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Crystallization and X-ray diffraction of an ectoine hydroxylase from *Bacillus pseudofirmus* OF4

Jiangli Yang[1,3#], Qingqing Wang[2#], Shujing Xu[2], Wanyi Guan[2], Baohua Zhao[2], Hui Dong[4*], Jiansong Ju[2*]

[1] College of Biotechnology, Tianjin University of Science & Technology, Tianjin 300222, China
[2] College of Life Science, Hebei Normal University, Shijiazhuang 050024, Hebei Province, China
[3] Tianjin International Joint Academy of Biotechnology & Medicine, Tianjin 300457, China
[4] Key Laboratory of Tianjin Radiation and Molecular Nuclear Medicine, Institute of Radiation Medicine, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin 300192, China

Abstract: [**Objective**] To study crystallization and X-Ray diffraction of ectoine hydroxylase from *Bacillus pseudofirmus* OF4. [**Methods**] We cloned the gene *Bpect*D from *B. pseudofirmus* OF4 with a His_6 tag and overexpressed it in *E. coli* BL21(DE3). Then, we purified protein *Bp*EctD by Ni^{2+}-chelating affinity and size-exclusion chromatography. After that, crystals were grown by the sitting-drop vapour-diffusion method at 289 K and diffracted at 100K using an in-house X-ray source. [**Results**] Protein *Bp*EctD was expressed and purified successfully. We obtained well diffracting crystals of about 360 μm × 240 μm × 60 μm in size using a solution consisting of 0.2 mol/L magnesium chloride hexahydrate, 0.1 mol/L bis-tris pH6.5, 25% (W/V) polyethylene glycol 3,350 at a protein concentration of 6.5 mg/mL, and collected X-ray diffraction data to 2.40 Å resolution in the anorthic space group *P*1, with unit-cell parameters $a = 45.18$Å, $b = 58.87$Å, $c = 68.81$ Å, $\alpha = 77.48°$, $\beta = 86.03°$, $\gamma = 66.97°$. The asymmetric unit contains two molecules of *Bp*EctD with a Mattews coefficient of about 2.44 Å^3/Da and a solvent content of 49.53%. [**Conclusion**] According to the X-ray diffraction data, the three-dimensional structure of *Bp*EctD from *B. pseudofirmus* OF4 soon will be analyzed, and it will provide insights into the biochemical properties of ectoine hydroxylase.

Keywords: *Bacillus pseudofirmus* OF4, ectoine hydroxylase, crystallization, X-ray diffraction
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Ectoine [(*S*)-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carbonic acid] and its derivative 5-hydroxyectoine [(*S*, *S*)-2-methyl-5-hydroxy-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid] are closely related in chemical

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* Corresponding author. Hui Dong, Tel/Fax: +86-22-85683033, E-mail: donghui@irm-cams.ac.cn; Jiansong Ju, Tel: +86-311-80787573, Fax: +86-311-80789710, E-mail: jujiansong@126.com
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structure and both function as compatible solutes which serve as protective substance by acting as an osmolyte and helps organisms survive in extreme osmotic stress, such as salt stress, temperature stress and dehydration[1-4]. Ectoine is synthesized in three successive enzymatic reactions starting from aspartic β-semialdehyde. Three enzymes involved in the biosynthesis are L-2, 4-diaminobutyric acid acetyltransferase (EctA; EC 2.3.2.178), L-2, 4-diaminobutyric acid transaminase (EctB; EC 2.6.1.76), and L-ectoine synthase (EctC; EC 4.2.1.108), respectively. In addition, 5-hydroxyectoine is catalyzed by the ectoine hydroxylase (EctD; EC 1.14.11) and yielded from ectoine through a stereo-specific hydroxylation[5].

Since the influence of 5-hydroxyectoine is usually superior to those of ectoine in stresses protecting of microorganisms[1,6], protein-stabilizing capacity[7-8], and melting temperature of DNA[9-10], it makes the research of ectoine hydroxylase more attractive for its fundamental investigation and practical application. However, there is few research work focusing on the ectoine hydroxylase, and the crystal structures of only two EctD from *Virgibacillus salexigens* (*Vs*EctD, PDB entry 3emr and 4nmi)[11-13] and *Sphingopyxis alaskensis* (*Sa*EctD, PDB entry 4mhr, 4mhu, and 4q5o)[14] have been deposited in the PDBs. Based on the crystal structure analysis of *Vs*EctD and *Sa*EctD, the iron and the co-substrate 2-oxoglutarate binding sites were clearly distinguished. However, several amino acid residues, which might involve in the substrate binding site, were still ambiguous.

Alkaliphilic *Bacillus pseudofirmus* OF4 with high optimal pH of 10.5 were isolated from limed garden soil[15]. The genome sequence analysis of *B. pseudofirmus* OF4 reveals that there are the compatible solute synthesis and uptake systems for synthesis of ectoine (the ectoine biosynthetic gene cluster, *ect*ABC) and hydroxyectoine (ectoine hydroxylase gene, *ect*D), which are part of a conserved response system of alkaliphile in stress[5,16]. In order to elucidate the biochemical properties of *Bp*EctD and its substrate recognition sites, a structural investigation was initiated. Here, we report our progress on the crystallization and preliminary X-ray diffraction data analysis of the recombinant *Bp*EctD protein carrying a His_6tag.

1 Materials and Methods

1.1 Cloning, expression and purification

The gene encoding ectoine hydroxylase *Bp*EctD (GenBank accession No. ADC50530.1) was amplified from *B. pseudofirmus* OF4 genomic DNA by PCR using the sense primer 5′-GCATATGCAAGATTTTTACCC TTC-3′ and the antisense primer 5′-GCTCGAGTTT AAGTACGGTTTCCT-3′, where the underlined sequences represent *Nde* I and *Xho* I restriction sites, respectively. The PCR product was digested with *Nde* I and *Xho* I and subsequently inserted into the expression vector pET-22b (+) (Novagen, USA). The recombinant pET-EctD plasmid was transformed into *Escherichia coli* BL21 (DE3) cells (Novagen, USA). The transformed cells were incubated in 800 mL LB medium containing 100 μg/mL ampicillin at 310 K. The culture was induced with 0.5 mmol/L isopropyl-β-D-1-thiogalactopyranoside (IPTG; final concentration) at 310K for a further 6 h incubation when the OD_{600} reached 0.6–0.8.

The cell pellet was harvested by centrifugation at 8600g and 277 K for 30 min and resuspended in 30 mL lysis buffer [50 mmol/L HEPES pH7.0, 200 mmol/L NaCl, 5% (V/V) glycerol]. Cells were disrupted by high-pressure homogenization (JNBIO, People's Republic of China) and cell debris was removed by centrifugation at 48400g for 30 min at 277 K. The cell-free extract containing crude protein was subjected to a Ni^{2+}-chelating affinity chromatography column (GE Healthcare, USA) and washed with 100 mL wash buffer [50 mmol/L HEPES pH7.0, 200 mmol/L NaCl, 5% (V/V) glycerol, 50 mmol/L imidazole] to remove unbound proteins. The His_6-tagged recombinant protein was eluted with 8 mL elution buffer [50 mmol/L HEPES pH7.0, 200 mmol/L NaCl, 5% (V/V) glycerol, 200 mmol/L imidazole]

and dialyzed against 50 mmol/L HEPES buffer pH7.0 with 5% (V/V) glycerol, and further loaded onto a size-exclusion chromatography column (Superdex 200 10/300 GL column, GE Healthcare, USA) and eluted using 50 mmol/L HEPES pH7.0, 50 mmol/L NaCl, 5% (V/V) glycerol (flow rate of 1.0 mL/min). The protein fraction was collected, and dialyzed against 50 mmol/L HEPES buffer pH7.0 with 5% (V/V) glycerol and concentrated to 20 mg/mL at 277 K using an Amicon Ultra centrifugal filter device (30 kDa molecular-weight cutoff, Millipore). The purity of *Bp*EctD was analyzed by 12.5% SDS-PAGE and the protein concentration was determined using a molar extinction coefficient of 34380 L/(mol·cm) at 280 nm with a NanoDrop device (Thermo Fisher, USA).

1.2 Crystallization

Initial crystallization screening was carried out at 289 K by the sitting-drop vapour-diffusion method in 96-well plates using the commercial kits including Crystal Screen, Crystal Screen 2, Index Screen, and PEG/Ion Screen (Hampton Research). For crystallization screening, the drop composed of 1 μL purified protein solution (5 or 10 mg/mL) in 50 mmol/L HEPES buffer pH7.0 with 5% (V/V) glycerol and 1 μL reservoir solution were equilibrated against 100 μL reservoir solution. Based on the initial screening results, the optimization of crystallization conditions was obtained by adjustment of the precipitant concentration, the protein concentration and the buffer pH.

1.3 X-ray diffraction data collection and processing

For the X-ray diffraction experiments, the crystals were soaked in reservoir solution supplemented with 25% (W/V) glycerol as a cryoprotectant for 15 s. Then the crystals mounted in nylon loops were flashed-cooled in a nitrogen stream at 100 K for data collection[17-18]. The X-ray diffraction data collection was performed at 100 K using a Rigaku MicroMax-007 HF desktop rotating-anode X-ray generator with a Cu target operated at 40 kV and 30 mA [Tianjin International Joint Academy of Biotechnology and Medicine (TJAB), People's Republic of China] and a R-AXIS HTC(IP) detector with a 200 mm crystal-to-detector distance at a wavelength of 1.5418 Å. The 720 diffraction frames were collected with 0.5° oscillation per image. All diffraction data were processed and scaled using the *HKL*-2000 program suite[19]. The data quality was appraised using SFCHECK[20] and the solvent content was calculated using MATTHEWS_COEF from CCP4[21].

2 Results

A 909 bp DNA fragment from *B. pseudofirmus* OF4 was successfully amplified and cloned into the expression vector pET-22b(+). The recombinant *Bp*EctD with a His_6 tag was overexpressed in *E. coli* BL21(DE3) and purified by Ni^{2+}-chelating affinity and size-exclusion chromatography. The molecular mass of *Bp*EctD was shown about 35 kDa by 12.5% SDS-PAGE analysis (Figure 1), which is consistent with the calculated molecular weight of 35.6 kDa for recombinant *Bp*EctD with a C-terminal His_6 tag.

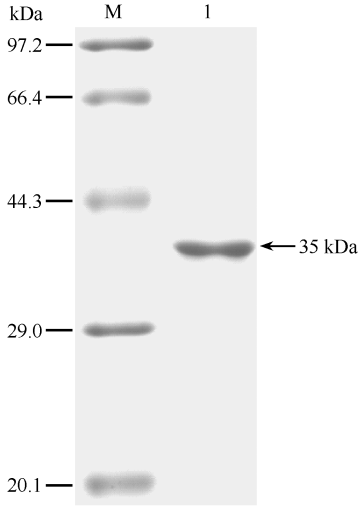

Figure 1. 12.5% SDS-PAGE analysis of purified protein *Bp*EctD. lane M, molecular-mass standards (labeled in kDa); lane 1, purified protein *Bp*EctD (35 kDa).

During initial crystallization screening, crystals of different shapes were obtained in PEG/Ion Nos. 4 [rhombuses; 0.2 mol/L lithium chloride, 20% (W/V) polyethylene glycol 3350], 40 [plates; 0.2 mol/L

ammonium phosphate dibasic, 20% (W/V) polyethylene glycol 3350], and Index Screen Nos. 81 [rods; 0.2 mol/L ammonium acetate, 0.1 mol/L tris pH8.5, 25% (W/V) polyethylene glycol 3350], 83 [lumps; 0.2 mol/L magnesium chloride hexahydrate, 0.1 mol/L bis-tris pH6.5, 25% (W/V) polyethylene glycol 3, 350]. Condition No. 83 of Index Screen was further optimized to obtain crystals suitable for structure analysis. By optimization of the crystallization conditions, well diffracting crystals of about 360 μm × 240 μm × 60 μm in size (Figure 2) were obtained using a solution consisting of 0.2 mol/L magnesium chloride hexahydrate, 0.1 mol/L bis-tris pH6.5, 25% (W/V) polyethylene glycol 3350 at a protein concentration of 6.5 mg/mL.

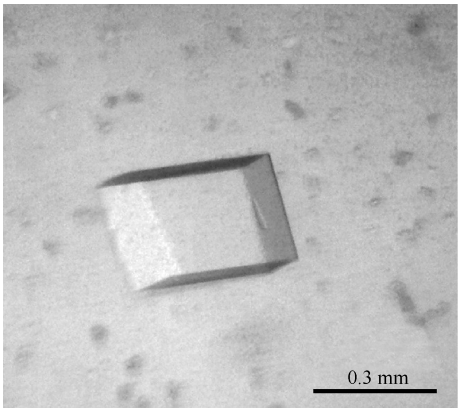

Figure 2. Typical crystals of *Bp*EctD (about 360 μm × 240 μm × 60 μm).

Crystallographic data statistics are shown in Table 1. The best *Bp*EctD crystal were diffracted to a resolution of beyond 2.40 Å in the anorthic space group *P*1 with the unit-cell parameters $a = 45.18$ Å, $b = 58.87$ Å, $c = 68.81$ Å, $\alpha = 77.48°$, $\beta = 86.03°$, $\gamma = 66.97°$ (Figure 3). The data set has 95.1% overall completeness and 72.3% completeness in the highest resolution shell, with an R_{merge} of 4.0%. The asymmetric unit contains two molecules of *Bp*EctD with a Mattews coefficient of about 2.44 Å^3/Da and a solvent content of 49.53%[22].

MOLREP was used to carry out the initial phases using the structure with PDB code 3emr as a search model. Using the data in the resolution range 50 – 2.40 Å, the best resolution was refined to an R_{work} of 23.74 % and an R_{free} of 29.80 %.

Table 1. Diffraction data statistics (values in parentheses are for the highest resolution shell)

Data collection	
Diffraction source	Rigaku MicroMax-007 HF
Wavelength (Å)	1.5418
Temperature (K)	100
Detector	R-AXIS HTC(IP) detector
Crystal-to-detector distance (mm)	200
Rotation range per image (°)	0.5
Total rotation range (°)	360
Exposure time per image (s)	60
Space group	*P*1
Unit-cell parameters (Å, °)	
	a=45.18, b=58.87, c=68.81
	α=77.48, β=86.03, γ=66.97
Mosaicity (°)	1.1
Resolution range (Å)	50 – 2.40 (2.44 – 2.40)
No. of observed reflections	90592(3545)
No. of unique reflections	23840(933)
Completeness (%)	95.1(72.3)
Redundancy	3.8(3.8)
Average I/σ (I)	39.6(14.7)
R_{merge} (%)	4.0(8.8)
No. of molecules in the asymmetric unit	2
V_M (Å^3 Da^{-1})	2.44
Solvent content (%)	49.53
Overall B factor from Wilson plot (Å^2)	37.50

$R_{merge} = \sum_{hkl} \sum_i | I_i(hkl) - \langle I(hkl) \rangle | / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith measurement of reflection hkl and $\langle I(hkl) \rangle$ is the mean intensity of all symmetry related reflection.

3 Discussion

Ectoine hydroxylase (EC 1.14.11), which catalyzes the conversion from ectoine to 5-hydroxyectoine, is a member of non-heme-containing iron (II)- and 2-oxoglutarate-dependent dioxygenase superfamily. Since the derivative 5-hydroxyectoine possesses superior stress protecting and function preserving properties than its precursor molecule ectoine, EctD has attracted considerable attention and are found widely in bacteria[6]. Based on the analysis of crystal structure and catalytic mechanism of *Sa*EctD (PDB entry 4mhr, 4mhu, and 4q5o) and *Vs*EctD (PDB entry 3emr and 4nmi), the iron (II), the co-substrate 2-oxoglutarate, substrate ecotion and the reaction product 5-hydoroxyectoine ligands were clearly determined[11, 13-14]. Alignments of the amino acid sequences of *Sa*EctD and *Vs*EctD have revealed that residues binding with iron and the 2-oxoglutarate are

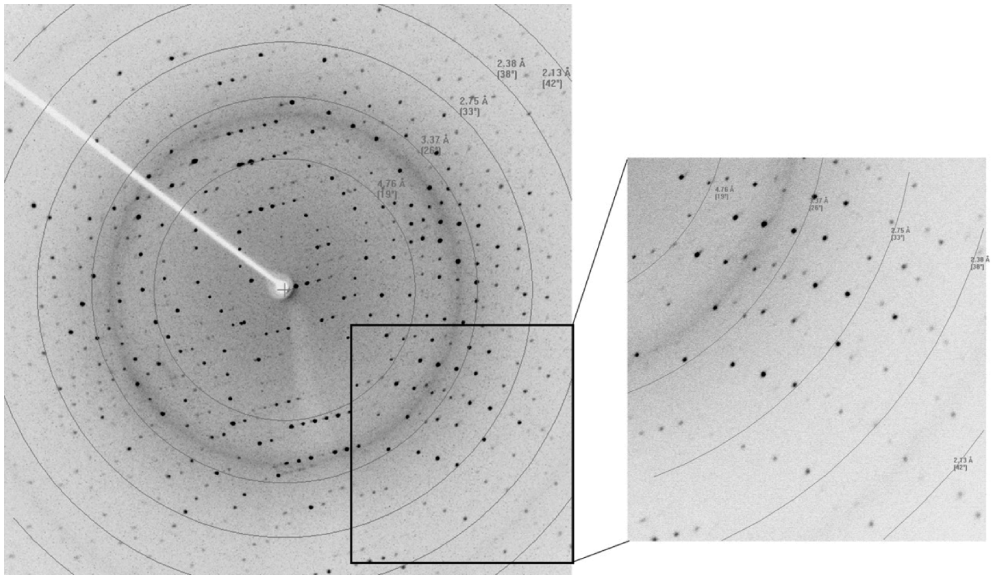

Figure 3. Typical X-ray diffraction pattern from *Bp*EctD crystal. The frame edge is at 2.40 Å resolution.

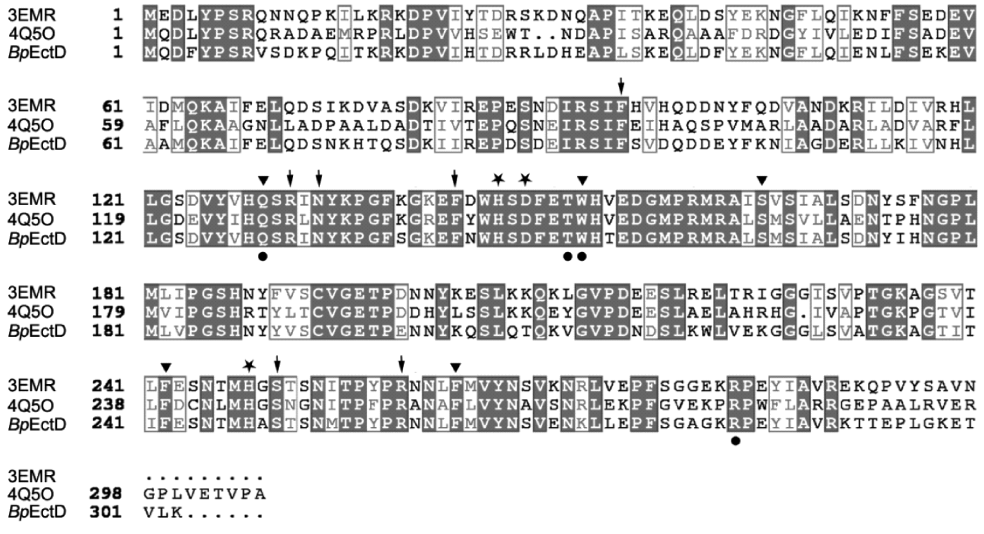

Figure 4. Multiple sequence alignment of ectoine hydroxylase from *V. salexigens* (3EMR), *S. alaskensis* (4Q5O) and *B. pseudofirmus* OF4 (*Bp*EctD). The stars (★) and arrows (↓) indicate the iron-binding residues and 2-oxoglutarate binding residues. The triangles (▼) and closed circles (●) indicate the putative substrate binding sites of 3EMR and 4Q5O, respectively.

fully consistent with each other (Figure 4), however, the ectoine/5-hydoroxyectoine binding sites in two proteins are varied somewhat. For *Sa*EctD, the possible 5-hydoroxyectoine binding site is composed of Gln-127, Thr-149, Trp-150 and Arg-280[14], whereas the ectoine ligand in protein *Vs*EctD might contact Gln-129, Trp-152, Ser-165, Phe-242 and Phe-263[13]. It is notable that all these residues binding with the substrate are highly conserved in members of EctD protein family[13].

In the present study, we have reported the cloning, purification, crystallization and X-ray diffraction analysis of ectoine hydroxylase (*Bp*EctD) from alkaliphilic *B. pseudofirmus* OF4. Based on its deduced amino acid sequence, the recombinant protein *Bp*EctD with a C-terminal His_6tag (LEHHHHHH) has a calculated molecular mass of 35.6 kDa, close to its apparent molecular mass of 35 kDa estimated by SDS-PAGE (Figure 1). From the diffraction data statistics,

calculation of a Matthews coefficient of 2.44 Å^3/Da implied one biological dimer per asymmetric unit, which is consistent with the dimeric state of protein *Vs*EctD and *Sa*EctD[12]. The protein *Bp*EctD displayed high sequence homology to the known ectoine hydorxylases in Protein Data Bank (PDB) from *V. salexigens* (*Vs*EctD, identity 72.8%; PDB entry 3emr)[11], *S. alaskensis* (*Sa*EctD, identity 48.3%; PDB entry 4q5o)[14], and carried all conserved residues binding with iron (His-146, Asp-148 and His-248), 2-oxoglutarate (Phe-95, Arg-131, Asn-133, Phe-143, Ser-250 and Arg-259), and ectoine/5-hydoroxyectoine (putative binding site: Gln-129, Thr-151, Trp-152, Ser-165, Phe-242, Phe-263 and Arg-283). The three-dimensional structure of *Bp*EctD will provide insights into the biochemical properties and the substrate binding sites of ectoine hydroxylase from *B. pseudofirmus* OF4.

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假坚强芽孢杆菌四氢嘧啶羟化酶的晶体制备及 X-射线衍射研究

杨江丽[1,3#], 王青青[2#], 徐书景[2], 关婉怡[2], 赵宝华[2], 董辉[4*], 鞠建松[2*]

[1]天津科技大学生物工程学院,天津 300222

[2]河北师范大学生命科学学院,河北 石家庄 050024

[3]天津国际生物医药联合研究院,天津 300457

[4]中国医学科学院放射医学研究所,天津市分子核医学重点实验室,天津 300192

摘要:【目的】假坚强芽孢杆菌四氢嘧啶羟化酶蛋白纯化、晶体制备及 X-射线衍射研究。【方法】通过 PCR 从假坚强芽孢杆菌 OF4 中克隆获得四氢嘧啶羟化酶基因,构建原核表达载体,经过原核表达,采用 Ni-NTA 亲和层析法和分子排阻色谱法纯化蛋白,289 K 下采用座滴法进行晶体筛选和制备,在低温 100 K 下通过 X-射线衍射仪(Rigaku MicroMax-007 HF)收集晶体衍射数据。【结果】通过原核表达及纯化成功获得了适合晶体生长的蛋白 *Bp*EctD。通过筛选最终在蛋白浓度为 6.5 mg/mL 及含有 0.2 mol/L $MgCl_2 \cdot 6H_2O$, 0.1 mol/L Bis-Tris pH6.5, 25% (W/V) 聚乙二醇 3,350 的缓冲液中获得了理想的蛋白晶体,其大小约为 360 μm×240 μm×60 μm,并在 100K 下成功收集了衍射数据,晶体衍射分辨率为 2.40 Å,空间群为三斜晶系 *P*1, 晶胞参数为 a=45.18 Å, b=58.87 Å, c=68.81 Å, α=77.48°, β=86.03°, γ=66.97°,每个不对称单位中含有 2 个 *Bp*EctD 单体,马修斯系数为 2.44 Å3/Da,溶剂含量约为 49.53%。【结论】衍射数据的成功收集为假坚强芽孢杆菌 OF4 四氢嘧啶羟化酶三维结构的解析奠定了前期基础,将有助于阐明四氢嘧啶羟化酶的催化机制。

关键词:假坚强芽孢杆菌 OF4,四氢嘧啶羟化酶,结晶,X-射线衍射

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*通信作者。董辉, Tel/Fax: +86-22-85683033, E-mail: donghui@irm-cams.ac.cn;鞠建松, Tel: +86-311-80787573, Fax: +86-311-80789710, E-mail: jujiansong@126.com

作者简介:#共同第一作者。杨江丽(1986-),女,山东菏泽人,硕士研究生,从事蛋白结构方面的研究,E-mail:1064102039@qq.com;王青青(1989-),女,河北沙河人,硕士研究生,从事分子酶学方面的研究,E-mail:43545565@qq.com

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