SHORT COMMUNICATIONS

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Crystallization and preliminary X-ray analysis of pectin lyase A from *Aspergillus niger*

JOHN JENKINS,[a]* MANDY SCOTT,[a] OLGA MAYANS,[a] RICHARD PICKERSGILL,[a] GILLIAN HARRIS,[a] IAN CONNERTON[a] AND TROELS GRAVESEN[b] *at* [a]*Department of Protein Engineering, Institute of Food Research, Earley Gate, Whiteknights Road, Reading RG6 6BZ, England, and* [b]*Danisco Ingredients, Edwin Rahrs Vej 38, DK-8220 Brabrand, Denmark*

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Abstract

The major secreted pectin lyase (E.C. 4.2.2.10) from *Aspergillus niger*, strain 4M-147, has been purified and crystallized by the hanging-drop method using polyethylene glycol as precipitant. The crystals belong to the space group $P2_12_12_1$ with cell dimensions $a = 45.2$, $b = 83.2$, $c = 93.1$ Å (1 Å = 0.1 nm) and a single molecule in the asymmetric unit. The crystals diffract to at least 2.0 Å resolution and are suitable for structure determination.

1. Introduction

Pectate and pectin lyases (E.C. 4.2.2.9 and E.C. 4.2.2.10, respectively) are produced by bacteria and fungi (Pilnik & Rombouts, 1981), whilst related genes are found in many plants (Wing, Yamaguchi, Larabell, Ursin & McCormick, 1990). These enzymes catalyze the degradation of pectate or pectin by β-elimination, breaking the α-glycosidic bond between O1 and C4 to leave an unsaturated 4–5 carbon–carbon bond. Pectate lyases are distinguished from pectin lyases by their specificity for pectin which has been demethylated by pectin methyl esterase. All pectate lyases require calcium for activity and most have a pH optimum near pH 8.5. In contrast pectin lyases generally have a much lower pH optimum near pH 5.0 and do not require calcium for activity.

Pectinase preparations containing pectin lyases from *Aspergillus spp.* are used on a very large scale as a processing aid in the food industry (Voragen & Pilnik, 1989; Whitaker, 1990). Pectin and pectate lyases from both bacteria and fungi play a major role in phytopathology (Crawford & Kolattukudy, 1987; Barras, van Gijsegem & Chatterjee, 1994) and in return evolution has endowed plants with both protein (Bugbee, 1993) and non-protein inhibitors (Wattad, Dinoor & Prusky, 1994) of these enzymes.

The crystal structures of three pectate lyases, PelC and PelE from *Erwinia chrysanthemi* (Yoder, Keen & Jurnak, 1993; Lietzke, Yoder, Keen & Jurnak, 1994) and the pectate lyase of *Bacillus subtilis* (Pickersgill, Jenkins, Harris, Nasser & Robert-Baudouy, 1994) have recently been determined, showing the new parallel β-helix architecture (Jurnak, Yoder, Pickersgill & Jenkins, 1994). Sequences show that the majority of pectate and pectin lyases, including those with known three-dimensional structures, form a single family of homologous enzymes despite rather low percentages of identical amino acids (Gysler, Harmsen, Kester, Visser & Heim, 1990; Kusters-van Someren, Harmsen, Kester & Visser, 1991; Kusters-van Someren, 1991).

We have obtained crystals from a pectin lyase occurring in *A. niger* strain 4M-147, which we show to be related to pectin lyase A of *A. niger* N400. The sequence of pectin lyase A from *A. niger* N400 is predicted to give a mature sequence of 359 residues (37.9 kDa). It shows approximately 20% identity with the bacterial pectate lyases and 45–60% identity with the other isozymes found in *Aspergillus spp.* (Kusters-van Someren, 1991).

2. Materials and methods

2.1. *Protein purification*

The pectin lyase secreted from *A. niger*, strain 4M-147 (Culture Collection of Grindsted Products, Brabrand, Denmark), was purified from the crude fermentation broth using successively Sephadex G-25 SF gel-filtration medium (50 × 200 mm) for desalting, Q-Sepharose High Performance anion exchanger (25 × 100 mm), Phenyl-Sepharose High Performance (25 × 100 mm) for hydrophobic interaction chromatography and Superdex 75 (50 × 600 mm) gel-filtration medium.

2.2. *Protein characterization*

Purity was checked by running the pectin lyase on Mono-Q (HR 5/5) and by isoelectric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis. (All media for chromatography and electrophoresis from Pharmacia-LKB Biotechnology.)

N-terminal amino-acid sequences were determined by gas-phase sequencing. A peptide was purified by high-pressure liquid chromatography after digestion with immobilized protease V8 (sequencing grade, Sigma Chemicals) for sequencing at an internal site.

2.3. *Crystallization*

The enzyme was placed in a 10 ml stirred-cell concentrator (Amicon Co.) with a 10 kDa cut-off membrane (Flowgen) and repeatedly washed with deionized water. The protein was then concentrated using a Centricon 10 (Amicon Co.) and protein concentrations were estimated using the OD_{280}, assuming an OD_{280} of 1.0 for a 1 mg ml^{-1} solution. Conditions were surveyed by the protocol of Jancarik & Kim (1991) using the hanging-drop method over 1 ml reservoirs on siliconized coverslips in Linbro trays. Crystallization trials were maintained at 291 K.

3. Results

3.1. *Identification as a pectin lyase A*

The N-terminal amino-acid sequence of the purified pectin lyase showed only two changes in 33 amino acids from the

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Table 1. *Data from native crystals and crystals soaked in heavy-atom containing solutions*

Crystal	Detector	Max. resolution (Å)	Observations	Unique refs.	Completeness (%)	$R_{sym}(I)$* (%)	mfid (*F*)† (%)
Native	Large MAR (SRS)	1.9	64360	24418	90.3 > 2.0 Å	5.4	–
K_2PtCl_4 10 m*M* 14 h	Weissenberg (PF)	2.8	29795	8599	92.9	6.1	10.4
K_2PtCl_4 20 m*M* 96 h	Siemens	2.9	21032	7285	95 > 3.4 Å 83 overall	6.9	29.3
$SmCl_3$ 0.5 m*M* 2 h	Siemens	2.9	25334	7360	96.5 > 3.4 Å 88 overall	7.1	33.1

*$R_{sym}(I) = \sum_h \sum_{i=1,N} |I(h)_{mean} - I(h)_i| / \sum_h \sum_{i=1,N} I(h)_i$. †mfid (*F*) is the mean fractional isomorphous difference calculated on *F*.

sequence predicted for pectin lyase A from the cloned DNA sequence of *A. niger*, strain N400 (Kusters-van Someren, Harmsen, Kester & Visser, 1991). A further 15 internal residues sequenced after digestion with immobilized protease V8 showed only one further change.

3.2. *Crystallization*

Using an estimated protein concentration of 22.4 mg ml^{-1} and adding 2.0 µl of protein and 2.0 µl of reservoir, crystals grew from several drops of the initial screen with polyethylene glycol (PEG) as precipitant and the conditions were refined using slightly more concentrated protein (30.6 mg ml^{-1}), and drops formed from 4.0 µl of protein with 2.0 µl of reservoir. The best reservoir contained PEG 6000 and 4000 at 26–32% with 100 m*M* sodium acetate, buffered by 100 m*M* Tris–HCl at pH 8.5. Crystals grew within a week from drops with PEG 6000 and a crystal was mounted from a drop equilibrated against 26% PEG 6000 and shown to diffract to at least 3 Å resolution.

Further investigation showed that crystals grew with between 0 and 200 m*M* acetate or formate added at 291 K and also with up to 400 m*M* acetate at 277 K and with PEG 8000 and 10000. Crystals grown at pH 9.0 were of inferior quality as judged by microscopy but good crystals could also be grown at pH 8.0. Most drops produced poorly formed crystals consisting of very thin plates or stacks of plates. However, it was possible to separate single crystals. The crystals were sensitive to warming (crystals sometimes dissolved after being photographed) and appeared to partially dissolve during heavy-atom soaking experiments.

The best formed crystal, Fig. 1, grew as a rectangular plate, 1.5 × 0.7 × 0.05 mm, much more slowly over 2 months from 28% PEG 4000 with 100 m*M* sodium acetate at pH 8.5.

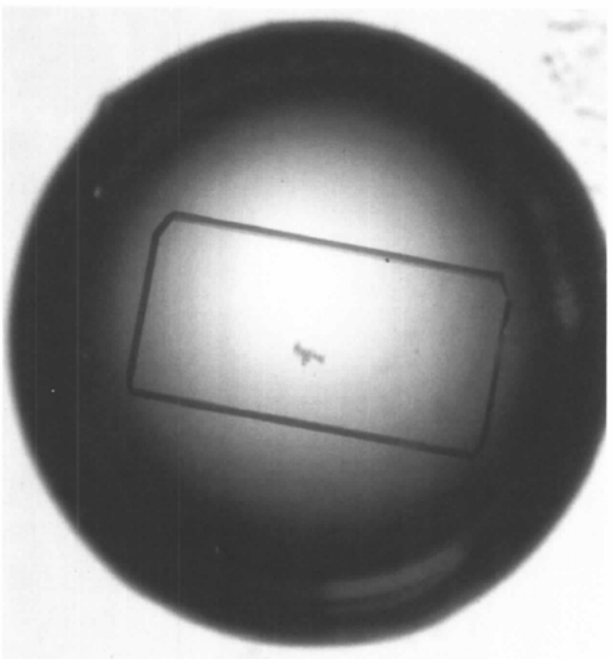

Fig. 1. The crystal of pectin lyase used to collect the high-resolution (2 Å) data before mounting. The crystal is approximately 1.5 × 0.7 × 0.05 mm with *a* as the long axis.

3.3. *Data collection*

Data from the best formed crystal were collected using a large MAR image plate on the wiggler beamline PX 9.6 at the SRS (Daresbury) with a wavelength of 0.882 Å. Processing of 59 images, each a 1.5° oscillation, with the program *MOSFLM* (A. G. W. Leslie, unpublished program) gave 24418 independent reflections to 1.9 Å resolution from 64360 fully recorded observations (after rejecting 76), with an $R_{sym}(I)$ of 5.4% overall (24.0% in the outer 1.93–1.86 Å shell). These data are 90.3% complete to 2 Å resolution (79.8% in the outer 1.93–1.86 Å shell). Overall 87% of reflections have intensities greater than 3 standard deviations (68% in the outer 1.93–1.86 Å shell).

Reasonably complete data sets (Table 1) have also been collected from three crystals soaked in heavy-atom containing solutions, one using BL6A, the Photon Factory, Tsukuba, Japan (Sakabe, 1991), processed using the program *DENZO* (Z. Otwinoski, unpublished program) and two with the Siemens area detector mounted on a three-axis camera using Cu $K\alpha$ radiation generated by a Siemens rotating-anode source running at 50 kV and 100 mA with a graphite monochromator processed by the program *XENGEN* (Howard *et al.*, 1987).

The space group can be established as orthorhombic by the $R_{sym}(I)$ on merging and identified as $P2_12_12_1$ using the pattern of systematically absent reflections along b^* and c^* in the data from the SRS whilst those along a^* could be identified from the data from the Siemens detector and the Photon Factory, as these crystals were reasonably isomorphous. The cell edges were determined from the MAR (SRS) data as $a = 45.4$, $b = 83.5$, $c = 93.4$ Å (1 Å = 0.1 nm) using *MOSFLM*. The molecular weight of 37.9 kDa gives a V_m of 2.3 Å^3 Da^{-1} within the normal range for protein crystals (Matthews, 1968).

4. Discussion

Crystals of an *A. niger* pectin lyase have been grown and are suitable for structure determination. Data have been collected and processed to better than 2.0 Å resolution. Solution of the structure will require either the refinement of a molecular-

replacement solution using the very distantly related model or the use of heavy-atom derivatives. Unfortunately, neither molecular-replacement solutions nor difference Pattersons have yet enabled us to find satisfactory positions for heavy atoms from any of the three available data sets.

Despite the availability of the crystal structures of three pectate lyases, rather little is known of the mechanism of these enzymes. Calcium is bound to three carboxylates in the structure of the pectate lyase of *Bacillus subtilis* and these residues are conserved in other pectate lyase sequences although one aspartate is sometimes substituted by glutamate. The extended binding site for pectate can probably be identified from a ribbon of positive potential with the calcium binding site at its centre. Conservation and their positive charges suggest that residues such as Lys247 and Arg279 and Arg282 (numbered as in the *B. subtilis* mature sequence) may be involved in binding and perhaps in catalysis.

The distant homology and the similar reaction suggest that pectin and pectate lyases share a related mechanism. Determination of the structure of a pectin lyase will allow an accurate alignment of pectin with pectate lyase, for example identifying the residues equivalent to the active-site carboxylates of pectate lyase, and reveal the similarities and differences in their respective active sites, which should clarify both mechanisms.

The structure may also reveal how the sequences of pectin lyases from various organisms relate to their specificities and roles in phytopathogenesis.

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