Enzyme kinetics of dUTPase from the planarian *Dugesia ryukyuensis*

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Abstract

**Objective:** Planarians including *Dugesia ryukyuensis* (Dr) have strong regenerative abilities that require enhanced DNA replication. Knockdown of the DUT gene in Dr, which encodes deoxyuridine 5′-triphosphate pyrophosphatase (dUTPase), promotes DNA fragmentation, inhibits regeneration, and eventually leads to death. dUTPase catalyzes the hydrolysis of dUTP to dUMP and pyrophosphate. dUTPase is known to prevent uracil misincorporation in DNA by balancing the intracellular ratio between dUTP and dTTP, and contributes to genome stability. Nevertheless, the catalytic performance of Dr-dUTPase has not been reported.

**Results:** To confirm the catalytic activity of Dr-dUTPase, we cloned and expressed Dr-DUT in *E. coli*. Then, we purified Dr-dUTPase using His-tag and removed the tag with thrombin. The resulting Dr-dUTPase had the leading peptide Gly–Ser–His– originating from the vector at the amino terminus, and a mutation, Arg66Lys, to remove the internal thrombin site. We observed the hydrolysis of dUTP by Dr-dUTPase using Cresol Red as a proton sensor. The $K_m$ for dUTP was determined to be 4.0 µM, which is similar to that for human dUTPase. Dr-dUTPase exhibited a preference for dUTP over the other nucleotides. We conclude the Dr-dUTPase has catalytic activity.

**Keywords:** dUTPase, Planarian, Regeneration

Introduction

The planarian *Dugesia ryukyuensis* is a model system for studying reproductive strategies for survival [1–3]. We identified key genes in its sexualization and regeneration, including a DNA repair gene, Dr-Rad51 [4], and a germ cell regulator gene, Dr-nanos [5]. Since these genes are also involved in genome stability, we hypothesized that the Drdut gene involved in nucleoside triphosphate biosynthesis [6] affects Dr-Rad51. The results of Dr-dut knockdown supported this hypothesis, upon which we observed alteration of the expression levels of Dr-rad51, Dr-rad51c, and the DNA damage response gene Dr-atm [7]. Nevertheless, the catalytic performance of Dr-dUTPase has not been reported.

The DUT gene encodes the enzyme deoxyuridine 5′-triphosphate pyrophosphatase (dUTPase; EC 3.6.1.23) [8]. Human (*Homo sapiens*, Hs) dUTPase is one of the most intensively studied enzymes [9] and is involved in pyrimidine metabolism. Specifically, Hs-dUTPase provides dUMP to thymidylate synthase [10] in the pathway of thymidine nucleotide biosynthesis [11]. By hydrolyzing dUTP, dUTPase decreases the intracellular concentration of dUTP, and hence lowers the probability of uracil being incorporated into DNA [9, 12]. In the biomedical field, Hs-dUTPase has been identified as a new target of cancer chemotherapy, and a dUTPase inhibitor (TAS-114) is under clinical study [13, 14].

Two major isoforms of Hs-dUTPase have been reported. Isoform 2 (164 amino acids; Fig. 1A) is localized in the nucleus, while isoform 3 (252 amino acids) is localized in the mitochondria [15, 16]. The 3D structure of isoform 2 was solved, revealing that the enzyme adopts a homo-trimer form [17]. However, in Dr-dUTPase, RNA sequencing revealed only one form of dUTPase [18].

Trimer dUTPases have three active sites at the subunit boundaries, consisting of five conserved motifs (Fig. 1A) [12, 19]. The $K_m$ of Hs-dUTPase for dUTP was reported to be 1.1 µM [20, 21]. To confirm the catalytic activity of Dr-dUTPase, we cloned, expressed, and purified the enzyme, and then measured its activity.
Main text

Materials and methods

1. Sequence analysis Sequences of trimer dUTPases from 
   Dugesia ryukyuensis (Dr; DDBI ID, LC421836), 
   Homo sapiens (Hs; UniProt ID, P33316-2), 
   Saccharomyces cerevisiae (Sc; P33317), 
   Escherichia coli (Ec; P06968), and 
   Plasmodium falciparum (Pf; Q8II92) 
   were used because of the availability of kinetic 
   data and 3D structures. Sequences were aligned by 
   MAFFT [22] using the L-INS-I method [23] and 
   BLOSUM62 scoring matrix [24].

2. Preparation of Dr-dUTPase The deduced amino 
   acid sequence of Dr-dUTPase contained an internal 
   thrombin cleavage site at Arg66 (Fig. 1A). To remove 
   the thrombin cleavage site, the Dr-dut 
   gene was mutated from G to A at position 197. 
   Codon utilization of Dr-DUT was optimized to the 
   expression host E. coli (Codon Optimization Tool, 
   https://www.idtdna.com/codonoptim; Additional file 1: Fig. S1, Table S1) 
   and synthesized (Integrated DNA Technologies, 
   Coralville, IA, USA). Dr-dut was cloned into the pET-15b vector (Novagen, 
   San Diego, CA, USA) using NdeI and BamHI sites. Dr-dUTPase was prepared 
   as described previously [19]. Briefly, E. coli BL21 (DE3) was transformed by 
   the construct and cultivated in LB medium. Expression of the Dr-dut gene 
   was induced by IPTG. His-tagged Dr-dUTPase was purified using a Ni–NTA column 
   (GE Healthcare, Chicago, IL, USA). Produced Dr-dUTPase contained the 
   leading peptide MGSSHHHHHHSSGLVPRGSH at the amino terminus. The purified fraction (Additional file 1: Fig. S2) was subjected to desalting by 
   dialysis against 1× thrombin buffer (150 mM NaCl, 
   1.5 mM CaCl2, 20 mM Tris–HCl, pH 8.0) before 
   the thrombin cleavage. After the thrombin cleavage, the 
   resulting Dr-dUTPase still contained the leading 
   peptide GSH at the amino terminus.

3. Kinetic measurements The enzymatic activity was 
   monitored by the color change of Cresol Red due 
   to the production of protons by the hydrolysis of 
   nucleotides [19] using a DU-640 UV/Vis spectrophotometer (Beckman Coulter, 
   Brea, CA, USA). A total of 990 µl of base buffer (100 mM KCl, 5 mM MgCl2, 
   0.25 mM Bicine, pH 8.2) containing final concentrations of 10 µM dUTP + 25 µM 
   Cresol Red was injected into a cuvette (optical path of 1 cm) containing 
   10 µl of 0.02 mM enzyme in the base buffer. Absorbance at 573 nm was recorded at intervals of 
   1.1 s (Additional file 1: Fig. S3). The recorded color 
   change as a function of time was converted to the 
   reaction product, and the $K_m$ values were calculated 
   by the integrated Michaelis–Menten method, as 
   described by Larsson et al. [20] and Inoguchi et al. 
   [19].

4. Structural bioinformatics The 3D structures of dUTPase from Hs (PDB ID, 3ehw) and Pf (1vyq) were 
   used. Homology modeling of intact Dr-dUTPase 
   was performed on the SWISS-MODEL server [25]. 
   The system ranked Hs-dUTPase (3ehw) as the tem-
   plate at the top, and the established model obtained a QMEAN value of $-0.21$ with the residue range 
   between 7 and 145. The root-mean-square distance 
   (RMSD) between the template and model was 0.1 
   Å in both monomer and trimer forms. Taking these 
   findings together, the model of D. ryukyuensis dUTPase was judged to be usable for structural mining. 
   Modeling for Dr-dUTPase Arg66Lys resulted in the 
   same folding as with the native Dr-dUTPase, with 
   RMSD against Hs-dUTPase in trimer of 0.1 Å. Struc-
   tural mining and graphics preparation were performed 
   using the PyMOL Molecular Graphics System, 
   Version v1.7.6.3 (Schrödinger, LLC, New York 
   City, NY, USA).

Results

1. Amino acid sequence of Dr-dUTPase. The planar 
   Dr-dut gene [7] contained an ORF of 462 bp encoding 
   a polypeptide of 153 amino acid residues (Fig. 1A; 
   Additional file 1: Fig. S1). The deduced molecular
mass and pl of Dr-dUTPase were 16,554 and 5.7, respectively. The amino acid sequence alignment revealed that Dr-dUTPase possesses the five conserved motifs unique to homotrimeric dUTPases, including Hs-dUTPase [12]. Dr-dUTPase and Hs-dUTPase share sequence identity of 70%.

2. Production of Dr-dUTPase After the Dr-dut gene had been mutated to exclude the internal thrombin site, its codon utilization was optimized to E. coli (Additional file 1: Fig. S1). The mutation Arg66Lys in motif 2 was chosen to retain the positive charge of the side chain. The pl of mutated dUTPase was thus predicted to remain at 5.7. Alteration of the enzyme may be unavoidable because the residue is located next to Ser67, which is a key residue for the active site. The optimization gave evenly distributed usage of codon utilization (Additional file 1: Table S1), and increased the GC content of the Dr-dut gene from 37 to 50%. The genomic GC content of E. coli was reported to be 50% [26]. The gene was cloned into the pET-15 vector and expressed in E. coli. After affinity purification (Additional file 1: Fig. S2A), the typical yield was about 20 mg of purified tagged protein from 250 ml of culture. Thrombin cleavage was performed to remove the His-tag. The resulting Dr-dUTPase contained the leading GSH peptide and was 156 amino acids in length. The deduced mass and pl were 16,807 and 5.9, respectively. The enzyme was purified uniformly showing the single band in the SDS-PAGE (Additional file 1: Fig. S2B). In the concentration by centrifugation using the 30 kDa-cut membrane (YM-30, MilliporeSigma, Burlington, MA, USA), Dr-dUTPase was retained. Therefore, Dr-dUTPase was potentially existed in oligomer form, including the trimer.

3. Enzymatic activity of Dr-dUTPase To assess the catalytic activity of Dr-dUTPase, hydrolysis of dUTP was monitored by the color change of Cresol Red. The change in absorbance upon multiple-turnover hydrolysis of dUTP as a function of time was converted to the amount of product at a certain concentration of substrate (Additional file 1: Fig. S3) [20]. To obtain \( K_m \) and \( V_{\text{max}} \), the reaction rate against the substrate concentration was fitted to the integrated Michaelis–Menten equation (Additional file 1: Fig. S3 inset). Dr-dUTPase exhibits enzymatic activity, with estimated \( K_m \) and \( V_{\text{max}} \) values of 4.0 \( \mu M \) and 20.2 \( \mu M \) s\(^{-1}\), respectively, at pH 8.2 and 25 °C (Table 1). \( k_{\text{cat}} \) was estimated to be \( 3.4 \times 10^2 \) s\(^{-1}\), assuming three active sites per trimer of dUTPase. The specificity constant, \( k_{\text{cat}}/K_m \), for dUTP was thus \( 8.5 \times 10^6 \) M\(^{-1}\) s\(^{-1}\). Dr-dUTPase exhibited catalytic activity for dUTP.

To analyze the substrate specificity of Dr-dUTPase, enzyme assays were performed using dATP, dTTP, dCTP, and dGTP as substrates at a final concentration of 100 \( \mu M \) and 4–6 \( \mu M \) dUTP (Table 1). The \( K_m \) obtained for the tested nucleotides ranged between 94 and 210 (Table 1). However, the performance of Dr-dUTPase for nucleotides other than dUTP was 2 orders of magnitude lower. Dr-dUTPase in the present format prefers dUTP as its substrate, while allowing the hydrolysis of dATP, dTTP, dCTP, and dGTP at lower levels.

To test the metal dependence of Dr-dUTPase, an enzyme assay was performed after the dialysis, without adding Mg\(^{2+}\). In the dialyzed enzyme, Dr-dUTPase activity was not detected, but the activity was regained as Mg\(^{2+}\) was added to the system. However, the \( K_m \) value remained unchanged at MgCl\(_2\) concentrations from 5 to 25 mM. When the magnesium was replaced with 5 mM MnCl\(_2\), Dr-dUTPase maintained its activity (Table 1). When magnesium was replaced with 5 mM CaCl\(_2\), enzymatic activity was not observed. These results suggest that the activity of Dr-dUTPase occurs in a manner dependent on the metal ions magnesium and manganese.

**Discussion**

Feeding based knockdown of Dr-dut invited planarian death. To obtain further knowledge about the mechanism of the cell death, we analyzed the catalytic performance of Dr-dUTPase.

Dr-dUTPase exhibited catalytic activity, as other trimer dUTPases do (Table 2). Interestingly, its tolerance for other nucleotides was unexpectedly high. For instance, the difference in the specificity constant between dUTP and other nucleotides, and Dr-dUTPase and other dUTPases were 100-fold and about 1000-fold, respectively (Table 2).

Potential causes of the differences in biochemistry included the difference in the amino acid sequence in key residues in conserved motifs (M1-5; Fig. 1A). In the case of Hs-dUTPase, the active site for the A chain

| Nucleotides | \( K_m \) (\( \mu M \)) | \( k_{\text{cat}} \) (s\(^{-1}\)) | \( k_{\text{cat}}/K_m \) (M\(^{-1}\) s\(^{-1}\)) |
|-------------|----------------|----------------|----------------|
| dUTP (n = 7) | 40 ± 0.4 | 3.4 × 10^3 ± 3.0 | 8.5 × 10^5 ± 1.1 × 10^6 |
| dATP | 9.4 × 10^1 ± 1.2 | 1.5 ± 0.2 | 1.6 × 10^4 ± 2.1 × 10^3 |
| dTTP | 1.1 × 10^2 ± 7.2 | 1.4 ± 0.3 | 1.3 × 10^4 ± 2.8 × 10^3 |
| dCTP | 7.7 × 10^1 ± 3.5 | 1.6 ± 0.2 | 2.1 × 10^4 ± 2.7 × 10^3 |
| dGTP | 2.1 × 10^2 ± 1.0 × 10 | 2.9 ± 0.5 | 1.4 × 10^5 ± 2.4 × 10^3 |
| dUTP, 5 mM MnCl\(_2\) | 7.3 ± 1.2 | 4.8 × 10^2 ± 6.8 | 6.5 × 10^4 ± 2.1 × 10^5 |

Measurements were triplicated (n = 3) except in the case of dUTP with MgCl\(_2\).
consists of Arg85, Ser86, and Gly87; for the B chain, Gly99, Asp102, and Gly110; and for the C chain Phe158 [9] (Fig. 1A, B). In the present form of Dr-dUTPase, substitutions were found as follows: Arg85Lys in the A chain and Phe158Tyr in the C chain. Among them, Phe158 in Hs-dUTPase was suggested to promote stacking enabling uracil recognition [27]. Phe158 in Hs-dUTPase is conserved in other dUTPases, including Sc-dUTPase, Ec-dUTPase, and Pf-dUTPase. By the substitution of Phe158Tyr, Dr-dUTPase potentially obtained alternative stacking between the aromatic ring in the residue and the uracil base. The alternative stacking could have allowed the larger ligand tolerance.

In motif 2 of trimer dUTPase, there is only one short α-helix, which holds two amino acid facing active site (Fig. 1A, B). One is the nucleophile Ser, and another is the four residues after it. Combination of the amino acid between the Ser and another residue seemed affect the affinity of the enzyme in terms of the initial capture of the substrate–water complex [19]. In hs-dUTPase and Sc-dUTPase have small hydrophobic residues, and they show a lower affinity than that of Ec-dUTPase which carry the positively charged residues at the corresponding site. In Dr-dUTPase has Ser and Leu on the α-helix. Our results of the activity assay showed Dr-dUTPase has lower affinity to dUTP than that of Hs-dUTPase (Table 2). Nevertheless, Pf-dUTPase had positively charge amino acid at the second position in the helix, this enzyme had a large insertion in the leading sequence of M2. Hence Pf-dUTPase is an exception case.

The present study shows that Dr-dUTPase has catalytic activity that preferentially favors dUTP, but also possesses high tolerance regarding substrate recognition. This result for Dr-dUTPase was unexpected because Hs-dUTPase can slightly recognize dTTP and dCTP, but not dATP and dGTP (Table 2). If this is also the case in vivo, the wide substrate acceptance of Dr-dUTPase would be notable as a planarian-specific phenomenon. Either way, the results of the present study confirm the catalytic activity of dUTPase, increasing our basic understanding of the roles of dUTPase in planarians.

**Limitations**

Dr-dUTPase has conserved motif of trimer dUTPase (M1-5), and has conserved residues for the inter-subunit interactions. Then we assumed that Dr-dUTPase keeps a trimer structure. To conform the oligomer state of Dr-dUTPase, we performed the size exclusion chromatography, but unfortunately it could not succeed.

The 3D structures of dUTPase showed that Dr-Tyr139 (B-chain) and Hs-Phe158 (B-chain) in motif 5 (M5), are involved in stacking between the U-base and aromatic ring of the side chain. The Dr-Phe29 (C-chain) and Hs-Tyr48 (C-chain) are located in structural motif 1 (M1), which is a part of the active sites. The aspartate in M1, next to the Dr-Phe29 (C-chain) and Hs-Tyr48, interacts with active site water molecules to stabilize the Mg ion. Therefore, Dr-Phe29 (C-chain) and Hs-Tyr48 are still involved in the substrate binding. Nevertheless, we do not have the crystal structure yet to support this idea.

**Additional file**

Additional file 1: **Fig. S1.** Nucleotide sequence of Dr-DUT gene. Shown sequences are RNA sequencing (Rseq), mutated (G197A), and codon optimized for E. coli. **Fig. S2.** Production of Dr-dUTPase analyzed by SDS-PAGE. **Fig. S3.** Hydrolysis of dUTP by Dr-dUTPase. **Table S1.** Michaelis–Menten constant, $K_m$ (μM), and specificity constant, $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$) of dUTPases from different origins.

| Substrate | D. ryukyuensis | H. sapiens | S. cerevisiae | E. coli | P. falciparum |
|-----------|----------------|------------|---------------|---------|--------------|
| dUTP      | 4.0 (8.5 × 10$^4$) | 1.1 (6.6 × 10$^3$) | 1.3 × 10$^4$ (7.4 × 10$^3$) | 0.2 (3.6 × 10$^3$) | 1.9 (7.1 × 10$^3$) |
| dATP      | 9.4 × 10$^3$ (1.6 × 10$^4$) | NR | NR | NR | 1.1 × 10$^3$ (2.7) |
| dTTP      | 1.1 × 10$^3$ (1.3 × 10$^4$) | 7.6 × 10$^2$ (2.0 × 10) | NR | > 2.0 × 10$^4$ (< 0.1 × 10$^5$) | 2.9 × 10$^3$ (9.2) |
| dCTP      | 7.7 × 10$^2$ (2.1 × 10$^3$) | 6.9 × 10$^2$ (1.6 × 10$^3$) | NR | 4.0 × 10$^4$ (1.0 × 10$^5$) | 4.7 × 10$^2$ (2.2 × 10$^3$) |
| dGTP      | 2.1 × 10$^2$ (1.4 × 10$^3$) | NR | NR | NR | 2.0 × 10$^3$ (1.3 × 10$^{-1}$) |

In dUTPase from E. coli, the $K_m$ for dTTP and dCTP were presented congruent to $K_d$ NR not reported

Abbreviations
dUTPase: deoxyuridine 5′-triphosphate pyrophosphatase; RMSD: root-mean-square distance.

Authors’ contributions
MSA, HM and MM designed the study. MSA performed all experiments and wrote the paper. All authors read and approved the final manuscript.
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Competing interests
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Availability of data and materials
The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

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Not applicable.

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