettor.

As shown in Figure 5, all requirements to setup crystallization, viz., buffers in deep well block, tips for pipetting protein and buffers and a block for washing tips are mounted on to the rotating disk. The reservoir solution (40 µl) was pipetted in reservoir well of 96-well crystallization plate using 96-channel pipettor. This was also used to mix the reservoir solution (0.3 µl) with the protein solution (0.3 µl) in the protein well pipetted using 8-channel pipettor. All liquid handling during setting up crystallization experiments like pipetting, changing and washing tips were executed by integrated user-friendly CyBio® Composer Software. The initial hits for crystallization of all four mutants were obtained in first round of screening. Except AsaP1E294A mutant, other mutants of Asap1 under investigation gave hits in subsequent rounds of screening at higher protein concentration. The first crystals of the mutant AsaP1-E294Q were obtained with the protein concentration of 12.5 mg/ml (Fig. 6). The condition with 2M $(NH_4)_2SO_4$ was further optimized to 1.6M $(NH_4)_2SO_4$ yielding crystals which diffracted to at least1.60 Å.

(a) (b) (c)

Figure 6. First crystals of Asap1-E294Q from crystallization screens (a) 15% PEG 400, 0.1M Na-HEPES pH 7.5, 0.2M $MgCl_2$; (b) 0.2M $(NH_4)_2SO_4$, 0.1M Tris-HCl pH 8.5; (c) 2.0M $(NH_4)_2SO_4$, 2% PEG 400, 0.1M Na-HEPES pH 7.5.

Data collection and refinement

The crystals of AsaP1-E294Q were flash frozen at 100 K under cryo-stream using 20% (v/v) glycerol in the reservoir solution as a cryo-protectant. The data collected at out home source Rikagu MSC MicroMax007 using Saturn-92 CCD detector. The collected data were indexed, integrated and scaled using CrystalClear. The data were processed in the monoclinic space group C2 with a=99.92Å, b=73.50Å, c=54.77Å, β=111.33˚. The structure of AsaP1-E294Q mutant was solved by molecular replacement using *PHASER*[5]. *Grifola frondosa* zinc peptidase (PDB entry 1G12) was taken as a model. The refinement of the structure was performed using *REFMAC5*[5].

The structure of AsaP 1

The overall structure of AsaP1 is clearly distinguished in two domains (see also Fig. 3). The N-terminal domain (NT) is mainly composed of 8 anti-parallel β-strands. In contrast the C-terminal domain (CT) is composed of 8 α-helices and 4 β-strands. The residues of N-terminal domain from 23 to 172 is a pro-peptide region. The residues from C-terminal domains 173 to 343 represent the catalytic domain.

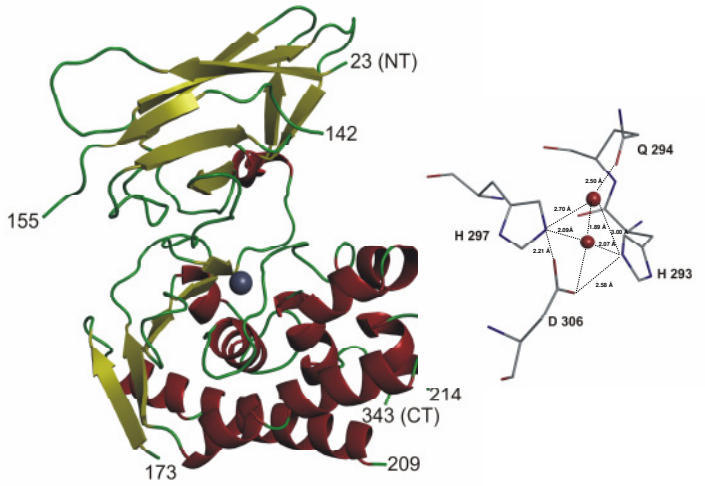


Figure 7. Ribbon plot of the overall structure of AsaP1 protein (left). The NT and CT indicate the position of the N- and C-terminus, respectively. The grey ball indicates the Zn^{2+}-ion of the active site. On the right, active site residues of the Zn^{2+}binding motif, where conserved E294 is replaced by Q294. A water molecule and H293, H297, D306 coordinate the Zn^{2+}-ion.

Most of the reported zinc metalloproteases in the PDB contains only this catalytic domain. Though the resolution of the collected data is 1.6Å, a few residues were found to be disordered for which no clear electron density were observed.

In the active site, the Zn^{2+}-ion was modelled in the electron density with lower occupancy. There is also a water molecule present at the Zn^{2+}-binding site. Because of presence of glutamine instead of glutamate, it is reasonable that the Zn^{2+} is present with the lower occupancy making the Asap1-mutant E294Q almost inactive.

Outlook

The information from the 3-dimensional structure of Asap1-E294Q may provide the better understanding towards the role of propeptide in the protein besides searching for inhibitors with improved activity and specificity.

References

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