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Expression, purification, crystallization and preliminary X-ray diffraction analysis of EtFPOX from *Eupenicillium terrenum* sp.

Keke Xing,[a,b] Weiqiong Gan,[a] Minze Jia,[a] Feng Gao[a] and Weimin Gong[a]*

[a]Laboratory of Noncoding RNA, Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road, Beijing 100101, People's Republic of China, and [b]Graduate University of Chinese Academy of Sciences, Beijing 100039, People's Republic of China

Correspondence e-mail: wgong@ibp.ac.cn

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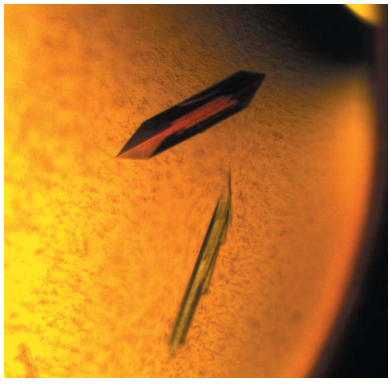


The flavoenzyme fructosyl peptide oxidase (FPOX) catalyses the oxidative deglycation of fructosyl amino acids or fructosyl dipeptides to produce amino acids, glucosone and hydrogen peroxide. In this study, FPOX protein from *Eupenicillium terrenum* sp. (EtFPOX) was expressed in *Escherichia coli* and purified by Ni-affinity and gel-filtration chromatography. EtFPOX crystals were obtained using the sitting-drop vapour-diffusion method with polyethylene glycol 3350 as precipitant. X-ray diffraction data were collected to 1.90 Å resolution using a synchrotron-radiation source. The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters a = 65.6, b = 80.0, c = 83.4 Å, and contained one molecule in the asymmetric unit. The calculated Matthews coefficient and solvent content were 2.22 Å^3 Da^{-1} and 44.62%, respectively.

1. Introduction

Fructosyl amino acid oxidase (FAOX), or amadoriase, is a flavoenzyme which belongs to the glucose–methanol–choline oxidoreductase family and catalyses the oxidation of the C—N bond linking the C1 atom of the fructosyl moiety and the N atom of the amino group of fructosyl amino acid to produce amino acids, glucosone and hydrogen peroxide.

FAOXs have been isolated from various microorganisms, such as *Corynebacterium* sp. (Sakaue *et al.*, 2002), *Arthrobacter* sp. (Ferri *et al.*, 2005), *Penicillium* sp. (Yoshida *et al.*, 1995), *Eupenicillium* sp. (Hirokawa *et al.*, 2003), *Pichia* sp. (Sode *et al.*, 2001; Miura *et al.*, 2006) and *Aspergillus* sp. (Takahashi *et al.*, 1997; Wu *et al.*, 2002; Collard *et al.*, 2008). Bacterial FAOXs are active against α-glycated amino acids such as fructosyl valine, f-$^{\alpha}$Val (Hirokawa *et al.*, 2003), while α- and/or ε-glycated amino acids such as fructosyl lysine (f-$^{\varepsilon}$Lys) are good substrates for eukaryotic FAOXs (Yoshida *et al.*, 1995, 1996). FAOX that also has activity towards fructosyl valyl histidine (f-$^{\alpha}$Val–His) is named fructosyl peptide oxidase (FPOX; Takahashi *et al.*, 1997; Hirokawa *et al.*, 2003; Kim *et al.*, 2012).

Much work on the mechanism of substrate specificity of FAOXs/FPOXs is under way. The crystal structure of FAOX has been determined for the protein amadoriase II from *Aspergillus fumigatus* sp. (Collard *et al.*, 2008), which has high activity towards ε-fructosyl amino acid and fructosyl propylamine, while having very low activity towards fructosyl peptides. The structure in complex with an analogue of f-$^{\alpha}$Gly highlights the critical role of Glu280 in fructosamine recognition and reveals a 12 Å deep catalytic site covered by two flexible loops, thereby providing a possible explanation for the inability of FAOX to oxidize large glycated peptides.

To investigate important regions that discriminate FPOX from FAOX, the FPOX protein from *Eupenicillium terrenum* sp. (EtFPOX) isolated by Hirokawa *et al.* (2003) was selected in this paper, which has 35% identity to the *A. fumigatus* sp. sequence. This enzyme has high specificity towards α-glycated compounds and especially shows activity towards f-$^{\alpha}$Val–His.

Here, we have constructed the recombinant EtFPOX and expressed it in *Escherichia coli* and we attempt to make clear, by means of X-ray crystallography, the reason why EtFPOX shows high activity towards α-fructosyl peptides. The crystal structure may provide useful information that can be applied to further improve the stability, substrate specificity or other properties of this enzyme. This

paper presents the initial steps towards three-dimensional structure determination of EtFPOX.

2. Materials and methods

2.1. Expression and purification of recombinant protein

The gene for EtFPOX (GenBank accession No. AB116146), inserted into pUC57, was obtained from GenScript (Nanjing, China) and subcloned into pET22b (Novagen) by digestion with *Nde*I and *Xho*I to construct pET22b-EtFPOX (pEEF). The plasmid pEEF containing EtFPOX was transformed into *E. coli* BL21 (DE3) strain and grown on an LB agar plate supplemented with 100 µg ml^{-1} ampicillin. A single colony was inoculated into 100 ml LB medium supplemented with 100 µg ml^{-1} ampicillin for overnight growth at 310 K to produce a primary culture. 8 ml of primary culture was then used to inoculate 800 ml LB medium supplemented with 100 µg ml^{-1} ampicillin. Cultures were grown to an optical density at 600 nm (OD_{600}) of 0.6–0.8, at which point the temperature was reduced to 289 K. After 2 h, protein production was induced by the addition of a 0.5 m*M* final concentration of isopropyl *β*-D-1-thiogalactopyranoside (IPTG) and cultures were grown for a further 20 h.

Bacteria were harvested at 4000 rev min^{-1} (4670*g*) at 277 K for 30 min and bacterial pellets were resuspended in buffer *A* (20 m*M* Tris–HCl pH 8.0, 40 m*M* NaCl) using 10 ml of buffer per 1 g of cells. The cells were homogenized by sonication and subsequently centrifuged at 20 000 rev min^{-1} (48 000*g*) at 277 K for 45 min. The supernatant containing 6×His-tagged EtFPOX was applied onto a 5 ml Ni–NTA agarose column (Qiagen) equilibrated with buffer *A* and washed with 20 m*M* Tris–HCl pH 8.0, 40 m*M* NaCl containing increasing concentrations of imidazole (0, 20, 50, 100 and 300 m*M*) at 277 K. The 100 m*M* imidazole elution was collected, concentrated by Amicon Ultra Ultracel 10K (Millipore) to a small volume, loaded onto a 120 ml HiLoad 16/60 Superdex 200 pg column (GE Healthcare) equilibrated with 20 m*M* Tris–HCl pH 8.0 containing 40 m*M* NaCl using an ÄKTA FPLC system (GE Healthcare) and eluted at a rate of 0.5 ml min^{-1} at 277 K. The fractions containing the protein were collected and analysed using 12% SDS–PAGE. A Gel Filtration Calibration Kit LMW (GE Healthcare) was employed to produce the standard curve for molecular-mass calculation. For crystallization, the purified 6×His-tagged EtFPOX solutions were concentrated to 15 mg ml^{-1} as determined using a Bio-Rad Protein Assay Kit.

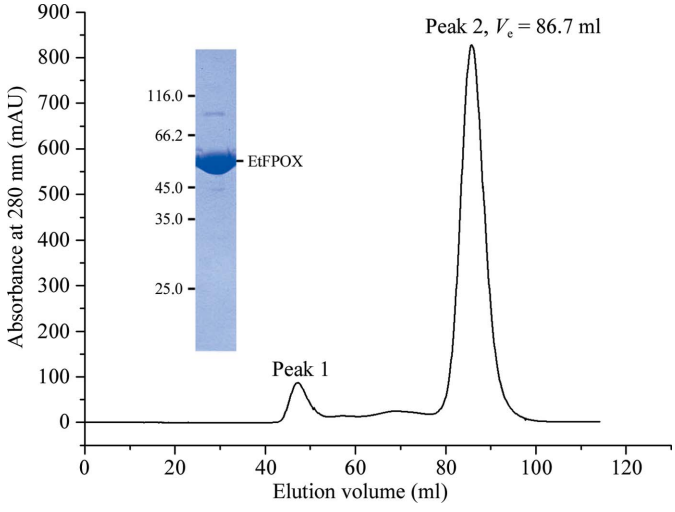
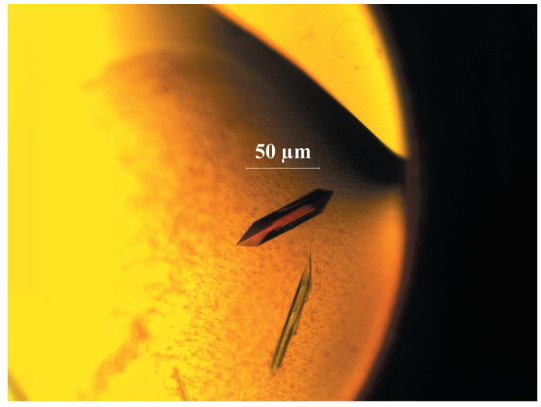


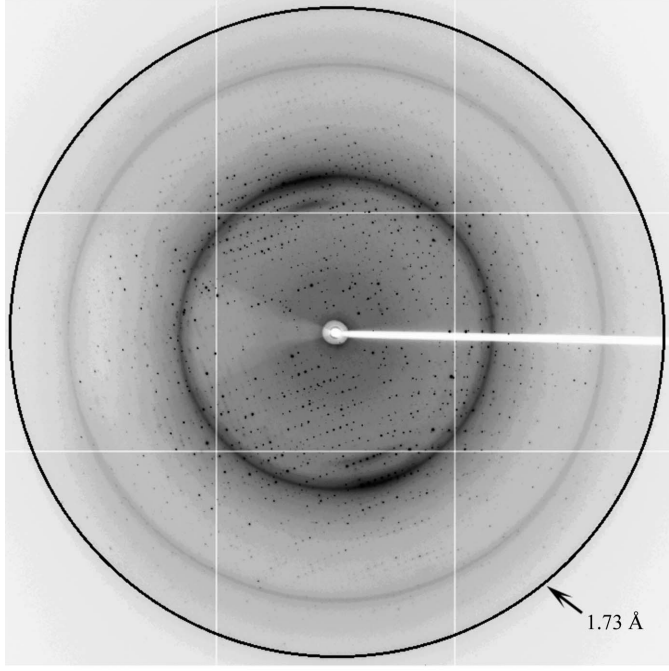
Figure 1
Purification of EtFPOX with a C-terminal 6×His tag by gel filtration. Gel-filtration chromatography profile and SDS–PAGE (12%) analysis (inset) of purified EtFPOX. Gel-filtration chromatography was carried out on a Superdex 200 column. Peak 1 corresponds to the void volume and peak 2 corresponds to monomeric EtFPOX. SDS–PAGE was carried out on a 12% polyacrylamide gel and the protein was visualized by Coomassie Brilliant Blue staining. The numbers indicate the migration of protein molecular-weight markers in kDa.

2.2. Crystallization and X-ray data collection

Initial crystallization screening was carried out at 277 and 298 K with Crystal Screen, Crystal Screen 2, Index and PEG/Ion kits (Hampton Research) using the sitting-drop vapour-diffusion method in 96-well plates (XtalQuest Co.). Each drop consisted of 0.3 µl protein solution in buffer *A* and 0.3 µl reservoir solution and was equilibrated against 40 µl reservoir solution. The crystals that gave the best X-ray diffraction pattern were obtained using solution No. 42 of the PEG/Ion kit [0.2 *M* potassium phosphate dibasic, 20%(*w*/*v*) polyethylene glycol 3350] after 5 d at 298 K.



(*a*)



(*b*)

Figure 2
(*a*) Typical crystals of EtFPOX with a C-terminal 6×His tag. (*b*) X-ray diffraction image of an EtFPOX crystal. The black ring represents 1.73 Å resolution.

Table 1
Data-collection and processing statistics for EtFPOX.

Values in parentheses are for the outermost resolution shell.

Beamline	SSRF BL17U
Wavelength (Å)	0.9793
Detector	ADSCQ315
Temperature of data collection (K)	100
Crystal-to-detector distance (mm)	250
Rotation range per image (°)	1
Total rotation range (°)	180
Exposure time per image (s)	1
Resolution range (Å)	30–1.90 (1.97–1.90)
Space group	$P2_12_12_1$
Unit-cell parameters (Å, °)	a = 65.6, b = 80.0, c = 83.4, $\alpha = \beta = \gamma = 90$
Total no. of measured intensities	206234
Unique reflections	35294
Multiplicity	5.8 (5.9)
Mean $I/\sigma(I)$	15.2 (4.2)
Completeness (%)	99.6 (100)
$R_{\rm merge}$† (%)	9.9 (45.5)
Overall B factor from Wilson plot (Å^2)	42.5

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith observation of unique reflection hkl and $\langle I(hkl)\rangle$ is the average over symmetry-related observations of the unique reflection hkl.

The crystals were flash-cooled in liquid nitrogen without any cryoprotectant and subjected to X-ray diffraction data collection at 100 K directly on beamline BL17U1 at the Shanghai Synchrotron Radiation Facility (SSRF) using an ADSQ 315 detector. A complete data set was collected to 1.9 Å resolution. The diffraction images were indexed, integrated and scaled using *DENZO* and *SCALEPACK* as implemented in *HKL*-2000 (Otwinowski & Minor, 1997).

3. Results and discussion

The expression plasmid encoded the fusion protein including the full-length EtFPOX and a C-terminal 6×His tag with a Leu–Glu linker (translated from the *Xho*I site) between them. The fusion protein contained a total of 445 residues with a molecular mass of 49.3 kDa. The His-tagged protein was overexpressed in *E. coli* and purified to homogeneity, yielding approximately 10 mg protein per litre of bacterial culture. The protein was estimated to be greater than 95% pure by SDS–PAGE stained with Coomassie Brilliant Blue (Fig. 1). According to the gel-filtration profile (Fig. 1), the second peak corresponds to an approximate molecular mass of 42 kDa, indicating that EtFPOX is a monomer in solution. A single crystal (Fig. 2*a*) was obtained after 5 d. The crystals were flash-cooled in liquid nitrogen without any cryoprotectant and several ice rings appeared. The diffraction pattern of the EtFPOX crystal reached 1.9 Å resolution (Fig. 2*b*). Data statistics are shown in Table 1. Assuming one monomer per asymmetric unit, the Matthews coefficient ($V_{\rm M}$) is 2.22 Å^3 Da^{-1}, corresponding to a solvent content of 44.62% (Matthews, 1968). There is 35% sequence homology between EtFPOX and the deglycating enzyme fructosamine oxidase from *A. fumigatus* (amadoriase II; PDB entry 3djd; Collard *et al.*, 2008). The structure of amadoriase II was used as the initial search model in the program *Phaser* (McCoy *et al.*, 2007). One monomer was found in the asymmetric unit and the *Z*-score was 13.1. The refinement of the structure is being processed.

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