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# Crystallization and preliminary X-ray diffraction studies of AsaP1_E294A and AsaP1_E294Q, two inactive mutants of the toxic zinc metallopeptidase AsaP1 from *Aeromonas salmonicida* subsp. *achromogenes*

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Two mutants of the toxic extracellular zinc endopeptidase AsaP1 (AsaP1_E294Q and AsaP1_E294A) of *Aeromonas salmonicida* subsp. *achromogenes* were expressed in *Escherichia coli* and crystallized by the vapour-diffusion method. Crystals were obtained using several precipitants and different protein concentrations. Protein crystals were found in a monoclinic (*C*2) as well as an orthorhombic ($P2_12_12_1$) space group. The crystals belonging to the monoclinic space group *C*2 had unit-cell parameters $a = 103.4$, $b = 70.9$, $c = 54.9$ Å, $\beta = 109.3^\circ$ for AsaP1_E294A, and $a = 98.5$, $b = 74.5$, $c = 54.7$ Å, $\beta = 112.4^\circ$ for AsaP1_E294Q. The unit-cell parameters of the orthorhombic crystal obtained for AsaP1_E294A were $a = 57.9$, $b = 60.2$, $c = 183.6$ Å. The crystals of the two different mutants diffracted X-rays beyond 2.0 Å resolution.

## 1. Introduction

*Aeromonas salmonicida* is a Gram-negative bacterium which has been known as a fish pathogen for over 90 years, but the mechanisms of its virulence are only partly understood. Both farmed and wild fish are affected in marine as well as freshwater environments. *A. salmonicida* has so far been divided into five subspecies (Pavan *et al.*, 2000; Holt *et al.*, 1994). *A. salmonicida* subsp. *salmonicida* is often referred to as the typical strain, whereas the others, based on the heterogeneity in this group, have been referred to as atypical strains (Wiklund *et al.*, 1994). One group of atypical *A. salmonicida*, *i.e.* subsp. *achromogenes*, has recently been found to form a homogeneous group of strains that cause atypical furunculosis, a systemic disease, in many species of fish (Austin *et al.*, 1998; Bjornsdóttir *et al.*, 2004; Gudmundsdóttir, Lange *et al.*, 2003; Gudmundsdóttir, Hvanndal *et al.*, 2003; Lund & Mikkelsen, 2004).

*A. salmonicida* subsp. *achromogenes* produces a toxic peptidase, AsaP1, which is a major virulence factor (Gudmundsdóttir *et al.*, 1990; Arnadóttir *et al.*, 2008). AsaP1 has not been detected in secretions from typical *A. salmonicida* (Gudmundsdóttir, Hvanndal *et al.*, 2003).

AsaP1 is a caseinolytic zinc metallopeptidase that is lethally toxic to both fish and mice (Gudmundsdóttir *et al.*, 1990; Gudmundsdóttir & Gudmundsdóttir, 2001). Sublethal doses of AsaP1 injected intramuscularly induce pathological changes in Atlantic salmon (*Salmo salar* L.) that are comparable to those seen in fish with atypical furunculosis (Gudmundsdóttir *et al.*, 1990).

Mature AsaP1 is composed of 172 amino acids and has a calculated molecular mass of 19 kDa. It is autocatalytically released from a 343-amino-acid proenzyme with a calculated molecular mass of 37 kDa (Arnadóttir *et al.*, 2008). AsaP1 contains the typical zinc-binding motif HE*xx*H, where *x* is any amino-acid residue and the two histidines coordinate the zinc ion. The glutamate is most probably involved in catalysis. Three families, aspzincins, metzincins and gluzincins, can be distinguished among these zinc peptidases, in which the third zinc ligand is aspartate, histidine or glutamate, respectively (Hooper, 1994). AsaP1 belongs to the family of aspzincins, in which a

GT*x*D*xx*YG loop is conserved and the aspartate within this sequence has been shown to act as a zinc ligand (Hori *et al.*, 2001; McAuley *et al.*, 2001).

For biochemical and structural characterization of AsaP1, an *Escherichia coli* expression system was established for two inactive mutants of AsaP1 created by site-directed mutagenesis (Hentschke, 2008). In this way, the glutamic acid at position 294 which is involved in the catalysis was replaced by an alanine (AsaP1_E294A) or by a glutamine (AsaP1_E294Q). Both mutants are inactive. Because of the inactivity of the mutants, the propeptide still remains as part of the protein but its function (whether it plays a role in protease folding and acts as an intramolecular chaperone or whether it inhibits proteinase activity in the intracellular space) still remains unclear.

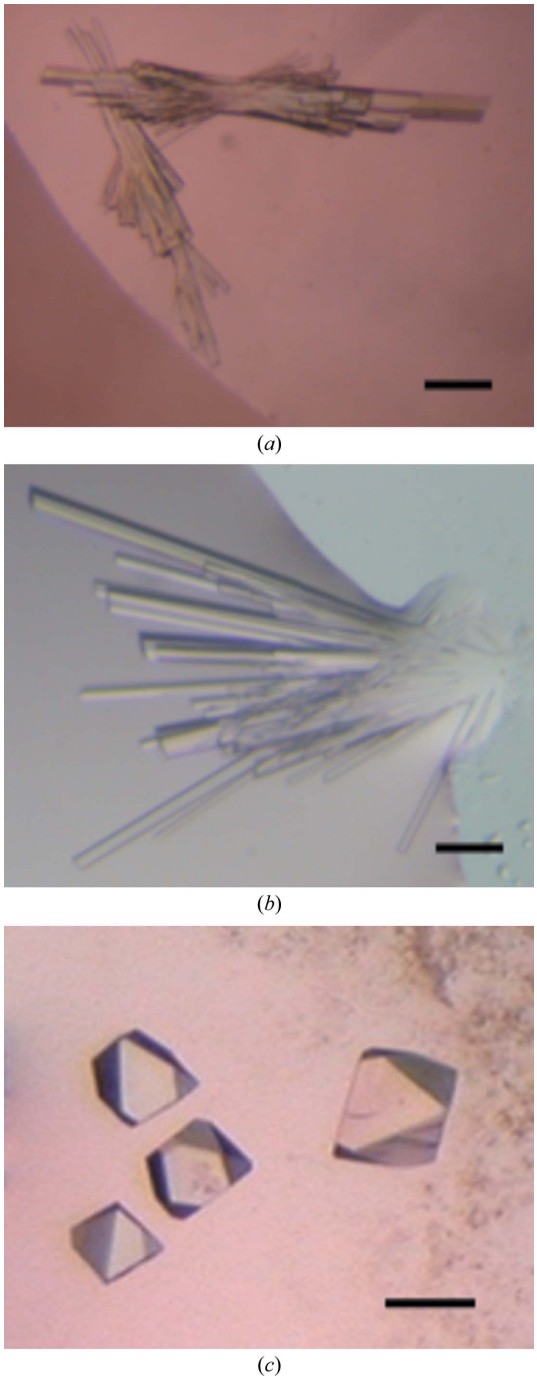

**Figure 1**
Crystals from the two different AsaP1 mutants E294Q and E294A grown by the hanging-drop vapour-diffusion method. (*a*) Monoclinic crystals of AsaP1_E294Q and (*b*) monoclinic crystals of AsaP1_E294A. (*c*) Orthorhombic crystals of AsaP1_E294A. The solid bar represents 200 µm.

## 2. Protein expression and purification

Starting from a glycerol stock frozen at 193 K of *E. coli* BL21 containing the expression vector pJOE E3075 (Stumpp *et al.*, 2000; Hentschke, 2008) encoding the His-tagged sequence for AsaP1_E294Q and AsaP1_E294A, a pre-culture was prepared with 5 ml Luria/Miller medium containing 0.1 mg ml$^{-1}$ ampicillin. Cultures were incubated overnight at 310 K with 220 rev min$^{-1}$ shaking.

1 l Luria/Miller medium containing 0.1 mg ml$^{-1}$ ampicillin was freshly inoculated with the overnight preculture at 310 K and 220 rev min$^{-1}$ shaking. Protein expression was induced at an $OD_{600\,nm}$ between 0.5 and 1.0 absorption units by adding L-rhamnose to a final concentration of 1 mg ml$^{-1}$ and cultures were incubated at 295 K and 220 rev min$^{-1}$ for 16 h.

Cells were harvested by centrifugation at 5000*g* and resuspended in 20 m*M* Tris–HCl pH 7.6 containing 300 m*M* NaCl. The cells were lysed by sonication and the homogenate was centrifuged for 1 h at 48 000*g* and 277 K. The supernatant was filtered through a 0.2 µm syringe filter and loaded onto an $Ni^{2+}$-charged immobilized metal-affinity (PorosMC) chromatography column with buffer *A* (20 m*M* Tris–HCl pH 7.6, 300 m*M* NaCl, 50 m*M* imidazole). The protein was eluted with buffer *B* (20 m*M* Tris–HCl pH 7.6, 300 m*M* NaCl, 200 m*M* imidazole) using a linear gradient from 0% to 100%. Fractions containing AsaP1 mutants were pooled and concentrated by ultrafiltration before loading onto a gel-filtration column (Superdex200). The buffer for gel filtration was composed of 20 m*M* Tris–HCl pH 7.6 and 300 m*M* NaCl. The protein concentration was determined by UV absorption ($\varepsilon_{280\,nm}$ = 31 400 $M^{-1}$ cm$^{-1}$).

## 3. Crystallization

Initial screening for crystallization conditions was performed using a CyBio crystallization robot with the sitting-drop vapour-diffusion method in 96-well plates (CrystalQuick Lp). For crystallization, 0.3 µl protein solution (concentrated to 5–25 mg ml$^{-1}$) and 0.3 µl reservoir solution were mixed in each drop and equilibrated against 40 µl reservoir solution. From these initial screens (JBScreen Classic 1–10, Jena Bioscience) the first hits were found (1.8 *M* ammonium sulfate, 0.1 *M* MES–NaOH pH 6.5 for the monoclinic crystals of AsaP1_E294Q, 2.0 *M* ammonium sulfate, 0.1 *M* Tris–HCl pH 8.5 for the monoclinic crystals of AsaP1_E294A and 16% PEG 4000, 10% 2-propanol, 0.1 *M* HEPES pH 7.5, 0.2 *M* ammonium sulfate for the orthorhombic crystals of AsaP1_E294A). These crystallization conditions were further optimized using 24-well crystallization plates (Greiner Bio-One). Optimization of crystallization conditions was carried out using the hanging-drop vapour-diffusion method. Each well contained 500 µl reservoir solution and the drop was a mixture of 1 µl protein solution and 1 µl reservoir solution. Crystallization of both inactive mutants AsaP1_E294A and AsaP1_E294Q occurred under several conditions and at different protein concentrations. Usually, colourless crystals appeared within 4 d.

Crystals of AsaP1_E294Q suitable for data collection were obtained from a protein solution at 7–10 mg ml$^{-1}$. Drops comprised of 2 µl protein solution and 2 µl reservoir solution (0.2 *M* MES–NaOH pH 7.5 and 1.6 *M* ammonium sulfate) were equilibrated against 500 µl reservoir solution (Fig. 1*a*).

**Table 1**
X-ray data-collection statistics of AsaP1_E294A and Asap1_E294Q.

Values in parentheses are for the outer resolution shell.

| Crystal | AsaP1_E294Q | AsaP1_E294A | AsaP1_E294A |
|---|---|---|---|
| X-ray source | EMBL X12 c/o DESY | Rigaku MicroMax007 | EMBL X12 c/o DESY |
| Detector | MAR CCD 225 | Rigaku CCD Saturn92 | MARCCD 225 |
| Wavelength (Å) | 0.95369 | 1.5418 | 0.97784 |
| Resolution range (Å) | 99.0–2.0 (2.05–2.00) | 35.2–2.1 (2.26–2.18) | 90.0–2.2 (2.25–2.20) |
| Space group | $C2$ | $C2$ | $P2_12_12_1$ |
| Unit-cell parameters | | | |
| $a$ (Å) | 98.5 | 103.4 | 57.8 |
| $b$ (Å) | 74.5 | 70.9 | 60.2 |
| $c$ (Å) | 54.7 | 54.8 | 183.6 |
| $\alpha$ (°) | 90.0 | 90.0 | 90.0 |
| $\beta$ (°) | 112.4 | 109.3 | 90.0 |
| $\gamma$ (°) | 90.0 | 90.0 | 90.0 |
| Total reflections | 92926 | 133664 | 204992 |
| Unique reflections | 24682 (1451) | 21701 (2103) | 33551 (7629) |
| Completeness (%) | 98.9 (86.3) | 98.9 (96.2) | 99.4 (93.1) |
| $R_{merge}$ (%) | 10.1 (39.3) | 9.7 (59.2) | 13.3 (60.2) |
| $R_{r.i.m.}$ (%) | 10.7 (53.3) | 11.2 (83.1) | 15.9 (50.7) |
| $R_{p.i.m.}$ (%) | 5.5 (27.8) | 4.4 (37.7) | 6.0 (18.5) |
| Average $I/\sigma(I)$ | 18.7 (3.3) | 8.8 (1.6) | 19.3 (2.69) |
| $d$-spacing (Å) | 2.05–2.00 | 2.26–2.18 | 2.25–2.20 |
| Mosaicity (°) | 1.5 | 0.7 | 0.9 |
| Redundancy | 3.8 (3.4) | 6.2 (4.5) | 6.1 (3.7) |
| Wilson $B$ factor (Å$^2$) | 23.7 | 44.5 | 41.7 |
| No. of images | 360 | 740 | 360 |

The crystallization condition for AsaP1_E294A (7–10 mg ml$^{-1}$) was found to be 0.1 *M* Tris–HCl pH 8.5 and 2.2 *M* ammonium sulfate (Fig. 1*b*). Bipyramidal shaped crystals appeared (Fig. 1*c*) using a crystallization condition composed of 15% PEG 4000, 0.1 *M* HEPES pH 7.5, 0.2 *M* ammonium sulfate and 10% 2-propanol.

## 4. Data collection and X-ray crystallographic analysis

Before mounting, crystals were transferred into and soaked with a suitable cryoprotectant for 30 s. For cryoprotection, the reservoir solution was mixed with glycerol to a final concentration of 20%(*v*/*v*) for the crystals of AsaP1_E294Q and AsaP1_E294A grown in 0.2 *M* MES–NaOH pH 7.5, 1.6 *M* ammonium sulfate and in 0.1 *M* Tris–HCl pH 8.5, 2.2 *M* ammonium sulfate, respectively. Crystals of AsaP1_E294A grown in 15% PEG 4000, 0.1 *M* HEPES pH 7.5, 0.2 *M* ammonium sulfate and 10% 2-propanol were transferred to a cryosolution containing the reservoir solution and 20% PEG 400. Crystals were then flash-cooled to 110 K (Oxford Cryosystems).

Diffraction data were collected on the home source, a Rigaku rotating-anode X-ray generator (MicroMax007) with Osmic multiple layer optics (beam size 0.3 × 0.3 mm), with a CCD detector (Saturn92) and also on the EMBL beamline X12 at DESY, Hamburg with a MAR CCD 225 (MAR Research, Germany).

The crystals of AsaP1_E294A (0.2 *M* MES–NaOH pH 7.5, 1.6 *M* ammonium sulfate) and AsaP1_E294Q (0.1 *M* Tris–HCl pH 8.5, 2.2 *M* ammonium sulfate) belonged to the monoclinic space group *C*2 and showed the same packing in general, with unit-cell parameters $a$ = 103.4, $b$ = 70.9, $c$ = 54.9 Å, $\beta$ = 109.3° for AsaP1_E294A and $a$ = 98.5, $b$ = 74.5, $c$ = 54.7 Å, $\beta$ = 112.4° for AsaP1_E294Q. The crystals of AsaP1_E294A grown in 15% PEG 4000, 0.1 *M* HEPES pH 7.5, 0.2 *M* ammonium sulfate and 10% 2-propanol belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a$ = 57.9, $b$ = 60.2, $c$ = 183.6 Å. The diffraction data from the crystals measured in-house were processed using the software *CrystalClear*1.3.6 (Pflugrath, 1999); the data from the crystal measured on EMBL beamline X12 were processed using *DENZO* and *SCALEPACK* from the *HKL* package (Otwinowski & Minor, 1997). To obtain values of $R_{p.i.m}$ and $R_{r.i.m.}$, the data were processed with *MOSFLM* and *SCALA* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

For data collection of AsaP1_E294Q one needle was broken off the crystal cluster shown in Fig. 1(*a*). This mechanical stress probably led to the high mosaicity of 1.5°. The needle-shaped crystals of AsaP1_E294A (Fig. 1*b*) were easier to handle.

Assuming the presence of one molecule in the asymmetric unit for the crystals belonging to the monoclinic space group, the Matthews coefficients $V_M$ for AsaP1_E294A and AsaP1_E294Q were calculated to be 2.58 and 2.53 Å$^3$ Da$^{-1}$, respectively. The Matthews coefficient $V_M$ for the crystal of AsaP1_E294A belonging to the orthorhombic space group was calculated to be 2.16 Å$^3$ Da$^{-1}$, assuming the presence of two molecules in the asymmetric unit.

Molecular replacement using the program *Phaser* (McCoy, 2007) was successful using the peptidyl-Lys metalloendopeptidase from *Grifola frondosa* (PDB entry 1g12) as a search model. The polypeptide of the search model consisted of only the C-terminal protease domain (amino acids 182–348), which shares a sequence identity of 43% with residues 171–343 of the AsaP1 protease domain. Initial phasing also revealed interpretable electron density for the propeptide domain of both inactive AsaP1 mutants. Currently, model building and refinement of all data sets is in progress.

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