SHORT COMMUNICATIONS

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Acta Cryst. (1993). D**49**, 349–352

Purification, crystallization and preliminary X-ray investigation of aqualysin I, a heat-stable serine protease.

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(*Received* 6 *April* 1992; *accepted* 17 *November* 1992)

Abstract

Aqualysin I, a thermostable protease found in the culture medium of *Thermus aquaticus* YT-1, has been purified to homogeneity using a combination of ion-exchange and affinity chromatography. It is a polypeptide with a molecular weight of 28 350 [Kwon, Terada, Matsuzawa & Ohta (1988). *Eur. J. Biochem.* **173**, 491–497] and is most active at 343–353 K and pH about 10.0 [Matsuzawa, Tokugawa, Hamaoki, Mizoguchi, Taguchi, Terada, Kwon & Ohta (1988). *Eur. J. Biochem.* **171**, 441–447]. Crystals of the enzyme are monoclinic, space group $P2_1$, with cell dimensions $a = 40.80$ (5), $b = 64.39$ (6), $c = 45.51$ (6) Å and $\beta = 109.1$ (1)°. The asymmetric unit consists of a single molecule ($V_m = 1.99$ Å^3 Da^{-1}). The crystals are stable to X-radiation and scatter to at least 2.8 Å resolution.

Introduction

Microbial serine proteases have been the subject of major research programs to determine their catalytic mechanism of action and to define their industrial applications. The subtilisin family of enzymes, produced by several strains of *Bacillus*, has been most extensively characterized. Complete amino-acid sequences of many subtilisins have been determined and shown to be homologous (Siezen, de Vos, Leunissen & Dijkstra, 1991). This homology has been shown by X-ray crystallography to extend also to the tertiary structures of these enzymes.

Recently, the subtilisins have been divided into two subfamilies, the cysteine-free and the cysteine-containing enzymes. Specificially, the amino-acid sequences and detailed three-dimensional structures of the cysteine-containing serine proteases, thermitase (Dauter, Betzel, Hohne, Ingelmann & Wilson, 1988) and proteinase K (Betzel, Pal & Saenger, 1988), show that they are closely related to the subtilisins.

The proteases from thermophilic bacteria are particularly interesting since they potentially offer insights into the mechanism of thermostability. One thermophilic bacterium that has been shown to produce extracellular proteases is *Thermus aquaticus* YT-1. During its growth, this bacterium secretes two proteases, an alkaline protease (aqualysin I) and a neutral protease (aqualysin II) (Matsuzawa, Hamakoi & Ohta, 1983). Aqualysin I is maximally active against various substrates at 343–353 K and in the pH range about 10.0 (Matsuzawa *et al.*, 1988). The gene for aqualysin I has been cloned and its amino-acid sequence has been determined from the gene (Kwon, Terada, Matsuzawa & Ohta, 1988). An alignment of its 281 amino-acid sequence (Kwon, Terada, Matsuzawa & Ohta, 1988) shows that aqualysin I is homologous to the subtilisin-type serine proteases with approximately 40% sequence identity to proteinase K. The primary structure of the enzyme also revealed that the enzyme contains four cysteine residues, thereby identifying it as a member of the cysteine-containing subfamily of the subtilisin-type proteases. By analyzing the fragments of a chymotryptic digest of aqualysin I, the four cysteine residues have been determined to form two disulfide bonds in the enzyme (Kwon, Matsuzawa & Ohta, 1988). These properties made aqualysin I an attractive candidate to purify and determine its crystal structure in order to investigate the structural origin of its thermostability. While the work at the Procter & Gamble Company (PRG and DRT) on purifying and crystallizing aqualysin I was in progress, a method for purifying aqualysin I using ion-exchange chromatography and pH elution from CM-Sepharose was reported (Matsuzawa *et al.*, 1988). We report here an independently developed purification method and the crystallization of aqualysin I.

Experimental

Thermus aquaticus YT-1 was purchased from The American Type Culture Collection. Bactopeptone and bactotryptone were from Difco. Urea and ammonium sulfate were enzyme grade from Bethesda Research Laboratories. Tris, glycine, sodium dodecyl sulfate, phenylmethylsulfonyl fluoride (PMSF) and *N*-benzoyloxyvsrbonyl-L-leucyl-*p*-nitrophenyl ester (*N*-Cbz-Leu-NPE) were purchased from Sigma Chemical Company. All other electrophoresis chemicals were from Bio-Rad. Sephadex G-75 Superfine and CM-Sepharose were obtained from Pharmacia and immobilized *N*-Cbz-D-phenylalanine and HPLC/Spectro Grade trifluoroacetic acid were purchased from Pierce Chemical Co. For HPLC, we used a Vydac Protein C4 column and HPLC solvents made by J. T. Baker, Inc. All other chemicals were reagent grade.

Assay of aqualysin I

The protease was assayed in two ways. First, protease was added to 0.5 ml of 50 m*M* potassium phosphate, pH 8.5, plus 0.6% casein at 343 K. The reaction was stopped with 0.5 ml of 10% trichloroacetic acid and placed

0907-4449/93/030349-04$06.00

on ice for 10 min. The sample was centrifuged at 277 K for 10 min and the absorbance of the supernatant at 280 nm was measured. A unit of activity was defined as 1.0 OD280 absorbing trichloroacetic acid-soluble material released per minute. Alternatively, the enzyme was assayed at 315 K in 1 ml of 0.5 m*M* *N*-Cbz-Leu-NPE, 50 m*M* potassium phosphate, pH 8.5. The reaction was stopped by adding 10 μl of 0.1 *M* PMSF and placed on ice. After centrifugation in a microfuge for 5 min at 277 K, the absorbance of the supernatant was measured at 400 nm. A unit of activity was defined at 1.00 OD400 absorbance increase per min.

Protein analysis

Samples were assayed for protein quantity using the method of Lowry, Rosebrough, Farr & Randall (1951) and absorbance values were compared to a bovine serum albumin standard curve. Purity was determined by discontinuous sodium dodecyl sulfate (SDS) electrophoresis (Laemmli, 1970). To prevent proteolysis, samples were treated with PMSF and precipitated in 5% trichloroacetic acid before suspension and boiling in sample buffer. A 5–15% gradient gel was used for analysis.

Growth of Thermus aquaticus

The cells were maintained in 5 ml cultures incubated at 333 K in media consisting of 0.4% bactopeptone and 0.2% bactotryptone brought to pH 7.2 with NaOH. For production of aqualysin I, a 5 ml culture that had been growing for 48–72 h was used to inoculate 1 l of the same media plus 10% 10X Castenholz salts (Ramaley & Hixson, 1970). These cultures were grown with shaking in a 2.8 l Fernbach flask at 333–336 K for 26 h. Growth of the inoculating cultures for a minimum of 48 h, addition of Castenholz salts and maintenance of a moderate (333 K) temperature were found to be essential for optimal recovery of activity.

Purification of aqualysin I

All steps were carried out at 277 K. Fractions were stored at 253 K between steps. Cultures of *Thermus aquaticus* were harvested by centrifugation in a Damon/IEC 981 rotor at 4000 r min^{-1} (4500 × *g*) for 10 min. The volume of supernatant was measured and poured into 4 l beakers. Solid ammonium sulfate was added to 95% saturation at 277 K (645 g l^{-1}) and stirred for 2 h. The suspension was centrifuged for 30 min as described above and the supernatant was discarded. The precipitate was dissolved in 15 ml of 50 m*M* Tris, pH 8.3 per litre of suspension and exhaustively dialyzed against 25 m*M* Tris, pH 8.3. A slimy precipitate was removed by centrifugation in a GSA rotor at 7000 r min^{-1} for 5 min. Supernatant from about 45 l of cell growth was then passed through a 5.6 × 41 cm column of CM-Sepharose. Due to residual suspended particulate, the column became fouled and the top was stirred every few hours. Loading the material took about 2 days. A portion of the activity did not bind to the column and the eluate was stored for reapplication to a fresh column. After the sample was loaded, the column was washed with 500 ml of 50 m*M* Tris, pH 8.3, and eluted with 3 l of 0–0.3 *M* NaCl gradient of the same buffer. Fractions of 25 ml were collected and assayed for activity and protein (Fig. 1). Combined active fractions from the CM-Sepharose column were applied to a 2.5 × 14 cm column of *N*-Cbz-D-phenylalanine agarose equilibrated with 50 m*M* Tris, pH 8.3. The column was then washed consecutively with 50 ml of buffer, 100 ml of buffer containing 2*M* NaCl, then 70 ml of buffer. The activity was eluted with 200 ml of buffer containing 2 *M* guanidine hydrochloride. Fractions of 5 ml were collected and assayed for activity and protein. For crystallization attempts, the protein was dialyzed against 20 m*M* 2-(*N*-morpholino)ethanesulfonic acid buffer, pH 6.0, and concentrated to 5.0 mg ml^{-1}.

Crystallization of aqualysin I

Crystals were obtained by vapor diffusion, initially on a small scale in hanging drops (4 μl) and subsequently by seeding (20 μl) sitting drops. The hanging-drop experiments were performed by mixing 2 μl of the protein solution that had been inhibited with PMSF and 2 μl of reservoir solution consisting of 21% polyethylene glycol 8000 in 100 m*M* p*H* 7.5 phosphate buffer. The droplets were stored over the reservoir solutions at room temperature (296 K) and plate-like crystals appeared at 1–2 weeks. Fresh droplets were then made by mixing equal volumes of the protein solution and a reservoir solution of the same composition as that of the hanging-drop experiment. After these droplets had equilibrated in a depression-spot plate/sandwich-box apparatus for several hours, they were seeded with the small crystals. The crystals grew to their final size during the next week. Most of the crystals were severely twinned and either cracked upon being mounted in glass capillaries or failed to diffract X-rays. After several disappointments of this type had occurred, we found that the problems could be diagnosed by carefully examining the crystals under a polarizing light microscope. Eventually one single crystal (0.70 × 0.22 × 0.02 mm) was obtained (*cf.* Fig. 2).

Results

Aqualysin I was purified to homogeneity using a combination of ion-exchange and affinity chromatography. The final preparation represented a 27.7-fold purification over ammonium sulfate-pelleted growth media with a yield of 34% (Table 1). An SDS-gel electrophoretic analysis of the purification is shown in Fig. 3. The enzyme migrated as a single M_r = 28 500 band whether reduced or not reduced with β-mercaptoethanol. Purity was confirmed by HPLC over a C4 reverse-phase column. A single peak was observed (data not shown).

For smaller preparations, it was possible to precipitate the activity from the cell culture media using a 50–75% ethanol cut. The pellet appeared much cleaner than when using ammonium sulfate and equivalent yields of activity were obtained; however, the use of ethanol as a precipitant was inconvenient for large preparations. The ammonium sulfate pellet was not compact, and it was sometimes difficult to completely separate it from the supernatant. When this happened, the supernatant was vacuum filtered and the filter paper was soaked in buffer to dissolve the protein. The immediate application of the dissolved protein to the CM-Sepharose may have been the best pro-

cedure; however, since we wanted to accumulate many litres of material, the samples were frozen until needed. Freezing caused a slimy precipitate to form which was not completely removed by centrifugation and thus fouled the CM-Sepharose. We had to occasionally stir the top of the column. The CM-Sepharose cleaned up the material sufficiently to apply it directly to the affinity column. Most of the activity was retained on the affinity column. If a small (2 ml) column was used for a small preparation, a few contaminants were not separated and eluted with the activity in the guanidine hydrochloride step. These could be separated from the activity using gel partition chromatography over Sephadex G-75 Superfine. By increasing the size of the affinity column, the activity was separated from the minor contaminants during the elution step.

The identity of aqualysin I was confirmed in several ways. First, the activity was measured at different temperatures and at various values of pH using casein as a substrate. Activity was maximal at 343–353 K and at pH 10.0–11.5. These results agreed with previously published results (Matsuzawa *et al.*, 1988). A sample of purified material was subjected to NH_2-terminal sequence analysis for 24 cycles (data not shown). The sequence matched exactly the reported sequence (Matsuzawa *et al.*, 1988). Thus, we concluded that we were able to affinity-purify aqualysin I.

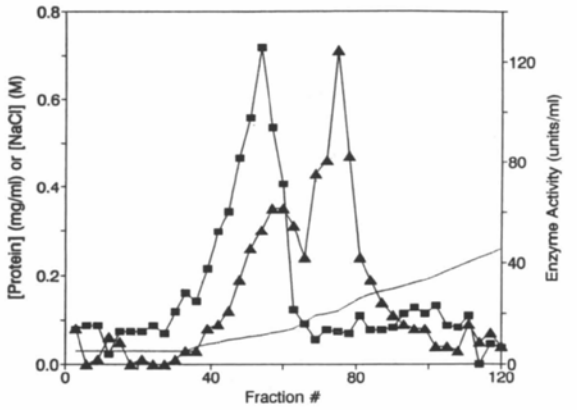

Fig. 1. Chromatography of aqualysin I over CM-Sepharose. Fractions 24–62 were pooled for further purification. Activity is indicated by ■, protein concentration by ▲, sodium chloride concentration by the dotted line.

Table 1. *Purification of aqualysin* I

Activity was measured using *N*-Cbz-L-Leu-NPE as substrate at 315 K.

Step	Protein (mg)	Activity (units)	Specific activity (units mg^{-1})	Purification (fold)	Yield (%)
Ammonium sulfate	6200	173000	27.9	1	100
CM-sepharose	350	95250	186	6.7	55
D-Phe agarose	77	59500	773	27.7	34

For the X-ray study the crystal of aqualysin I was mounted in a glass capillary along with a small quantity of its mother liquor and sealed with dental wax. The crystal was then transferred to the goniostat of a Rigaku AFC-5/RU-200 diffractometer. The X-ray generator was operated at 50 kV and 180 mA. A systematic search for reflections in the range 5–15° 2θ provided 25 reflections that defined a primitive monoclinic unit cell with the dimensions a = 40.80 (5), b = 64.39 (6), c = 45.51 (6) Å, and β = 109.1 (1)°. The $2/m$ diffraction symmetry was determined by comparing the intensities of 25 mirror-related pairs of reflections. A quadrant of X-ray diffraction data within the range h = 0 to 15, k = 0 to 15, and l = −15 to 15 were collected using the ω-scan method at 16° min^{-1} and a maximum of four repeated scans per reflection. Examination of these data revealed the crystal scattered to at least 2.8 Å resolution. By using the data in the resolution range 4–8 Å, preliminary solutions to the structure by the molecular-replacement technique as implemented in the *MERLOT*

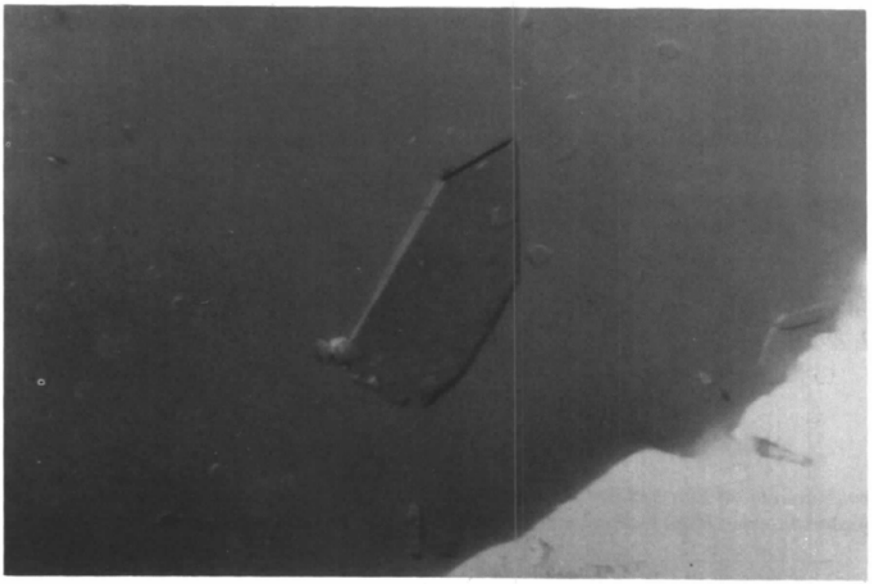

Fig. 2. Photomicrograph of a crystal of aqualysin I showing a similar morphology to that of the crystal used for data collection.

(Fitzgerald, 1988) suite of programs were obtained with both subtilisin BPN′ (Bott *et al.*, 1988) ($R = 0.46$) and proteinase K (Betzel, Pal & Saenger, 1988) ($R = 0.40$) as the search model. In both cases, the complete model for the enzyme was employed. Efforts to refine the structure by both simulated annealing [*X-PLOR* (Brünger, Kuriyan & Karplus, 1987)] and restrained least-squares [*PROFFT* (Finzel, 1987)] methods produced poor-quality electron-density maps that were not suitable to fit to the aqualysin I sequence. Work is currently underway to obtain crystals suitable for higher-resolution X-ray data collection and the determination and refinement of the structure of the enzyme.

Note: The protein sample used for the crystallization experiments was recently analyzed by laser desorption mass spectrometry (LDMS) by T. Keough and M. Lacey (Procter & Gamble Company). The measured M_r of the intact protein is 28 296 Da and is approximately 51 Da less than the M_r calculated from the gene sequence of aqualysin I (Kwon, Terada, Matsuzawa & Ohta, 1988). This discrepancy in M_r suggests that the loss of a glycine residue has occurred. Additional LDMS analyses of a CNBr-treated sample and a carboxypeptidase Y-treated fragment from the CNBr treatment of the enzyme have shown the protein specimen is a mixture of the intact enzyme (~ 20% abundance) and the enzyme with its C-terminal glycine removed (~ 80% abundance). The cause of this loss of the C-terminal residue could not be determined.

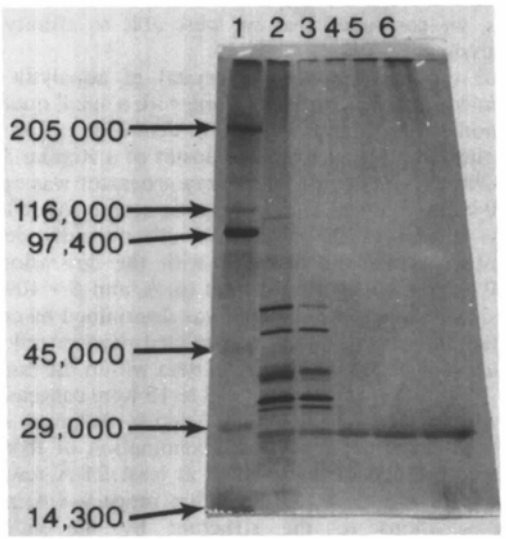

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis. Lane 1, standards: myosin, 205 000; α-galactosidase, 116 000; phosphorylase B, 97 400; ovalbumin, 45 000; carbonic anhydrase, 29 000; lysosyme, 14 300. Lane 2, ammonium sulfate pellet. Lane 3, pooled fractions from the CM-Sepharose column. Lanes 4–6, affinity purified aqualysin I (1, 6 and 12 μg of protein, respectively).

The authors thank Raymond A. Grant of the Procter & Gamble laboratories for performing the sequence analysis experiments.

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Acta Cryst. (1993). D**49**, 352–354

Crystallization of a *Humicola lanuginosa* lipase-inhibitor complex with the use of polyethylene glycol monomethyl ether.

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(*Received* 5 *November* 1992; *accepted* 26 *January* 1993)

Abstract

The fungal *Humicola lanuginosa* lipase complexed with the inhibitor *n*-dodecylphosphonate ethyl ester was crystallized in space group $P2_12_12_1$ with pseudotetragonal unit-cell parameters of $a = 131.7\,(2)$, $b = 131.3\,(1)$, $c = 75.4\,(1)$ Å. 92% of X-ray diffraction data to 2.8 Å resolution were collected with a final $R_{merge} = 8.5\%$. The crystals were grown using a new kind of precipitant – polyethylene glycol monomethyl ether (Peg-mme) of molecular weight 5000.

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0907-4449/93/030352-03$06.00