

Cloning, Expression, Purification, Characterization, Crystallization and X-Ray Diffraction of Bifunctional Pyrimidine Deaminase/Reductase from *Shigella flexneri 2a*

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Abstract: Bifunctional pyrimidine deaminase/reductase (RibD) plays an important role during riboflavin biosynthesis in many microorganisms. The 40.4 kDa RibD from *Shigella flexneri 2a* has been cloned, expressed, purified and characterized. Three Crystals of RibD have been obtained by the hanging-drop technique at 291 K using PEG 20k or NaCl as precipitant. The RibD crystal using PEG 20k as precipitant diffracted to 2.5Å.

Keywords: RibD, *Shigella flexneri 2a*, bifunctional pyrimidine deaminase/reductase, riboflavin biosynthesis, expression, crystallization.

1. INTRODUCTION

Riboflavin, the precursor molecule for synthesis of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), both essential for basic functions of the cell metabolism, is synthesized by plants and many microorganisms [1]. Therefore, the enzymes involved in riboflavin biosynthesis have the potential to become attractive candidates for the design of new therapeutic agents. The production of riboflavin from GTP is catalyzed by the enzymes GTP cyclohydrolase II, riboflavin deaminase/reductase, 3, 4-dihydroxy-2-butanone-4-phosphate synthase, lumazine synthase and riboflavin synthase [1]. The deamination and reduction steps have been shown to proceed in the opposite order in yeast and *Escherichia coli* [2, 3]. Most eubacteria contain a bifunctional protein, for instance *Bacillus subtilis* RibD (BsRibD) is composed of an N-terminal deaminase domain (D domain) and a C-terminal reductase domain (R domain) [4]. In contrast, these two enzymes are separate in fungi, plants and most archaea [5-7]. To date, the three-dimensional structure of bifunctional pyrimidine deaminase/reductase from *Bacillus subtilis* has been determined [8].

Here, we report the cloning, expression, purification, characterization, crystallization and X-ray diffraction for the bifunctional enzyme from *Shigella flexneri 2a*. The present study may thus further lead to the development of new therapeutic agents.

2. MATERIALS AND METHODS

2.1. Cloning

Cloning of the RibD gene took place using the forward primer 5'-GGATTCCATATGGTGCAGGACGAGTATTAC-

3' and the reverse primer 5'-GGAATCCTCGAGTCATGCACCCACTAAATG-3'. These primers introduced NdeI and XhoI restriction sites. The PCR used a *Shigella flexneri 2a* (strain 301) genomic DNA template. The PCR DNA fragment digested with the NdeI/XhoI enzymes was ligated to the vector pET-22b (+) digested with the same enzymes to obtain pET-22b-RibD. The plasmid was sequenced to confirm the insertion of the gene.

2.2. Expression and Purification

1L culture of E.coli BL21 (DE3) cells harbouring pET-22b-RibD was grown at 310 K in a shaking flask with LB medium. When the culture reached $A_{600nm}=0.5-0.6$, the lac promoter was induced with 0.8 mM isopropyl- β -D-thiogalactopyranoside (IPTG). After 3 h induction, cells were harvested by centrifugation. Cell pellets were resuspended in 30 ml buffer A (25 mM Tris-HCl pH 8.0, 100 mM NaCl, 10% glycerol). After sonication, soluble and insoluble fractions were separated by centrifugation. A 3ml Nickelating Sepharose column was washed with 20 ml buffer A and the supernatant was applied onto the column and eluted with buffer B (25 mM Tris-HCl pH 8.0, 100 mM NaCl) containing 20, 50, 100 and 150 mM imidazole in turn. The fractions eluted with buffer B containing 100 mM imidazole were concentrated to 1 ml and was applied onto a HiLoad 16/60 Superdex G-75 column (prep grade; Amersham Pharmacia Biotech), which was washed with buffer consisting of 10 mM Tris-HCl pH 8.0, 100 mM NaCl, 3 mM DTT. After concentrating the fractions containing the enzyme, we obtained protein solution at a concentration of 25 mg/ml in 10 mM Tris-HCl pH 8.0, 100 mM NaCl, 3 mM DTT buffer (Fig. 1). The protein solution was frozen in liquid nitrogen and stored at 200 K.

2.3. Characterization of the Recombinant Protein

The oligomerization state of the protein in buffer was examined using DynaPro dynamic light scattering (Protein

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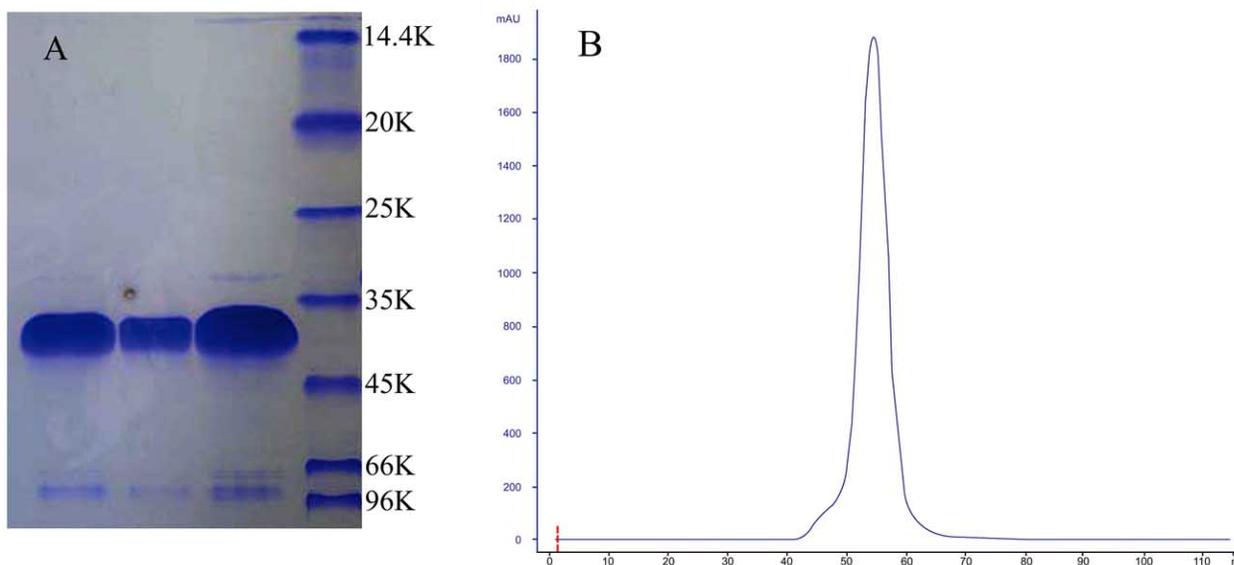


Figure 1. A) SDS-PAGE of the RibD. B) Purification profile of the RibD using chromatography on a size-exclusion column Superdex G-75.

Solutions Co.) and the data were analyzed using DYNAMICS v.5.26.39. An analysis of light scattering signal provides quantitative information about the behavior of protein in solution [9]. All DLS measurements were performed on a DynaPro DLS instrument with a 781.8nm wavelength laser (Protein Solutions).

2.4. Crystallization and X-Ray Diffraction

RibD was crystallized by the hanging-drop vapour-diffusion method using Linbro plates at 291 K. 1 μ l protein solution with concentration of about 10 mg/ml was typically mixed with 1 μ l reservoir solution and suspended on a cover slip over 0.45 ml reservoir solution. The protein was initially screened with Crystal Screens I and II and Index Screen (Hampton Research). Initial microcrystals appeared after only 7d from a few conditions. Through optimization, the

diffraction-quality crystals were grown under the following two conditions: 0.2 M NaCl, 0.1 M MES PH 5.7, 4% PEG 20K and 1, 2, 3-Heptanetriol as additive agent; 0.1M Tris-HCl PH 7.5, 3M NaCl. In the end, we get the best crystals for X-ray diffraction using micro-seeding technique with the first condition.

X-ray diffraction was performed on a Rigaku R-Axis IV^{++} image-plate detector using rotating copper-anode ($\lambda=1.5418$) operating at 40KV and 30mA. The data were collected at 100K with crystal-to-detector distance of 200mm, $\Delta\phi=1^\circ$, exposure time was 5 min per frame, respectively. A diffraction image is shown in (Fig. 4).

3. RESULTS AND DISCUSSION

The RibD was successfully cloned into pET22b(+) with a hexahistidines tag for efficient purification. Recombinant

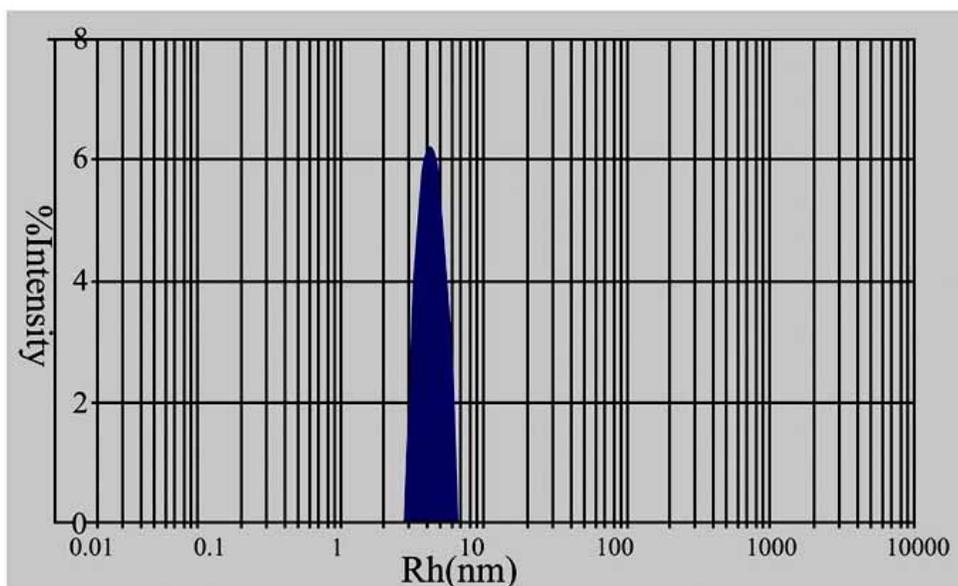


Figure 2. DLS regularization histograms for the RibD protein.

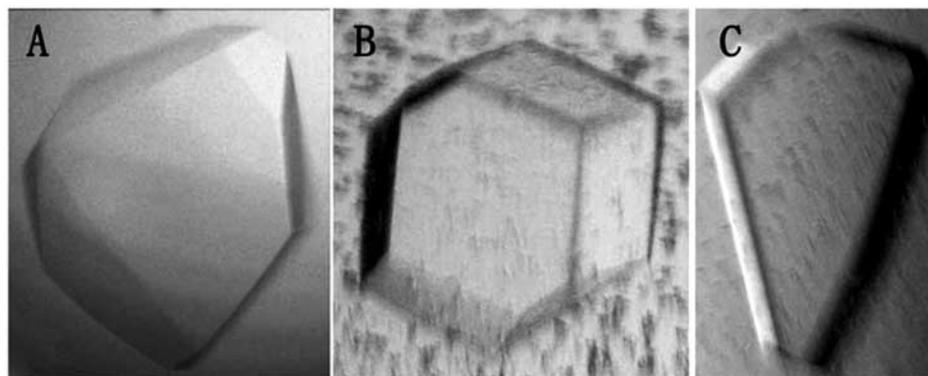


Figure 3. Crystals obtained from different crystallization conditions. A) 0.1M Tris-HCl pH 7.5, 3M NaCl (0.4×0.4×0.2 mm³). B) 0.2 M NaCl, 0.1 M MES pH 5.7, 4% PEG 20K and 1, 2, 3-heptanetriol as additive agent (0.05×0.05×0.05mm³). C) Micro-seeding technique in the second condition (0.2×0.1×0.05mm³).

protein was over-expressed and purified. The purity of RibD was estimated to be greater than 90%. The molecular weight of the recombinant protein was determined by SDS-PAGE and a band was located at around 41kDa corresponding to the recombinant protein with a His tag (Fig. 1).

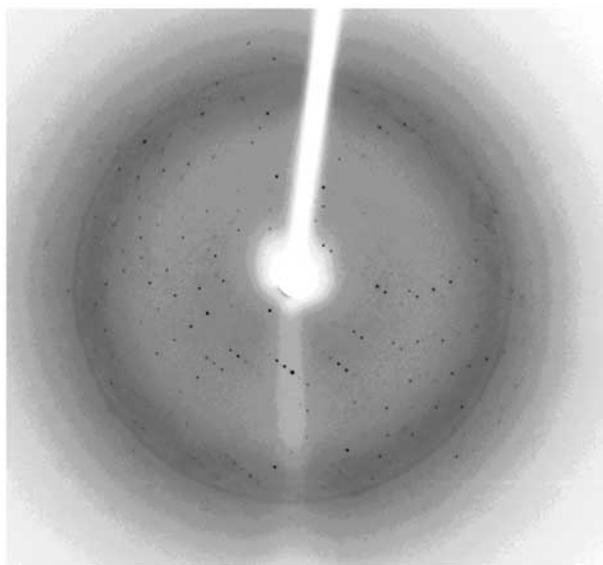


Figure 4. The image of the RibD diffraction pattern of a resolution 2.5Å.

The results of DynaPro dynamic light scattering showed that the protein was monomer in the buffer and did not aggregate, furthermore, the estimated molecular weight is in accord with that derived from SDS-PAGE (Fig. 2). After a month, the reserved recombinant protein was examined again and had the same result of a monomer in the buffer and no aggregation. This shows that the protein was stable in the buffer under the storage conditions.

At beginning, RibD crystals grown were not good enough for X-ray diffraction. However, through conditional optimization and micro-seeding, the qualified crystals were obtained (Fig. 3).

To date, although the three-dimensional structure of RibD from *Bacillus subtilis* has been determined, its sequence identity is only 40% to the protein from *Shigella flexneri 2a* which may cause bacillary dysentery or shigellosis in man. The structural determination of the bifunctional enzyme from *Shigella flexneri 2a* may make an important contribution to the designing of new therapeutic agents.

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