Acta Cryst. (1998). D**54**, 647–649

Purification, crystallization and preliminary X-ray analysis of the *Escherichia coli* phytase

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(*Received 21 July 1997; accepted 10 November 1997*)

Abstract

A recombinant form of *Escherichia coli* phytase, which hydrolyzes phytic acid into phosphate and myo-inositol, has been expressed, purified and crystallized. Crystals have been obtained by the method of bulk crystallization in 10 m*M* sodium acetate buffer (pH 4.5) without using a conventional precipitant. The enzyme crystallized in space group $P2_1$, with unit-cell dimensions a = 74.9, b = 72.2, c = 82.4 Å, and $\beta = 92.0^\circ$. Crystals diffract to at least 2.2 Å at a rotating-anode X-ray source and a 2.3 Å resolution data set has been collected, giving completeness of 98.0% and an $R_{\rm sym}$ of 0.072. Assuming there are two phytase molecules in the asymmetric unit, the solvent content is calculated to be 42.1%. A self-rotation function shows a clear twofold non-crystallographic symmetry relating two molecules of *E. coli* phytase in the asymmetric unit.

1. Introduction

Phytase (myo-inositol hexaphosphate hydrolase) catalyzes the hydrolysis of phytic acid into inorganic phosphate and myo-inositol with intermediates from inositol mono- to penta-phosphates. Phytic acid (phytate, myo-inositol 1,2,3,4,5,6-hexaisophosphate) is the most abundant inositol phosphate in nature and is found in a variety of microbial, plant and animal cells. Phytate accounts for 60–90% of the total phosphorus in plant seed (Reddy *et al.*, 1982). However, in this form the phosphate remains largely unavailable to simple-stomached (monogastric) animals, because these species are devoid of sufficient endogenous phytase activity to degrade phytic acid. Phytate has also long been characterized as an anti-nutritional factor since it can decrease the bioavailability of proteins and nutritionally important minerals such as calcium, zinc, magnesium and iron (Graf, 1986). Two classes of phytases exist: 3-phytase (E.C. 3.1.3.8) which initially removes *ortho*-phosphate from the 3-position of phytic acid and 6-phytase (E.C. 3.1.8.26) which first acts on the 6-position of phytate. Phytases are present in plants, microorganisms and some animal tissues (Wodzinski & Ullah, 1996). Usually 3-phytases are found in microorganisms and filamentous fungi and 6-phytases in plant tissues.

We were interested in crystal structure of phytase isolated from the periplasm of *E. coli* (Greiner & Jany, 1991). It is a small (42 kDa) acidic phytase with a pH optimum at 4.5, with higher specific activity (Greiner *et al.*, 1993) than commercially used *Aspergillus ficuum (niger)* phytase (Gibson & Ullah, 1990). It will be interesting to identify features responsible for the high activity of the *E. coli* phytase. Based on a partial amino-terminal sequence and molecular and catalytic properties, *E. coli* phytase is identical to the previously cloned pH 2.5 acid phosphatase (appA) from *E. coli* (Dassa *et al.*, 1982, 1990). Strong biochemical evidence shows that high molecular weight acid phosphatases including phytases are histidine phosphatases with a phosphorylated histidine as an intermediate in the reaction pathway (Van Etten, 1982). The crystal structure of a high molecular weight acid phosphatase was determined for rat acid phosphatase (Schneider *et al.*, 1993). More recently, the crystal structure of a 3-phytase, namely the phytase from *A. ficuum*, was reported at 2.5 Å resolution (Kostrewa *et al.*, 1997), although the crystallization conditions have not been documented. However, no structure has been determined so far for any 6-phytase. The primary sequence identity between *E. coli* and *A. ficuum* phytases is rather low, being only ∼15% (Wodzinski & Ullah, 1996). It is not clear whether these two enzymes possess the same tertiary fold. Crystallographic studies of *E. coli* phytase would provide the first three-dimensional structure of 6-phytase, which would in turn reveal the underlying structural basis for the different catalytic properties between 3- and 6-phytases. Furthermore, the industrial use of phytases is limited by the low thermostability of the enzymes; the crystal structure of the phytase should provide new insights into the protein stability which would be useful for engineering an enzyme with improved properties.

2. Results and discussion

2.1. *Cloning and protein purification*

We isolated the *appA* gene by PCR from *E. coli* strain ATCC 33965 which was used by Greiner *et al.* (1993) for purification of phytase. The gene was sequenced and found to be identical to that reported by Dassa *et al.* (1990). We expressed the gene in *E. coli* using pET21a(+) expression system and purified the appA protein based upon the following modified procedure originally established by Ostanin *et al.* (1992).

An overnight culture of *E. coli* BL21 (DE3) carrying the pET expression vector was inoculated into 4 l of LB medium with ampicillin (100 µg ml^{-1}) and incubated with shaking for 24 h at 310 K. The cells were collected, washed with cold water and resuspended in 200 ml of cold 20%(*w*/*v*) sucrose, 33 m*M* Tris–HCl (pH 8.0), 1 m*M* EDTA and 1 mg ml^{-1} of lysozyme. After 20 min of incubation, supernatant was collected by centrifugation at 8000 rev min^{-1} for 15 min and concentrated by ultrafiltration through a PM30 membrane (Amicon). This and all subsequent steps were performed at 277 K. The solution was loaded on a DEAE–Sepharose Cl-6B column (2.6 × 30 cm) equilibrated with 50 m*M* Tris–HCl (pH 7.8). After washing with 800 ml of the same buffer, the phytase was eluted with a linear gradient of sodium chloride (0–0.2 *M*) at a flow rate of 30 ml h^{-1}. Enzyme eluted at 0.09–0.1 *M* NaCl. The fractions were collected and assayed for phytase activity and analyzed on a 10% SDS–PAGE gel. Fractions with the highest

activity and smallest number of contaminated proteins were combined and concentrated by ultrafiltration through a PM30 membrane (Amicon). The concentrated solution was loaded on a Sephadex G-75 column (2.6 × 90 cm) pre-equilibrated with 50 m*M* Tris–HCl (pH 7.8) and 150 m*M* NaCl and eluted with the same buffer at a flow rate of 10 ml h^{-1}. The fractions were again collected and assayed for phytase activity and analyzed on a 10% SDS–PAGE gel. The fractions with the highest activity and free from contaminating proteins were concentrated and dialyzed by ultrafiltration through a PM30 membrane (Amicon). Protein purity was checked using SDS–PAGE.

2.2. *Crystallization*

The enzyme was dialyzed into 10 m*M* sodium acetate buffer pH 4.5 and then concentrated in a 10 ml cell concentrator (Amicon) using a 30 kDa cut-off membrane. The protein was concentrated to 10 mg ml^{-1} in 10 m*M* sodium acetate pH 4.5 for crystallization trials. Initial standard crystal screening using a sparse-matrix screen (Jancarik & Kim, 1991) at room temperature (293 K) was carried out using a Hampton Research screen kit (Hampton Research, California, USA). After a couple of months very small crystals were obtained. However, we were not able to improve the crystals by varying various parameters. Because of the small size, we were not even able to ascertain whether these small crystals were protein or salt by either diffraction or colouring using organic dye. A separate tube containing the same protein sample (10 mg ml^{-1} in 10 m*M* sodium acetate, pH 4.5), which was placed on ice in a refrigerator (277 K) for about two weeks produced a large number of visible crystals. Upon examining the crystals under the microscope, it was found that most of these crystals possessed a curved shape and lacked regular faces and sharp edges (Fig. 1). After carefully examination of these crystals, only a few regularly shaped crystals were found suitable for diffraction studies. The phytase crystals were very sensitive to temperature change; they quickly shattered or dissolved at room temperature. Hence, all crystal observation and manipulation were performed in the cold room (277 K). Furthermore, these crystals were only stable in the protein solution from which they grew. In the protein-free buffer, they also cracked or dissolved.

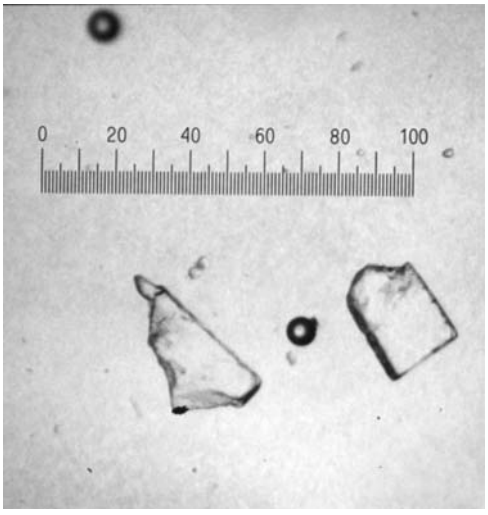

Fig. 1. Typical crystals of *E. coli* phytase from bulk crystallization at 277 K. Scale bar gradation represents 1 mm.

Table 1. *X-ray data statistics*

No. of measured reflections	116905
No. of independent reflections	37498
Resolution (Å)	2.3
R_{sym} (%)†	7.2 (24.2)‡
Completeness (%)	98.0 (97.1)‡
Average $I/\sigma(I)$	15.3

† Defined as $R_{sym} = \sum |I(k) - \langle I \rangle| / \sum I(k)$, where $I(K)$ and $\langle I \rangle$ represent the diffraction intensity values of the individual measurements and the corresponding mean values. The summation is over all measurements. ‡ Values given in parentheses refer to reflections in the outer resolution shell, 2.4–2.3 Å.

2.3. *X-ray diffraction*

A crystal was mounted in a capillary tube and transported on ice from the cold room to the X-ray facility for data collection. Data collection was carried out at 277 K using a cryo-cooling apparatus (Oxford Cryosystems Cryostream, Oxford, UK) to maintain the integrity of the crystal. The best crystal of *E. coli* phytase diffracted to 2.2 Å resolution and a data set was collected to 2.3 Å resolution. The data statistics are summarized in Table 1. There was no apparent decay of the phytase crystals upon X-ray radiation over the course of the data collection at 277 K. Data collection was carried out on a Mar Research imaging plate equipped with a Rigaku rotating-anode X-ray generator operated at 50 kV, 100 mA. Data were processed using *DENZO* (Otwinowski, 1993) and *CCP*4 (Collaborative Computational Project, Number 4, 1994).

The crystals belong to space group $P2_1$, based on the systematic absence observed for the reflections $0k0$. The cell dimensions are: $a = 74.9$, $b = 72.2$, $c = 82.4$ Å, and $\beta = 92.0^\circ$. The data set is 98.0% complete, with $R_{sym} = 0.072$ (Table 1). Given that the molecular weight of *E. coli* phytase is 44 644 Da (410 amino acids), the calculated solvent content is 42.1% by assuming that there are two enzyme molecules in the asymmetric unit. In order to determine whether there is non-crystallographic twofold symmetry that relates each monomer in the asymmetric unit, a self-rotation function calculation was performed using the program *POLARRFN* in the *CCP*4 package. The self-rotation function was calculated using a Patterson radius of 20 Å and data between 15 and 4 Å resolution. At $\kappa = 180^\circ$ section, there is a strong peak at ($\psi = 90$, $\varphi = 0$, $\kappa = 180^\circ$), which is 74.6% of the origin peak, clearly indicating a non-crystallographic twofold axis relating two molecules of *E. coli* phytase in the asymmetric unit. In the crystal structure of the *A. ficuum* phytase, there is only one enzyme molecule in the asymmetric unit. The *E. coli* phytase is a monomer, as shown by gel filtration.

Finally, given the low sequence similarity between the *E. coli* and *A. ficuum* phytases, it is not clear whether the *E. coli* phytase structure can be solved by molecular replacement using the known crystal structure of *A. ficuum* phytase which is now available from the Protein Data Bank (Brookhaven National Laboratory, NY, USA). If molecular replacement is unsuccessful, we will attempt to screen heavy-atom derivatives and solve the structure by multiple isomorphous replacement.

The authors would like to thank Mr Steffen Graether for carrying out the initial crystallization screening in the early stages of this work.

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