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Procedia Chemistry 18 (2016) 185 – 189

Procedia Chemistry

Molecular and Cellular Life Sciences: Infectious Diseases, Biochemistry and Structural Biology 2015 Conference, MCLS 2015

Characterization of Tryptophanase from *Vibrio cholerae* O1

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Abstract

Tryptophanase (Trpase) encoded by the *tnaA* gene catalyzes the conversion of tryptophan to indole, which is an extracellular signaling molecule detected in various bacteria including *Vibrio cholerae*. Indole has been demonstrated to regulate biofilm formation, drug resistance, plasmid maintenance and spore formation of bacteria. In the present study, the *tnaA* gene from *V. cholerae* O1 (VcTrpase) was cloned and expressed in *E. coli* BL21(DE3) *tn5:tnaA* (a Trpase-deficient competent). VcTrpase was purified by Ni^{2+}-NTA chromatography. The obtained VcTrpase had a molecular mass of approximately 49 kDa, a specific activity of 3 U/mg protein, and absorption peaks at 330 and 435 nm. Using a site-directed mutagenesis technique, replacement of Arg419 by Val resulted in a VcTrpase completely devoid of activity. Thus, this site can be a target for drug design for controlling *V. cholerae.*

Peer-review under responsibility of the organizing committee of the Molecular and Cellular Life Sciences: Infectious Diseases, Biochemistry and Structural Biology 2015 (MCLS 2015)

Keywords: V. cholerae; tryptophanase; indole

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doi:10.1016/j.proche.2016.01.003

1. Introduction

Tryptophanase (Trpase) is detected in a wide variety of bacteria. *V. cholerae* and many other Gram-negative bacteria use this enzyme to catalyze the conversion of L-tryptophan into indole, ammonium and pyruvate (Fig. 1)[1]. This enzyme is of particular interest because indole has been demonstrated to act as an extracellular signaling molecule to control virulence and biofilm formation[2]. Thus, tryptophanase is a useful target for novel potential inhibitors to inhibit *V. cholerae* biofilm formation. The aim of this work was to investigate amino acid residues in tryptophanase that could abolish its activity. Arg419 of tryptophanase has been demonstrated to bind the tryptophan substrate. Thus, in this study, arginine 419 was replaced with valine using site directed mutagenesis and the R419V mutant was evaluated.

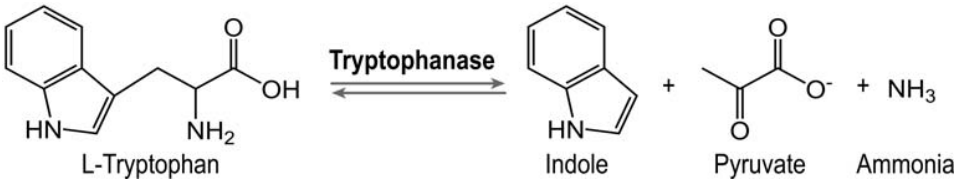

Fig. 1. Reaction catalyzed by Trpase.

2. Methods

2.1. Strains and plasmid

V. cholerae PSU966 serogroup O1 was used for PCR amplification of the *tnaA* gene. *E. coli* BL21 (DE3) *tn5:tnaA* was used as Trpase-deficient competent cells for expression of recombinant VcTrpase[3]. Plasmid pET20b (+) vector containing a hexa-histidine tag (Novagen, Germany) was used for cloning and expression of the *tnaA* gene.

2.2. Cloning of tnaA gene

An overnight culture of *V. cholerae* PSU966 in Luria Bertani (LB) broth was harvested by centrifugation at 12,000x*g* for 5 min and chromosomal DNA was extracted by the phenol-chloroform method[4]. The *tnaA* gene was amplified from the total DNA of *V. cholerae* by the polymerase chain reaction (PCR) with primers tnaA-VC-F 5'-TACATATGGAAAATTTTAAACACTTACCAGAACC-3' and tnaA-VC-R 5'-TTGTCGACGGCTTTTTCTT TTAAGCG-3'. The amplification cycles were 35 cycles of denaturation at 95°C for 45 sec, annealing at 53°C for 30 sec, and extension at 72°C for 2 min. The PCR product was digested with *Nde*I and *Sal*I enzymes and purified from agarose gel with the QIAquick Gel Extraction Kit (Qiagen, Germany). Then it was ligated to the pET20b(+) vector. The recombinant plasmid pET20b(+)-VcTrpase was transformed into *E. coli* BL21(DE3) *tn5*:*tnaA* host cells by the $CaCl_2$ technique[5]. The transformants were selected on LB agar plates containing kanamycin (50 μg/ml) and ampicillin (100 μg/ml), and screened for indole production. Then the recombinant plasmid (pET20b(+)-VcTrpase) was extracted and confirmed for the presence of the *tnaA* gene by PCR and DNA sequencing (Macrogen, Korea).

2.3. Site-directed mutagenesis

For amplification of the *tnaA* gene, site-directed mutagenesis was performed using the pET20b(+)-VcTrpase as a template. To replace arginine 419 with valine, the primer pair R419V_F 5′-CCAGCCGAATTGCT CGCGTTAACCATTCCACGC-3' and R419V_R-5'-CGCTGGAATGGTTAACGCGAGCAATTCGGCTGG-3' with mutagenized codon (underlined) was used. Then the template plasmid was removed by *Dpn*I restriction enzyme digestion. After that the R419V plasmid was transformed into *E. coli* BL21 (DE3) *tn5:tnaA*. The plasmid was extracted and the presence of the correct codon substitution in the *tnaA* gene was confirmed by DNA sequencing (Macrogen, Korea).

2.4. Preparation of crude enzyme extract

Wild type and mutated *tnaA* gene *E. coli* was grown to mid-log phase at 37ºC in 1 L of LB broth supplemented with kanamycin (50 μg/mL) and ampicillin (100 μg/mL). After induction of protein expression with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), the bacterial cells were incubated at 30ºC for an additional 16-18 h. Cells were collected by centrifugation at 10,000x*g* for 10 min at 4ºC, and washed twice with 0.1 M potassium phosphate buffer, pH 7.8, 5 mM mercaptoethanol, 50 μM pyridoxal 5'-phosphate (PLP), and then resuspended in the same buffer. After that, the cells were sonicated five times for 30 s with 3 min intervals. The crude enzyme was collected after centrifugation at 14,000x*g* for 20 min at 4ºC and stored at -20ºC. The protein concentration of the crude enzyme was determined using the Bradford method[6] with BSA as a standard.

2.5. Purification of VcTrpase enzyme

Crude enzyme was loaded on an Ni^{2+}-NTA agarose column (Qiagen, Germany) and eluted with a 20 – 400 mM imidazole gradient. The fractions with high protein concentration and enzymatic activity were pooled and dialyzed overnight at room temperature against 0.1 M potassium phosphate buffer, pH 7.8, 5 mM mercaptoethanol, 50 μM PLP to remove residual imidazole. The concentration of purified VcTrpase was quantified at 278 nm taking an absorbance $A^{1\%}$ value of 9.19[7]. The purity and molecular mass of the purified VcTrpase were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli[8]. The purified VcTrpase was kept at 4ºC until further analysis.

2.6. Measurement of enzymatic activity and kinetic parameters

VcTrpase activity was monitored by a lactate dehydrogenase coupled (LDH) assay by measuring the decrease in NADH absorption at 340 nm (ε = 6220 M^{-1} cm^{-1})[9]. Briefly, 10 μg of VcTrpase in 0.1 M potassium phosphate buffer, pH 7.8, 5 mM mercaptoethanol, 50 μM PLP, was mixed with an appropriate concentration of L-tryptophan and then incubated at optimal temperature for 1-10 min. The reaction mixture was stopped by boiling at 95ºC for 5 min and chilling on ice prior to the addition of 5 U LDH and 1 mM NADH. One unit of Trpase activity was defined as the amount of enzyme that produces 1 μmol of product per min.

Kinetic parameters were determined using L-tryptophan, L-phenylalanine, L-serine, *S*-methyl-L-cysteine and *S*-benzyl-L-cysteine at varying substrate amounts. The kinetic parameters were determined by the Michaelis–Menten equation, using a Lineweaver Burk plot for calculating the k_{cat} and K_m values for each substrate.

2.7. Spectral studies of the enzymes

Absorption spectra of VcTrpase were taken using a UV-Vis spectrophotometer (Shimadzu UV-1800 Japan). The concentration of the enzymes was adjusted to 10 μM and the spectra were taken in 0.1 M potassium phosphate buffer, pH 7.8, 5 mM mercaptoethanol, 50 μM PLP. The buffer was scanned as the baseline.

3. Results and Discussion

3.1. Enzyme purification

The *tnaA* gene of *V. cholerae* PSU966 was amplified and the PCR product was ligated into the pET20b (+) vector and cloned into *E. coli* BL21 (DE3) *tn5:tnaA*. Around 1.4 kb PCR product of the *V. cholerae tnaA* gene obtained from a transformant was sequenced. After cloning this gene into *E. coli*, tryptophanase was purified using a Ni^{2+}-NTA column, resulting in a VcTrpase yield of around 45% of the total protein. For the mutant strain, the yield was around 57%. Purity and molecular mass of recombinant VcTrpase were analyzed by SDS-PAGE. A single protein band of approximately 49 kDa corresponding to the plausible size of the 472-amino acid protein was visualized (Fig. 2). Two absorption peaks at 330 and 435 nm were detected in the spectra of wild type and mutant VcTrpase at pH 7.0, which possibly corresponded to the enolimine and ketoenamine tautomeric forms, respectively (Fig. 3). Those are analogous to Trpase from *E. coli* (EcTrpase) and *P. vulgaris* (PvTrpase)[10, 11].

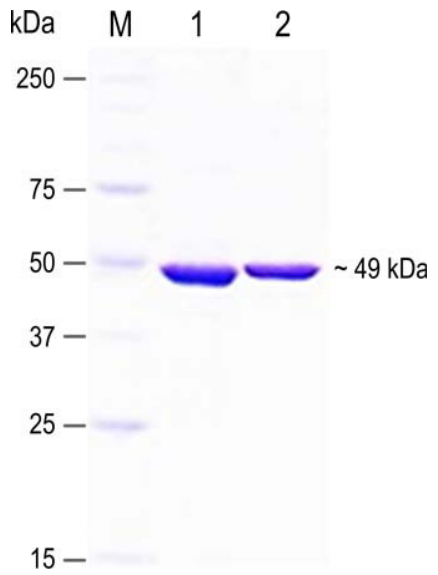

Fig. 2. SDS-PAGE gel of VcTrpase after protein purification; (M) molecular mass markers; (1) wild-type VcTrpase; (2) mutant VcTrpase.

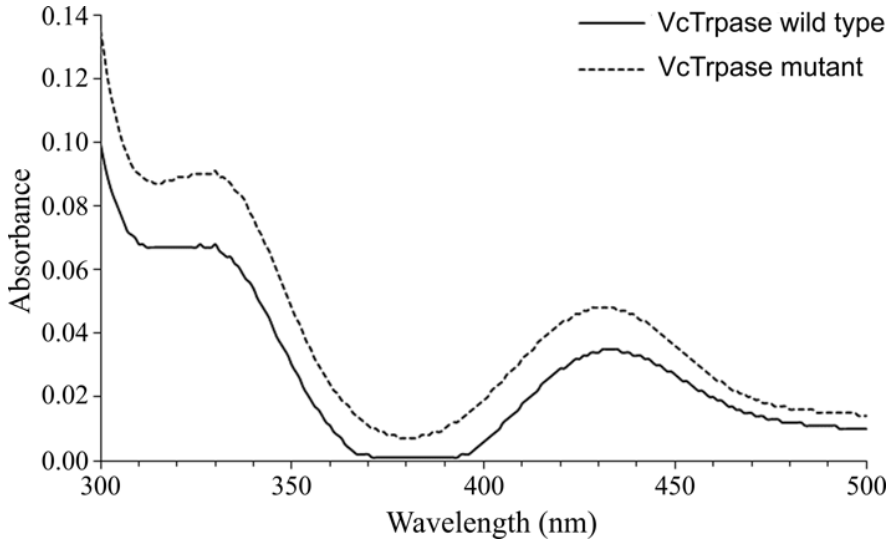

Fig. 3. Absorption spectra of wild-type and mutant VcTrpase.

3.2. Trpase activity of wild type and mutant VcTrpase

The activity of the recombinant *tnaA* gene product from *V. cholerae* in *E. coli* BL21 (DE3) *tn5:tnaA* was evaluated. Wild type VcTrpase possessed a specific activity of 3.0 U/mg protein. Unlike *E. coli* Trpase (EcTrpase), the enzyme was not inactivated at cold temperature[12]. Therefore, VcTrpase did not undergo dissociation into inactive dimers or monomers upon storage at 4ºC. In this work, no activity of the mutant VcTrpase was observed because the conserved Arg419 residue was mutagenized to valine. This amino acid substitution completely destroyed VcTrpase activity. This confirms the significant role of this residue for substrate-binding.

3.3. Kinetic parameters and substrate specificity

A kinetic study of wild type VcTrpase was performed under optimal conditions as described above at various concentrations of L-tryptophan, *S*-benzyl-L-cysteine, *S*-methyl-L-cysteine, L-phenylalanine, and L-serine substrates. The catalytic parameters of L-tryptophan, K_m and k_{cat} were 0.61 mM and 5.25 s^{-1}, respectively. It was found that the affinity of VcTrpase for *S*-benzyl-L-cysteine (30 mM) was 150 times higher than that for *S*-methyl-L-cysteine (0.2 mM), which is similar to the enzyme activities reported for *E. coli* (EcTrpase) and *P. vulgaris* (PvTrpase)[13, 14]. However, the catalytic activity on L-phenylalanine and L-serine was undetectable. This may be due to the structural and chemical properties of enzyme active site, which provides a preferential interaction with polar aromatic substrates such as L-tryptophan, *S*-benzyl-L-cysteine, and *S*-*o*-nitrophenyl-L-cysteine.

4. Conclusion

Recombinant Trpase from *V. cholerae* O1 was successfully cloned, purified, and characterized. Amino acid substitution of Arg419 with valine completely destroyed VcTrpase activity. Thus, this site can be a target for drug design for controlling *V. cholerae*, a serious diarrheal pathogen.

Acknowledgements

We thank Prof. Dr. Robert S. Phillips for generous providing *E. coli* BL2 DE3 *tn5*:*tnaA.* This work was supported by the Department of Microbiology, Faculty of Science, Prince of Songkla University (PSU Ph.D. Scholarship grants No. PSU 0994/2555, research assistant grant number 1-2553-02-008, and research grant from Prince of Songkla University grant no. MET5302285).

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