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Preliminary X-ray crystallographic analysis of a novel phytase from a *Bacillus amyloliquefaciens* strain

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A novel bacterial phytase from a *Bacillus amyloliquefaciens* strain was crystallized using the hanging-drop vapour-diffusion method. The amino-acid sequence of the enzyme does not show any homology to those of other known phytases or phosphatases, with the exception of a phytase from *Bacillus subtilis*. The enzyme exhibits a thermal stability which is strongly dependent on calcium ions. High-quality single crystals of the enzyme in the absence of calcium ions were obtained using a precipitant solution containing 20% 2-methyl-2,4-pentanediol and 0.1 *M* MES (pH 6.5). Native diffraction data to 2.0 Å resolution were obtained from a flash-frozen crystal at 110 K using a rotating-anode X-ray source. The crystals belong to space group $P2_12_12_1$ with unit-cell dimensions a = 50.4, b = 64.1, c = 104.2 Å and contain one monomer per asymmetric unit. Structure determination using heavy-atom derivative crystals is in progress, along with an effort to crystallize the calcium ion bound form of the enzyme.

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1. Introduction

Phytases hydrolyze phytic acid, *myo*-inositol-hexakisphosphate, to less phosphorylated *myo*-inositols and inorganic phosphate (Ullah, 1988). Phytase activity in microorganisms has been found most frequently in fungi, in particular *Aspergillus* species such as *A. ficuum* (Gibson, 1987; Ullah & Gibson, 1987) and *A. niger* (Volfova *et al.*, 1994). Phytases are different from other phosphatases in that they prefer phytic acid as a substrate. Two fungal phytases, phyA and phyB, from *A. ficuum* have been studied extensively (Ullah, 1988; Ullah & Dischinger, 1993; Ullah & Sethumadhavan, 1998). The two enzymes exhibit no apparent sequence similarity to other phosphatases except for the RHG*x*R*x*P motif. The tripeptide RHG is the most highly conserved sequence motif, and is found at the active sites of both microbial and mammalian acid phosphatases and phosphoglycerate mutase (Ullah *et al.*, 1991). The arginine residue was postulated to interact directly with the phosphate group of substrates making it susceptible to nucleophilic attack (Ostanin *et al.*, 1992), while the histidine residue has been proposed to serve as a nucleophile in the formation of covalent phosphoenzyme intermediates (MaTigue & Van Etten, 1978; Van Etten, 1982). Recently, a mammalian enzyme, rat hepatic multiple inositol polyphosphate phosphatase, was cloned and expressed. The recombinant enzyme exhibits phytase activity and contains an 18 amino-acid region that aligns with approximately 60% identity with the catalytic domain of phyA (Craxton *et al.*, 1997). The similarity encompassed conservation of the RHG*x*R*x*P motif. Outside this region, the sequence of the enzyme does not show any significant similarity to the fungal enzymes. This enzyme is considered to provide the cell with the only known means of dissipating the pools of Ins(1,3,4,5,6)P5 and InsP6. Very recently, the crystal structure of the deglycosylated phyA was determined at 2.5 Å (Kostrewa *et al.*, 1997). The structure revealed an α/β-domain, similar to that of rat acid phosphatase, and a novel α-domain. These phosphatases represent an unusual example of evolutionary pressure leading to the conservation of only a small selective segment for similar catalytic activity. This class of phosphatases containing the RHG sequence is designated as 'histidine phosphatase', and is distinguished from alkaline, protein and purple acid phosphatases.

Phytases are of significant interest for biotechnological applications, and in particular for the reduction of phytic acid in feedstuff. Phytic acid is the major storage form of phosphorus in seeds, but has poor availability to monogastric animals owing to their lack of digestive enzymes which can hydrolyze the substrates (Reddy *et al.*, 1982). Since phytases dephosphorylate phytic acid, the enzyme activity not only renders animal feed more nutritious, but also reduces excretion of phosphate to the environment. Due to concerns about environmental pollution, the use of the fungal phytases as a feed additive has been cleared in 22 countries (Wodzinski & Ullah,

1996). The native phyA has a temperature optimum at 331 K, which is in the sub-optimal range for processing animal feed. There is ongoing interest in discovering or engineering thermostable phytases. Recently, a bacterial phytase was isolated and cloned from a *Bacillus amyloliquefaciens* strain (Kim, Kim *et al.*, 1998; Kim, Lee *et al.*, 1998). This gene comprises 1152 nucleotides and encodes a polypeptide of 383 amino acids including a signal sequence, in contrast to the fungal phytases which comprise about 450 amino acids. The deduced amino-acid sequence does not align with those of any other known phytases, nor does this enzyme contain the RHG*x*R*x*P motif. Another phytase from *Bacillus subtilis* was independently cloned recently (Kerovuo *et al.*, 1998) which is highly homologous (93% sequence identity) to the phytase from *Bacillus amyloliquefaciens*. These two bacterial enzymes may represent a novel phytase whose active site and possibly protein fold are different from those of all the other phosphatases. Phytases are often classified as 3- or 6-phytases: 3-phytases initially remove the phosphate group from the 3-position of phytic acid, while 6-phytases remove the phosphate group from the 6-position. The fungal phyA, the structure of which is available, belongs to the 3-phytases, and an *Escherichia coli* phytase which belongs to the 6-phytases was recently cloned and crystallized (Jia *et al.*, 1998). For the *Bacillus* phytase, the order of phosphoester bond cleavages has not yet been determined. Another notable characteristic of the enzyme is a calcium-dependent thermal stability. About 50% of the original enzyme activity remained after incubation at 363 K for 10 min in the presence of 5 m*M* $CaCl_2$. However, in the absence of $CaCl_2$, the enzyme stability fell drastically above 323 K (Kim, Kim *et al.*, 1998). The calcium ion was also found to be critical for the enzyme activity, since the enzyme is severely inhibited in the presence of EDTA (Kim, Kim *et al.*, 1998). We sought to understand, by determining the three-dimensional structure of the enzyme, how the unrelated amino-acid sequences of the enzyme could exhibit the same catalytic activity with the other known phytases and how the calcium ion plays a critical role in enhancing the thermal stability drastically. The structure will also help the elucidation of the catalytic mechanism of the enzyme. Here, we report the crystallization of this themostable phytase (TS-Phy) from a *Bacillus amyloliquefaciens* strain and preliminary X-ray crystallographic analysis of the crystals.

2. Protein purification

For overproduction of TS-Phy, a DNA fragment encoding the open reading frame of TS-Phy was cloned in the *Hin*dIII site of the pJH27 vector containing a strong promoter. The vector was introduced into *Bacillus subtilis* DB104, and TS-Phy was overexpressed in modified LB media containing 10 g l^{-1} of tryptone, 5 g l^{-1} of yeast extract and 5 g l^{-1} NaCl, 10 g l^{-1} K_2HPO_4, 0.45 g l^{-1} $MgSO_4$, 40 g l^{-1} glucose and 2 ml l^{-1} of trace elements solution. The enzyme was secreted as an extracellular enzyme into the culture media, and was precipitated by the addition of acetone [20–50%(*v*/*v*)]. Under these conditions TS-Phy was overexpressed, consisting of more than 80% of the precipitated proteins. After addition of a buffer containing 1 m*M* $CaCl_2$ and 10 m*M* Tris–HCl (pH 7.0), insoluble contaminant was removed by centrifugation. The soluble fraction was yellow, most probably owing to contamination by heterogeneous Schiff base compounds in the culture media. It was noticed that these compounds precipitated when thawing the soluble fraction which was frozen at 293 K and could be removed efficiently by centrifuging the frozen sample at 277 K. The precipitant contained only a small amount of TS-Phy, reflecting the high solubility of the enzyme. The soluble fraction was recovered and subsequently incubated at 343 K for 5 min in the presence of 20 m*M* $CaCl_2$. This heat treatment precipitated most contaminant proteins. As a final purification step, gel-permeation chromatography was employed using Superdex 200 HR (Pharmacia-Upjohn, USA), which yielded an apparently homogeneous protein on sodium dodecylsulfate polyacrylamide-gel electrophoresis. Using a vacuum-dialysis membrane (Schleicher & Schuell, Germany), the protein solution was dialyzed and concentrated to 40 mg ml^{-1} against a buffer containing 150 m*M* NaCl, 10 m*M* Tris–HCl (pH 7.0).

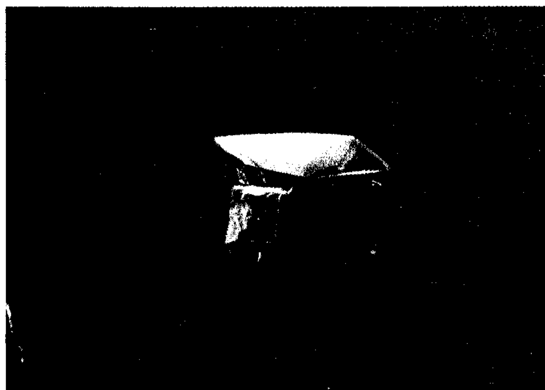

Figure 1
A photograph of a TS-Phy crystal. The crystal size is 0.4 × 0.4 × 0.5 mm. Although the surfaces of the crystal are rough, it is a single crystal.

3. Crystallization and X-ray studies

Crystals of TS-Phy were obtained by the hanging-drop vapor-diffusion method using 24-well Linbro plates at 277 K. The first crystallization screening was performed with Crystal Screen, the sparse-matrix screening kit (Hampton Research, USA). Under one crystallization condition, small plate-shaped crystals were found which were unbreakable by a cat's whisker. They were identified as protein crystals of TS-Phy, as a dissolved crystal showed a protein band on a SDS-PAGE gel. Subsequently, the initial crystallization condition was optimized to produce high-quality single crystals (0.4 × 0.4 × 0.5 mm, Fig. 1) in droplets containing 2 μl protein sample (40 mg ml^{-1}) and an equal volume of precipitant solution (20% 2-methyl-2,4-pentanediol, 0.1 *M* MES pH 6.5). The droplets were equilibrated with 0.5 ml of the same precipitant solution at 277 K and the crystals grew to a maximum size in 4 d. For data collection, crystals were frozen at 110 K using a Cryostream cooler (Oxford Cryosystems, UK) after briefly being immersed in a cryoprotectant solution containing 25% 2-methyl-2,4-pentanediol, 0.1 *M* MES (pH 6.5). The crystals diffracted very well and a native data set (93.8% completeness at 2.0–20 Å, R_{sym} = 3.1%; 85.5% completeness at 2.0–2.15 Å, R_{sym} = 15.9%) was obtained using Cu $K\alpha$ radiation on a MacScience DIP2020 imaging-plate system mounted on a M18XHF X-ray generator operated at 50 kV and 90 mA. Using an autoindexing program provided with the *HKL* program suite (Otwinowski & Minor, 1997) and examining the diffraction data set, we found that the crystals belong to the space group $P2_12_12_1$ with unit-cell dimensions a = 50.4, b = 64.1 and c = 104.2 Å. One molecule of TS-Phy is contained in the asymmetric unit, corresponding to a crystal volume per unit molecular weight (V_m) of 2.17 $Å^3$ Da^{-1} and a solvent content of 43.3% (Matthews, 1968). The relatively low solvent content appears to correlate with the hardness of the crystals. It was confirmed that dissolved crystals exhibited enzyme activity

and had the same molecular weight as the mature protein (residues 31–383) as judged by SDS–PAGE.

4. Discussion

TS-Phy could represent a novel fold for the phosphatase function, considering the low molecular weight of the protein, the lack of sequence similarity and the absence of the RHG sequence compared with the fungal high-molecular weight phytases and all other known phosphatases (Ullah & Wodzinski, 1996). Though the crystal of TS-Phy is sturdy as mentioned above, inclusion of 1 m*M* $CaCl_2$ in the crystallization conditions causes the crystals to dissolve completely. The calcium ion appears to induce a conformational change which affects the crystal packing. Thus, the substantial increase in the thermal stability of the enzyme may be accompanied by the conformational change of the protein. A detailed view of the structural basis for the induced thermal stability awaits the structure determination of both the apo- and the calcium ion bound enzyme. In attempts to determine the structure by the molecular-replacement method using the coordinates of phyA, no promising solution was found despite the employment of several different molecular-replacement protocols. Structure determination using heavy-atom derivative crystals is in progress, along with efforts to crystallize the calcium ion bound form.

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References

Craxton, A., Caffrey, J. J., Burkhart, W., Safrany, S. T. & Shears, S. B. (1997). *Biochem. J.* **328**, 75–81.

Gibson, D. (1987). *Biotechnol. Lett.* **9**, 305–310.

Jia, Z., Golovan, S., Ye, Q. & Forsberg, C. W. (1998). *Acta Cryst.* D**54**, 647–649.

Kerovuo, J., Lauraeus, M., Nurminen, P., Kalkkinen, N. & Apajalahti, J. (1998). *Appl. Environ. Microbiol.* **64**, 2079–2085.

Kim, Y. O., Kim, H. K., Bae, K.-S., Yu, J. H. & Oh, T. K. (1998). *Enzyme Microb. Technol.* **22**, 2–7.

Kim, Y. O., Lee, J. K., Kim, H. K., Yu, J. H. & Oh, T. K. (1998). *FEMS Microbiol. Lett.* **162**, 185–191.

Kostrewa, D., Gruninger-Leitch, F., D'Arcy, A., Broger, C., Mitchell, D. & van Loon, A. P. (1997). *Nature Struct. Biol.* **4**, 185–190.

MaTigue, J. J. & Van Etten, R. L. (1978). *Biochim. Biophys. Acta*, **523**, 407–421.

Matthews, B. (1968). *J. Mol. Biol.* **33**, 491–497.

Ostanin, K., Harms, E. H., Stevis, P. E., Radoslawa, K., Zhou, M.-M. & Van Etten, R. L. (1992). *J. Biol. Chem.* **267**, 22830–22836.

Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.*, **276**, 307–326.

Reddy, N. R., Sathe, S. K. & Salunkhe, D. K. (1982). *Adv. Food Res.* **28**, 1–92.

Ullah, A. H. (1988). *Prep. Biochem.* **18**, 459–471.

Ullah, A. H., Cummins, B. J. & Dischinger, H. C. Jr (1991). *Biochem. Biophys. Res. Commun.* **178**, 45–53.

Ullah, A. H. & Dischinger, H. C. Jr (1993). *Biochem. Biophys. Res. Commun.* **192**, 747–753.

Ullah, A. H. & Gibson, D. M. (1987). *Prep. Biochem.* **17**, 63–91.

Ullah, A. H. & Sethumadhavan, K. (1998). *Biochem. Biophys. Res. Commun.* **243**, 458–462.

Ullah, A. H. & Wodzinski, R. J. (1996). *Adv. Appl. Microbiol.* **42**, 263–302.

Van Etten, R. L. (1982). *Ann. NY Acad. Sci.* **390**, 27–51.

Volfova, O., Dvorakova, J., Hanzlikova, A. & Jandera, A. (1994). *Folia Microbiol. (Praha)*, **39**, 481–484.

Wodzinski, R. J. & Ullah, A. H. (1996). *Adv. Appl. Microbiol*, **42**, 263–302.