# Crystallization and preliminary X-ray diffraction analysis of proline iminopeptidase from *Xanthomonas campestris* pv. *citri*

F.J. Medrano[a,*], J. Alonso[b], J.L. García[b], W. Bode[a], F.X. Gomis-Rüth[a]

[a] *Max-Planck-Institut für Biochemie, Abteilung Structurforschung, Am Klopferspitz 18A, D-82152 Martinsried, Germany*
[b] *Departamento de Microbiología Molecular, Centro de Investigaciones Biológicas, C.S.I.C., Velázquez 144, 28006 Madrid, Spain*

Received 14 November 1996

**Abstract Proline iminopeptidase from *Xanthomonas campestris* pv. *citri*, displaying no significant sequence homology to any protein previously analyzed by X-ray crystallography, has been crystallized using the vapour diffusion method. Two different orthorhombic crystal forms (space group C222 and I222) were obtained from a solution containing NaCl or polyethylene glycol monomethyl ether (MW 5000) as precipitating agent for the native and lanthanum-derivatized protein, respectively. Complete diffraction data sets have been collected up to 2.6 Å (native) and 3.0 Å (lanthanum derivative) resolution. Cell dimensions are $a = 147.2$ Å, $b = 167.8$ Å, and $c = 85.6$ Å (C222) and $a = 146.7$ Å, $b = 167.7$ Å, and $c = 171.4$ Å (I222), respectively. Considerations of the possible values of $V_m$ and analysis of the self-rotation function of the native crystals account for the presence of one dimer per asymmetric unit, whereas a tetramer probably would occupy the smallest crystallographically independent crystal portion in the lanthanum-derivatized protein crystals.**

*Key words:* Protein crystallization; X-ray diffraction analysis; Proline iminopeptidase; *Xanthomonas campestris* pv. *citri*

## 1. Introduction

*Xanthomonas campestris* is a Gram-negative bacterium belonging to the family Pseudomonaceae which is phytopathogenic for cruciferous plants. *X. campestris* pv. *citri* is associated with various citrus bacterial diseases [1]. It is known that protease-deficient mutants of *X. campestris* pathovars induce only mild symptoms and show reduced bacterial counts in plants [2].

Proline iminopeptidase (PIP; EC 3.4.11.5) activity was first reported by Sarid et al. [3]. Different sources of the enzyme have been described implying its wide distribution in nature. However, this enzyme has been found mainly in bacteria [4–12] and in some plants [13]. The failure to isolate the enzyme from mammalian tissues, together with the fact that other aminopeptidases like leucine aminopeptidase [14] and even carboxylesterases [15,16] have a weak activity on the amide substrate used in the proline iminopeptidase assay, suggests the absence of a true PIP activity in the latter sources.

PIP catalyzes the removal of amino-terminal proline from peptides with high specificity. Several PIP genes have been cloned and sequenced to date: *Bacillus coagulans* [17], *Neisseria gonorrhoeae* [4], *Lactobacillus delbrueckii* ssp. *bulgaricus* [6], *L. delbrueckii* ssp. *lactis* [11], *Aeromonas sobria* [9], and *Mycoplasma genitalium* [18]. These enzymes show a high degree of sequence homology, and share similar characteristics with regard to substrate specificity and molecular weight, and can therefore be grouped together in a PIP family.

In order to analyze the structural determinants by which PIP cleaves their substrates, we have undertaken an X-ray study of *X. campestris* pv. *citri* PIP and here we report the crystallization and preliminary X-ray diffraction analysis. As no phase information of any sequentially related protein is available, the structural analysis will require use of the multiple isomorphous replacement method.

## 2. Materials and methods

### 2.1. Protein purification

*Escherichia coli* HB101 harboring the plasmid pJJ185 [19] was grown in LB medium. All purification procedures were performed at 4°C. The washed cells were suspended in 20 mM Tris-HCl buffer, pH 8.0 (buffer A), and disrupted with a French pressure cell (Aminco). Unbroken cells and cell debris were removed by centrifugation at 35000×*g* for 1 h. Ammonium sulfate was added to the supernatant to make it 40% saturated, the mixture was allowed to stand for 1 h, and the precipitated proteins were recovered by centrifugation. The pellet obtained was dissolved in buffer A, and dialyzed against the same buffer. The protein solution was applied to a DEAE-cellulose column (5×15 cm) equilibrated with buffer A, elution being carried out with a linear gradient of 0.0–0.5 M sodium chloride. The active fractions were combined and desalted by dialysis. The protein solution was then applied to a Sephadex G-200 column (2.5×80 cm). The protein was followed by monitoring the enzymatic activity (see below) and purity was checked by SDS-PAGE [20].

### 2.2. Enzyme activity assay

Proline iminopeptidase activity was assayed by measuring the amount of *p*-nitroanilide (*p*-NA) liberated from *p*-NA substrates as described previously [11].

### 2.3. Crystallization

Crystallization experiments were carried out at a constant temperature of 20°C using the sitting-drop vapour diffusion method. All reservoir solutions contained 0.02% (w/v) sodium azide. The initial PIP concentration was 10 mg/ml in 20 mM Tris-HCl buffer, pH 8.0. Drops were prepared by mixing 5 μl of PIP solution with 5 μl of the reservoir solution. The mixture was allowed to equilibrate against 0.4 ml reservoir solution.

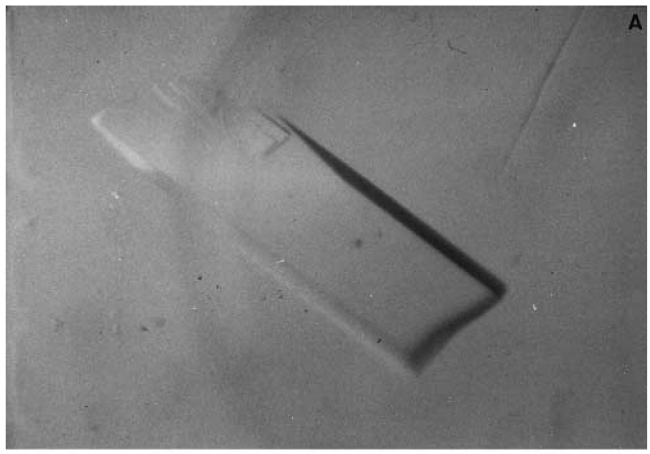
### 2.4. X-ray diffraction analysis

Crystals were harvested using the reservoir solution and mounted in thin-walled glass capillary tubes. X-ray diffraction data were collected at −15 to −20°C (in order to prevent diffraction decay due to radiation damage) on a 300 mm MAR-Research image plate detector attached to a Rigaku RU200 rotating Cu-anode X-Ray generator operated at 120 mA and 45 kV. The data were evaluated with the MOSFLM package [21], and loaded, scaled and merged with CCP4 suite [22].

Local symmetry axis search was performed by means of the GLRF program [23], using data between 20 and 3.5 Å resolution, and sampling the rotating space in steps of 2° at the beginning and of 0.1° around the initially found peaks in the last stage of calculations.

*Corresponding author. Fax: (49) (89) 85783516.
E-mail: medrano@alf.biochem.mpg.de


*PII* S0014-5793(96)01363-4



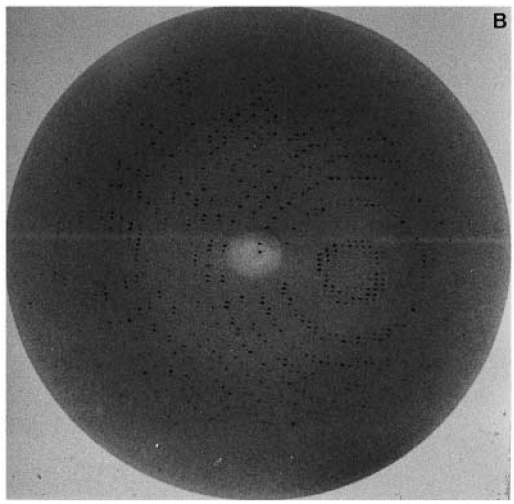




Fig. 1. (A) Orthorhombic crystal, belonging to the space group C222, of the proline iminopeptidase grown in 0.1 M sodium citrate buffer, pH 6.0 and 4.0 M NaCl. Crystal size is 0.8 mm×0.4 mm×0.2 mm. (B) X-ray diffraction pattern recorded on an MAR Research Image Plate detector at 220 mm crystal-to-detector distance.

## 3. Results and discussion

Initial crystallization experiments were carried out using the screening protocols described by Jankarik and Kim [24] with protein obtained after DEAE-cellulose chromatography (see Section 2). At this stage, PIP is not completely pure, and accounts for approx. 75–80% of the total protein present in the preparation. Small crystals and needles were obtained when sodium acetate, sodium tartrate, sodium formate, ammonium sulfate, magnesium sulfate or jeffamine M-600 were used as precipitating agents. Larger crystals grew in 1–2 months when polyethylene glycol monomethyl ether (MW 5000) or NaCl were used as precipitating agents. Using the latter, crystals grew over a wide range of pH (5.6–8.0) and in different buffers, namely sodium citrate, Tris, and HEPES. Best results were obtained employing 0.1 M sodium citrate buffer, pH 6.0 and 4.0 M NaCl. In order to reduce the time of growth of the crystals, different ratios of protein to reservoir solution volume were used. Optimal conditions were achieved using a ratio of 3:1 (v/v). Under these conditions, large crystals (0.9 mm×0.4 mm×0.2 mm) grew in 2–4 weeks (Fig. 1). These crystals belong to the orthorhombic space group C222, have unit cell dimensions $a=147.16$ Å, $b=167.84$ Å, $c=85.58$ Å, and diffract beyond 2.6 Å resolution (Table 1). When polyethylene glycol monomethyl ether was used as the precipitating agent best results were obtained using 0.2 M Tris-HCl pH 8.1 and 12% polyethylene glycol monomethyl ether. These crystals are isomorphous to those obtained with NaCl and diffract to the same maximum resolution.

Similar results were obtained when pure protein was used for crystallization. These crystals have the same morphology and share space group and cell unit constants with those obtained with less pure protein. Crystals were washed and dissolved displaying only one band on SDS-PAGE of approx. 37 kDa corresponding to the PIP.

Due to the fact that $HgCl_2$ and $LaCl_3$ are inhibitors (data not shown) of the enzymatic activity of the proline iminopeptidase, and in order to obtain isomorphous heavy-atom derivatives, crystallization experiments were carried out in the presence of 0.1–5 mM of each metal. Two types of crystals grew in the presence of $LaCl_3$ when NaCl was used as precipitant agent. One form belonged to the same space group and had similar unit cell dimensions to those obtained in its absence, whereas the other form belonged to the orthorhombic space group I222, had unit cell dimensions $a=146.63$ Å, $b=167.68$ Å, $c=171.37$ Å, and diffracted to 3.0 Å resolution (Table 1). Only crystals isomorphous to the native ones grew in the presence of $HgCl_2$.

From the native C222 crystal form two data sets from one single crystal each were collected, integrated, loaded, scaled and merged. From the I222 crystal form one complete data set from a single crystal was collected, loaded, scaled and merged. Data collection and statistics are summarized in Table 1.

Analysis of the self-rotation function (Fig. 2) reveals the presence of one non-crystallographic 2-fold axis at polar an-

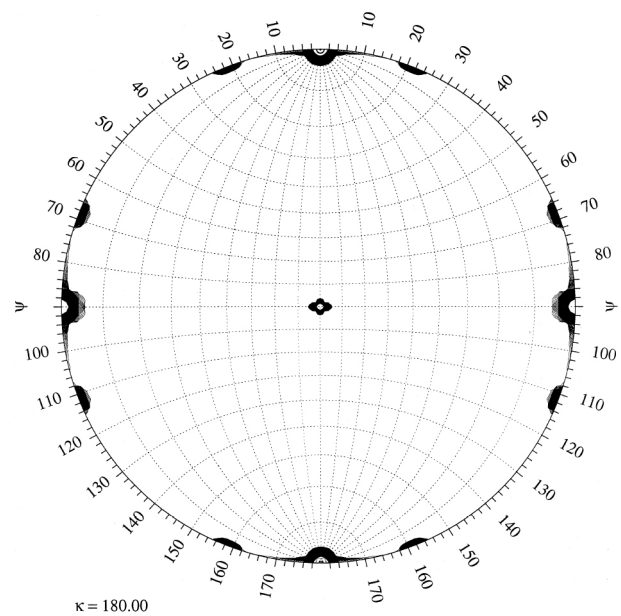



Fig. 2. Stereographic projection of the self-rotation function calculated by GLRF using data from 20 to 3.5 Å resolution. Section $\kappa=180°$, showing the 3 crystallographic 2-fold axes and one additional local axis at $\phi=359.0°$, $\varphi=21.4°$ parallel to the *ab* plane. The crystal axes run horizontally (a), vertically (b), and out of the plane towards the reader (c).

Table 1
Data collection and processing statistics

| | C222 crystal form | I222 crystal form |
|---|---|---|
| Cell constants | $a = 147.2$ Å<br>$b = 167.8$ Å<br>$c = 85.6$ Å | $a = 146.7$ Å<br>$b = 167.7$ Å<br>$c = 171.4$ Å |
| Space group | C222 | I222 |
| Crystal-to-detector distance | 220 mm | 220 mm |
| Maximum resolution | 2.6 Å | 3.0 Å |
| Rotation for each exposure | 1° | 1° |
| Time for each image | 1500 s | 1500 s |
| Total rotation for a data set | 90° | 90° |
| Number of measured reflections | 154900 | 108912 |
| Number of unique reflections | 29854 | 34586 |
| $R_{merge}$[a] | 9.6% | 15.4% |
| Completeness | 93.9% (9.85–2.63 Å) | 81.7% (11.23–3.00 Å) |
| Last resolution shell | 65.6% (2.71–2.63 Å) | 52.3% (3.08–3.00 Å) |

[a] $R_{merge} = \Sigma\Sigma|I(h)_i - <I(h)>|/\Sigma <I(h)>$; $I(h)_i$ is the observed intensity of the $i$-th measurement of reflection $h$, and $<I(h)>$ the mean intensity of reflection $h$; calculated after loading, and scaling.

gles $\phi = 359.0°$, $\varphi = 21.4°$ and $\kappa = 180.1°$ that is lies almost in the *ab* plane. The most probable calculated packing parameter $V_m$ [25] is 3.6 Å$^3$/Da. This accounts, together with the analysis of the self-rotation function, for the presence of one dimer per asymmetric unit. Data for several heavy-atom derivatives are being collected and their difference Patterson maps analyzed.

*Acknowledgements:* We thank Prof. Robert Huber for the possibility of performing this work in his department. Financial help was provided by the European Union (Grant CHRX-CT 94 0535) and by a grant from the F.P.I. program of the Ministerio de Educación y Ciéncia to F.X.G.-R. (EX 94 46121143).

## References

[1] Verniere, C., Provost, O., Civerolo, E.L., Gambin, O., Jacquemoud-Collet, J.P. and Luisette, J. (1993) Appl. Environ. Microbiol. 59, 243–249.
[2] Dow, J.M., Fan, M.J., Newman, M.-A. and Daniels, M.J. (1993) Appl. Environ. Microbiol. 59, 3996–4003.
[3] Sarid, S., Berger, A. and Katchalski, E. (1959) J. Biol. Chem. 234, 1740–1744.
[4] Albertson, N.H. and Koomey, M. (1993) Mol. Microbiol. 9, 1203–1211.
[5] Allaker, R.P., Young, K.A. and Hardie, J.M. (1994) FEMS Microbiol. Lett. 123, 69–74.
[6] Atlan, D., Gilbert, C., Blanc, B. and Portalier, R. (1994) Microbiology 140, 527–535.
[7] Ehrenfreund, P., Mollay, C. and Kreil, G. (1992) Biochim. Biophys. Res. Commun. 184, 1250–1255.
[8] Gilbert, C., Atlan, D., Blanc, C. and Portalier, R. (1994) Microbiology 140, 537–542.
[9] Kitazono, A., Kitano, A., Tsuru, D. and Yoshimoto, T. (1994) J. Biochem. 116, 818–825.
[10] Kitazono, A., Ito, K. and Yoshimoto, T. (1994) J. Biochem. 116, 943–945.
[11] Klein, J.R., Schmidt, U. and Plapp, R. (1994) Microbiology 140, 1133–1139.
[12] Yoshimoto, T., Saeki, T. and Tsuru, D. (1983) J. Biochem. 93, 469–477.
[13] Ninomiya, K., Kawatani, K., Tanaka, S., Kawata, S. and Makisumi, S. (1982) J. Biochem. 92, 413–421.
[14] Turzynski, A. and Mentlein, R. (1990) Eur. J. Biochem. 190, 509–515.
[15] Heymann, E. and Mentlein, R. (1981) Methods Enzymol. 77, 333–344.
[16] Matsushima, M., Inoue, H., Ichinose, M., Tsukada, S., Miki, K., Kurokawa, K., Takahashi, T. and Takahashi, K. (1991) FEBS Lett. 293, 37–41.
[17] Kitazono, A., Yoshimoto, T. and Tsuru, D. (1992) J. Bacteriol. 174, 7919–7925.
[18] Fraser, C.M., Gocayne, J.D., White, O., Adams, M.D., Clayton, R.A., Fleischmann, R.D., Bult, C.J., Kerlavage, A.R., Sutton, G., Kelley, J.M., Fritchman, J.L., Weidman, J.F., Small, K.V., Sandusky, M., Fuhrmann, J., Nguyen, D., Utterback, T., Saudek, D.M., Phillips, C.A., Merrick, J.M., Tomb, J.F., Dougherty, B.A., Bott, K.F., Hu, P.-C., Lucier, T.S., Peterson, S.N., Smith, H.O., Hutchison III, C.A. and Venter, J.C. (1995) Science 270, 397–403.
[19] Alonso, J. and García, J.L. (1996) Microbiology 142, 2951–2957.
[20] Laemmli, U.K. (1970) Nature 227, 680–685.
[21] Leslie, A.G.W. (1991) in: Crystallographic Computing V (Moras, D., Podjarny, A.D. and Tierry, J.P. eds.) pp. 27–38, Oxford University Press, Oxford.
[22] Collaborative Computational Project no. 4 (1994) Acta Crystallogr. D50, 760–763.
[23] Tong, L. and Rossmann, M.G. (1990) Acta Crystallogr. A46, 783–792.
[24] Jankarik, J. and Kim, S.M. (1991) J. Appl. Crystallogr. 24, 409–411.
[25] Matthews, B.W. (1968) J. Mol. Biol. 33, 491–497.