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Postprint: Crystal Structure of Dehalogenase DehDIV-R Determined by X-ray Crystallography

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Abstract

R-2-haloacid dehalogenase can stereoselectively hydrolyze R-2-haloacids. Resolving the single-crystal structure of enzymes provides direct structural guidance for improving enzyme selectivity and activity, representing the forefront of research in the field of enzyme structures. Using the R-2-chloropropionic acid dehalogenase (DehDIV-R) from Pseudomonas ZJU26 obtained previously in our laboratory as the research subject, the structure was determined using X-ray crystallography. The DehDIV-R protein was expressed as a fusion with the pp-SUMO vector, and purified through sequential Ni-NTA affinity chromatography, dialysis and cleavage, second Ni-NTA affinity chromatography, and gel filtration chromatography to obtain a protein with a single band and good homogeneity. Subsequently, crystallization conditions were initially screened and optimized, yielding the optimal crystallization condition of 0.1 mol/L HEPES pH 7, 12% PEG 6000, 0.2 mol/L MgCl2, and 8 mmol/L CHAPS. Diffraction data for the crystals were collected at the BL18U1 beamline of the Shanghai Synchrotron Radiation Facility, and the crystal structure of DehDIV-R was successfully determined at a resolution of 2.35 Å using molecular replacement. Ramachandran plot analysis indicated that 98.02% of the amino acids were located in the most favored regions, confirming the rationality of the structure. The purification, crystallization, and structural determination of DehDIV-R lay the foundation for further in-depth understanding of its structure and function.

Full Text

Crystal Structural Analysis of DehDIV-R by X-ray Crystallography

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Abstract

R-2-haloacid dehalogenase selectively hydrolyzes R-2-haloacids and holds significant potential for the synthesis of chiral compounds. Determining the crystal structure of such enzymes provides direct structural guidance for improving their selectivity and catalytic activity, representing a frontier area in enzymatic structure research. This study focuses on the R-2-chloropropionic acid dehalogenase (DehDIV-R) from *Pseudomonas* ZJU26, previously isolated in our laboratory. The DehDIV-R protein was expressed in *E. coli* BL21(DE3) using the ppSUMO fusion vector and purified through a multi-step protocol involving Ni-NTA affinity chromatography, dialysis with ULP1 protease cleavage, a second Ni-NTA affinity chromatography step, and final gel filtration chromatography. High-quality crystals were obtained under optimized conditions of 0.1 mol/L HEPES pH 7.0, 12% PEG 6000, 0.2 mol/L MgCl , and 8 mmol/L CHAPS. Diffraction data were collected at the BL18U1 beamline of the Shanghai Synchrotron Radiation Facility, and the crystal structure of DehDIV-R was successfully solved by molecular replacement at a resolution of 2.35 Å. The Ramachandran plot analysis revealed that 98.02% of residues reside in the most favored regions, confirming the structural model' s validity. The successful purification, crystallization, and structural determination of DehDIV-R establish a solid foundation for further investigation of its structure-function relationships.

Keywords: R-2-haloacid dehalogenase; purification; crystallization; X-ray diffraction

Introduction

2-Haloacid dehalogenases (EC 3.8.1.X) are widely distributed in nature and represent the earliest discovered enzymes capable of catalyzing carbon-halogen bond cleavage in 2-haloacids [1]. Based on genetic homology, these enzymes are classified into Group I and Group II [2-3]. Group I includes both R,S-2-haloacid dehalogenases and R-2-haloacid dehalogenases, while Group II comprises S-2-haloacid dehalogenases. Both groups act on the -carbon of small-molecular-weight haloacids through an SN2 hydrolytic substitution reaction that results in product inversion [4]. Among these, S-2-haloacid dehalogenases are the most extensively studied class, with numerous reports on their genes, structures, and catalytic mechanisms. The catalytic process of S-2-haloacid dehalogenases involves a two-step nucleophilic attack: first, an aspartate residue acts as a nucleophile to attack the carbon-halogen bond, forming a covalent enzyme-ester intermediate; subsequently, a neighboring water molecule attacks this intermediate, hydrolyzing the bond to release the halide ion and generate the inverted product [5-7].

In contrast, structural information for Group I enzymes remains limited. Schmidberger et al. [8-9] determined the crystal structure of an R,S-2-haloacid

dehalogenase from *Pseudomonas putida* PP3 and elucidated its catalytic mechanism at the molecular level. Their work revealed that an aspartate-activated water molecule directly performs nucleophilic attack on the carbon-halogen bond, displacing the halogen atom without forming a covalent enzyme-ester intermediate. R-2-haloacid dehalogenases have only been identified in a few microorganisms, including *Rhizobium* sp., *Pseudomonas putida* AJ1/23, and *Agrobacterium* sp. NH3 [10-12], and remain poorly characterized. Despite their high sequence homology with R,S-2-haloacid dehalogenases, R-2-haloacid dehalogenases exhibit substantially different substrate selectivity. The absence of crystal structures for R-2-haloacid dehalogenases has prevented direct molecular-level explanations for these selectivity differences and has hindered subsequent structure-based enzyme engineering efforts.

Our laboratory previously isolated *Pseudomonas* ZJU6 from sludge near a pesticide manufacturing plant, which exhibits dehalogenase activity toward R-2-chloropropionic acid [13]. This strain can specifically dehalogenate R-2-chloropropionic acid, enabling the resolution of racemic 2-chloropropionic acid to prepare the valuable chiral intermediate S-2-chloropropionic acid for pharmaceutical and agrochemical synthesis. Lin et al. [14] successfully cloned the R-2-chloropropionic acid dehalogenase gene (*dehDIV-R*) from this strain and expressed it in *E. coli* BL21(DE3) using the pET30a(+) plasmid. To further investigate the structure of DehDIV-R, we undertook its purification, crystallization, and X-ray diffraction analysis. This study reports the successful purification and crystallization of DehDIV-R, yielding protein crystals that diffracted to 2.35 Å resolution. The determined structure not only provides a basis for deeper investigation of this enzyme' s structure and function but also expands the structural database for R-2-haloacid dehalogenases, offering crucial data for molecular-level exploration of substrate selectivity mechanisms.

Materials and Methods

1.1.1 Strains and Plasmids

The expression host *E. coli* BL21(DE3) and the recombinant plasmid DehDIV-R-ppSUMO were maintained in our laboratory.

1.1.2 Reagents and Instruments

Common chemical reagents were of analytical grade from domestic suppliers, while biochemical reagents were purchased primarily from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. and Majorbio Bio-Pharm Technology Co., Ltd. Ni-NTA affinity resin and gel filtration media were obtained from General Electric Company. Crystallization screening kits including PEGIIs and PACT were from Qiagen; Wizard I/II and Wizard III/IV from Rigaku; HR2-110, HR2-112, HR2-144, and optimization kits Additive and Detergent from Hampton Research.

1.2.1 Expression of DehDIV-R

The recombinant plasmid DehDIV-R-ppSUMO was transformed into *E. coli* BL21(DE3). A single colony was inoculated into 5 mL LB liquid medium containing 30 mg/L kanamycin and cultured at 37°C with shaking at 200 rpm for 6 h. This culture was then transferred to 1 L LB medium and grown under the same conditions until OD reached 1.5. The temperature was lowered to 16°C, and IPTG was added to a final concentration of 0.4 mmol/L to induce expression for 16 h. Cells were harvested by centrifugation and stored at -80°C until use.

1.2.2 Purification of DehDIV-R

The *dehDIV-R* gene was cloned into the ppSUMO vector, yielding a fusion protein with an N-terminal hexahistidine tag and SUMO protein. The purification strategy involved initial Ni-NTA affinity chromatography to remove most contaminating proteins, followed by ULP1 protease cleavage to remove the tag, a second Ni-NTA step to separate the cleaved protein, and final gel filtration chromatography to obtain crystallization-grade DehDIV-R.

Cell lysis: Cell pellets were resuspended in Buffer A (25 mmol/L Tris-HCl, 500 mmol/L NaCl, 50 mmol/L imidazole, 5 mmol/L -mercaptoethanol, pH 8.0) and lysed by high-pressure homogenization at 4°C. The lysate was centrifuged, and the supernatant was retained for purification.

First Ni-NTA affinity chromatography: The supernatant was loaded onto a Ni-NTA column pre-equilibrated with Buffer A. Unbound proteins were washed away with 10 column volumes of Buffer A, followed by gradient elution with mixtures of Buffer A and Buffer B (25 mmol/L Tris-HCl, 500 mmol/L NaCl, 250 mmol/L imidazole, 5 mmol/L -mercaptoethanol, pH 8.0) at ratios of 10% and 30% Buffer B. Finally, 15 column volumes of 100% Buffer B were applied. All fractions were analyzed by SDS-PAGE to assess purity.

ULP1 digestion and dialysis: ULP1 protease was added to the pooled eluate, and the mixture was dialyzed overnight at 4°C against dialysis buffer (25 mmol/L Tris-HCl, 50 mmol/L NaCl, pH 8.0) using a 7 kDa molecular weight cutoff membrane.

Second Ni-NTA affinity chromatography: This step followed the same procedure as the first Ni-NTA chromatography, but the flow-through and gradient elution fractions containing the tag-free DehDIV-R were collected. Purity was verified by SDS-PAGE.

Gel filtration chromatography: The protein was concentrated to 2 mL using a 30 kDa molecular weight cutoff centrifugal filter and loaded onto a HiLoad 26/600 Superdex S200 prep grade column pre-equilibrated with Buffer C (25 mmol/L Tris-HCl, 150 mmol/L NaCl, 2 mmol/L DTT, pH 8.0). Elution was performed at 1 mL/min, and 1 mL fractions (designated AX, where X indicates elution volume) were collected based on UV absorbance. Fractions were

analyzed by SDS-PAGE, and those showing high purity and homogeneity were pooled, concentrated, and quantified by A measurement before storage at -80°C.

1.2.3 Crystallization Screening and Optimization

Purified DehDIV-R was screened for crystallization conditions using the sitting-drop vapor diffusion method. In 96-well plates, 75 L of reservoir solution was added to the well, and the drop was prepared by mixing 1 L protein solution (diluted to 10 mg/mL in Buffer C) with 1 L reservoir solution. Plates were sealed and incubated at 18°C. Crystals were examined every two days under a microscope to identify initial hits and monitor growth. Promising conditions were further optimized by varying protein concentration, precipitant concentration, buffer pH, additives, and drop setup (sitting-drop vs hanging-drop) in 24-well plates. For optimization, 500 L reservoir solution was used, and drops consisted of 2 L protein plus 2 L reservoir solution. For additive screening, 1.8 L protein, 1.8 L reservoir solution, and 0.4 L additive were used. Optimization was iterated until high-quality crystals suitable for X-ray diffraction were obtained.

1.2.4 X-ray Diffraction Data Collection, Processing, and Structure Determination

Single crystals were cryoprotected using reservoir solution supplemented with glycerol, ethylene glycol, low-molecular-weight PEG, or 2-methyl-2,4-pentanediol (MPD) at appropriate concentrations, or using heavy oil. Crystals were looped and flash-cooled in liquid nitrogen. Diffraction data were collected at the BL18U1 beamline of the Shanghai Synchrotron Radiation Facility. Diffraction images were processed using the HKL3000 software suite. The CCP4 Online server (http://www.ccp4.ac.uk/ccp4online) was employed for molecular replacement phasing, and initial models were built. Structure refinement was performed iteratively using PHENIX and COOT.

Results

2.1 Purification of DehDIV-R

DehDIV-R was expressed as a fusion protein with both a hexahistidine tag and SUMO protein using the ppSUMO vector. The SUMO fusion partner aids proper folding and enhances protein stability and homogeneity [15-16]. The theoretical molecular weight of the fusion protein is 47.97 kDa, and DehDIV-R was expressed solubly at high levels. The first Ni-NTA affinity chromatography results are shown in [Figure 1: see original paper]. The target protein band migrated between 66.2 kDa and 45 kDa, consistent with the theoretical size. The 30% Buffer B and 100% Buffer B elution fractions contained relatively pure target protein at high concentration with minimal contaminants, and these fractions were pooled for further processing.

Following overnight dialysis with ULP1 protease, the theoretical molecular weight of cleaved DehDIV-R is 33.9 kDa. The digested protein was subjected to a second Ni-NTA affinity chromatography step, with results shown in [Figure 2: see original paper]. Comparison of lanes 1 and 2 demonstrates the shift of the target band to between 35.0 kDa and 25.0 kDa, confirming complete removal of the histidine tag and SUMO fusion partner. The tag-free DehDIV-R lacks the hexahistidine tag and therefore does not bind to Ni-NTA resin, eluting in the flow-through fraction. This fraction was collected and concentrated to 2 mL using a 30 kDa molecular weight cutoff filter.

Gel filtration chromatography of the concentrated sample yielded a symmetric UV absorption peak ([Figure 3: see original paper]), indicating good protein homogeneity. SDS-PAGE analysis of fractions A71-A87 showed only the target protein band with virtually no detectable contaminants ([Figure 4: see original paper]), demonstrating that the multi-step purification protocol produced highly pure and homogeneous protein suitable for crystallization trials.

2.2 Crystallization Screening and Optimization

Initial crystallization screening of DehDIV-R was performed using commercial kits including PEGIIs, HR2-110, HR2-112, HR2-144, PACT, Wizard I/II, and Wizard III/IV, with crystals examined every two days. Several conditions produced crystals: HR2-144 H8 (0.1 mol/L magnesium formate, 15% PEG 3350) yielded numerous small rod-shaped crystals; PEGIIs H10 (0.2 mol/L magnesium acetate, 10% PEG 8000) produced few clustered crystals; and PACT C10 (0.2 mol/L MgCl , 0.1 mol/L HEPES pH 7.0, 20% PEG 6000) generated abundant needle-shaped crystals ([Figure 5: see original paper]).

Conventional gradient optimization of buffer pH and precipitant concentration based on these initial hits did not substantially improve crystal quality. Subsequent additive screening using Hampton Research Additive and Detergent kits identified Detergent 86# (80 mmol/L 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, CHAPS) as significantly improving crystal morphology, producing spindle-shaped crystals. After extensive iterative optimization, high-quality single crystals were obtained in the final condition: 0.1 mol/L HEPES pH 7.0, 12% PEG 6000, 0.2 mol/L MgCl , 8 mmol/L CHAPS. These crystals exhibited a well-defined spindle shape with sharp edges ([Figure 6: see original paper]).

2.3 Single Crystal X-ray Diffraction

Crystals were cryoprotected with 22.5% PEG 400, which prevented ice formation during flash-cooling and maintained excellent diffraction quality. X-ray diffraction data were collected at the BL18U1 beamline of the Shanghai Synchrotron Radiation Facility. Data collection parameters were: wavelength 0.97776 Å, oscillation range 0.5°, exposure time 1.5 s, detector distance 350 mm, and 360 frames collected. A representative diffraction image is shown in [Figure 7: see

original paper]. Data processing with HKL3000 involved indexing, integration, and scaling, revealing that the crystals belonged to space group P31 with a resolution of 2.35 Å. Detailed diffraction statistics are presented in .

2.4 Structure Determination and Evaluation

The processed diffraction data and protein sequence were submitted to the CCP4 Online server for molecular replacement phasing, yielding an initial model. Inspection of the electron density maps confirmed correct tracing of the polypeptide backbone and proper placement of side chains. Structure refinement was performed through iterative cycles using COOT for manual rebuilding and PHENIX for automated refinement, including addition of water molecules and correction of unreasonable dihedral angles. The refinement converged with satisfactory R-work and R-free values, as summarized in .

The Ramachandran plot ([Figure 8: see original paper]) shows that 98.02% of residues lie in the most favored regions, with only 0.37% in disallowed regions. Among the four residues in disallowed regions, three are glycines. Since glycine is achiral and often occurs at secondary structure transitions, its presence in disallowed regions is not uncommon and does not necessarily indicate model errors. Overall, the refinement statistics and Ramachandran plot analysis confirm the validity and high quality of the DehDIV-R structure.

2.5 Overall Structure of DehDIV-R

The DehDIV-R structure forms a pseudo-dimer composed entirely of -helices ([Figure 9a: see original paper]). This pseudo-dimeric arrangement generates a strong dipole moment and positive electrostatic potential that facilitates binding of the negatively charged substrate in the active site cavity. The substrate access channel is primarily lined with hydrophobic residues, while the catalytic pocket contains key residues Trp48, Gly50, Asn131, Tyr134, Asn203, Ser204, and Asp205. The halide-binding site is formed by Val51, Phe281, Leu284, and Leu285. Notably, Asp205 forms a hydrogen bond with Asn131, which likely positions Asp205 to activate a water molecule for nucleophilic attack on the carbon-halogen bond ([Figure 9b: see original paper]).

Conclusion

This study successfully determined the crystal structure of DehDIV-R from *Pseudomonas* ZJU6 using X-ray crystallography. The recombinant protein was efficiently expressed and purified to high homogeneity through a multi-step chromatographic protocol. Crystallization conditions were systematically screened and optimized, yielding high-quality single crystals in 0.1 mol/L HEPES pH 7.0, 12% PEG 6000, 0.2 mol/L MgCl , and 8 mmol/L CHAPS. Using 22.5% PEG 400 as cryoprotectant, a complete X-ray diffraction dataset was collected at the Shanghai Synchrotron Radiation Facility BL18U1 beamline to 2.35 Å resolution. Molecular replacement phasing on the CCP4 Online server successfully yielded

the DehDIV-R structure, which was refined to excellent geometry as validated by comprehensive refinement statistics and Ramachandran plot analysis. The enzyme adopts a pseudo-dimeric -helical fold, with Asp205 positioned through hydrogen bonding with Asn131 to activate the nucleophilic water molecule. The determination of the DehDIV-R crystal structure provides a crucial foundation for future complex structure analysis and molecular-level elucidation of its substrate selectivity.

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