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Engineering of *Escherichia coli* β-lactamase TEM-1 variants showing higher activity under acidic conditions than at the neutral pH

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ABSTRACT

Escherichia coli β-lactamase TEM-1 is potentially useful in the antibody-directed enzyme/prodrug therapy (ADEPT), converting nontoxic prodrugs to toxic agents. The produced toxin would kill cancer cells, when the enzyme is attached to a tumor-antigen-specific antibody. However, the off-site reaction possibly occurring in the blood or normal tissues raises safety concern. In the present study, we engineered TEM-1 variants preferentially active at pH 5.8–6.2, near the pH of the acidic microenvironment of tumor. A library of randomly mutagenized variants was screened for the ability to confer an antibiotic resistance on *E. coli* cells in acidic growth media and not in neutral media, to isolate a variant with a Thr-to-Ile substitution at position 160. An extensive mutagenesis study was then conducted in the proximity of this position, to show that a Leu162Glu mutation also causes the acid preference. Kinetic analyses indicated that the overall activity of the wild-type TEM-1 hardly changes over a pH range from 5.8 to 7.0, whereas TEM-1(T160I) is 1.5-times as active at pH 6.2 than pH 7.0, and TEM-1(T160I) is 3.1-fold as active at pH 5.8 than pH 7.0. A further mutagenesis study suggested that a change in the overall structure of the enzyme underlies the pH dependency of the variants.

1. Introduction

β-Lactamases are potentially useful in the antibody-directed enzyme/prodrug therapy (ADEPT) [1,2]; the enzymes are conjugated with tumor-antigen-specific antibodies and catalyze the conversion of non-toxic prodrugs into toxic agents to attack cancer cells [3–11]. If this toxic reaction takes place in the blood and normal tissues, it raises safety concern, and the quick clearance of the enzyme—antibody conjugates from the non-tumor sites has been a challenge [1]. The extracellular environments of tumor tissues are often acidic, approaching pH 6.0, due to the high glycolytic activities of cancer cells [12]. If a β-lactamase was activated under acidic conditions and not active near the neutral pH, the distribution of the enzyme—antibody conjugate outside tumors would not be problematic. Although a great number of lactamase mutants were reported previously [13–22], no variants showed a preference for acidic pH. In the present study, we attempted to alter the pH profile of the catalytic activity of the TEM-1 β-lactamase from *Escherichia coli* [EC 3.5.2.6] in favor of acidic conditions.

2. Materials and methods

2.1. TEM-1 mutagenesis and plasmids

A GeneMorph II Random Mutagenesis kit (Agilent Technologies Japan, Co. Ltd.) was used to incorporate random mutations. *E. coli* MegaX DH10B T1 cells (Invitrogen) were transformed with the resulting variant genes to create a variant library. Site-directed mutagenesis was performed using a PrimeSTAR mutagenesis kit (Takara Bio Inc., Japan). TEM-1 was expressed under the control of the T7 promoter from a variant of the vector pET26b (+) (Novagen), which carried a chloramphenicol resistant gene in place of the kanamycin resistant gene. The TEM-1 gene included the 23-residue signal peptide, and a C-terminal 7-residue linker (GSSSGSS) followed by a TEV protease recognition sequence and a hexahistine tag in this order. This expression plasmid was used for either of *in vivo* screening and variant overproduction.

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2.2. In vivo assays

The library of TEM-1 variant genes were introduced into the *E. coli* B-95.ΔA strain [23] with pCDF-IYN3 [24], which conferred kanamycin resistance. The growth medium at pH 5.8 contained LB broth, 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), 3-nitro-L-tyrosine (Watanabe Chemical Industries, Ltd., Japan) at a final concentration of 1 mM, and carbenicillin and kanamycin at final concentrations of 100 μg/ml and 30 μg/ml, respectively, and buffered by MES [pH5.8] at a final concentration of 100 mM. The other medium at the neutral pH was prepared similarly, except for the absence of the MES buffer. 3-Nitrotyrosine should be incorporated into TEM-1 variants with in-frame UAG codons under these conditions. We initially intended to obtain also TEM-1 variants containing the tyrosine derivative at any site. However, the isolated variants turned out to contain no in-frame UAG. The *in vivo* activities of the TEM-1 variants with the mutations in the proximity of position 160 were assayed by introducing these variant genes into the B-95.ΔA strain, not harboring pCDF-IYN3. The used LB plates were prepared in a similar manner to that used for the screening, except for the absence of kanamycin and 3-nitrotyrosine in the media. For comparing the *in vivo* activities of the wild-type enzyme, TEM-1(T160I), and TEM(L162E), *E. coli* DH5α cells were transformed with their genes, and grown on the LB plates at the different pH containing the indicated final concentrations of carbenicillin. The bacteria were grown overnight at 37 °C or kept at room temperature for a few days.

2.3. Purification of TEM-1 variants

The B-95.ΔA cells expressing TEM-1 from the pET vector were cultured in a 100 ml volume of the 2 × YT auto-induction medium at 25 °C for 24 h. The auto-induction method was reported previously [25]. The cells were lysed in a PBS buffer (Takara Bio, Inc., Japan) containing imidazole (20 mM), and the supernatant was then applied to a Ni Sepharose 6 Fast Flow column (GE healthcare), followed by elution in the PBS buffer containing 250 mM imidazole. TEM-1 was digested by TEV protease during the dialysis against PBS at 4 °C overnight, and then applied to the Ni Sepharose 6 Fast Flow column again, to keep the flow-through. To further purify the protein, the second chromatography on a HiLoad 16/60 superdex75 column (GE healthcare) was performed with PBS using an ÄKTA chromatography system (GE healthcare). The purified sample was stored at −25 °C in the PBS buffer containing 50% (v/v) glycerol.

2.4. In vitro assays

The catalytic activity of TEM-1 was analyzed *in vitro* using a Beta Lactamase activity assay kit (Abcam) with nitrocefin. The assay buffer was replaced by a phosphate buffer at a final concentration of 50 mM, which was previously used for TEM-1 assay [26] and was adjusted at pH5.8—pH7.0. TEM-1 was diluted with a 50 mM phosphate buffer containing 1% (w/v) bovine serum albumin (BSA), and each reaction contained BSA at a final concentration of 0.02% (w/v) as a result. The optimal density at 490 nm was measured using a SpectraMax i3x spectrometer (Molecular Devices). To obtain the time courses of the reaction, TEM-1 was included at a final concentration of 600 pM in the presence of nirocefin at a final concentration of 40 μM. For determining kinetic parameters, the wild-type TEM-1 was included at 150 pM, and the two variants were each included at 600 pM. The substrate concentration varied from 10 to 40 μM, while nitrocefin was diluted by DMSO and the DMSO concentration was 2% in the reaction. Reactions were performed at 37 °C, in triplicate for observing time courses shown in the main text and for determining kinetic parameters.

3. Results and discussion

3.1. Amino acid replacements at position 160 in TEM-1 cause a preference for acidic conditions

E. coli TEM-1 β-lactamase comprises 286 amino acids including a 23-residue signal peptide. We created a library of TEM-1 variants mutagenized at random positions, with the mutation rate of about one base per gene. *E. coli* cells were transformed with these variant genes, to form 10^6–10^7 colonies on non-selective growth media plates. One thousand and fifty-six clones of them formed colonies on carbenicillin-containing media plates adjusted at pH 5.8. Replica plates showed that 32 clones of them were incapable of growing at the neutral pH. By contrast, *E. coli* cells expressing the wild-type TEM-1 were able to grow with the antibiotic of a concentration of 100 μg/ml at either pH (Fig. 1A). Eight of the isolated 32 clones each contained one amino-acid replacement, and only the Thr-to-Ile substitution at position 160 (T160I) occurred in more than one clone (in four clones). The pH-dependent activity of this TEM-1 variant, TEM-1(T160I), was confirmed with the cells freshly transformed with the variant gene (Fig. 1B). These were able to grow with the antibiotic of a concentration of 10 μg/ml, and thus, the *in vivo* activity of TEM-1(T160I) was weaker than that of the wild-type TEM-1.

We purified the wild -type TEM-1 and TEM-1(T160I) from overproducing cells and subjected them to *in vitro* assays. The hydrolytic activity of the enzyme to break a β-lactam ring was measured using nitrocefin, a chromogenic cephalosporin, as the substrate. The wild-type molecules showed similar reaction rates at pH 5.8 and 7.0 under the conditions described in “Materials and methods”, whereas TEM-1(T160I) showed a significantly slower rate at pH 7.0 than pH 5.8 (Fig. 2). The initial velocity of the reaction for the variant was slower than the wild-type enzyme at either pH.

Next, we replaced Thr160 with the other 18 amino acids in TEM-1, to conduct *in vitro* assays (Fig. S1). The variants with Gly, Ala, Val, Ser, Met, and Phe at this position were almost equally active at pH 5.8 and pH 7.0, whereas those with Asp, Asn, and Gln showed lower activities at the neutral pH. The other mutations impaired the activity at both pH. These preliminary data suggested that T160I was not the only mutation to generate the desirable pH profile of the activity.

3.2. Extensive survey in the proximity of position 160 for further TEM-1 variants with a preference for acidic conditions

In the reported crystal structures [27,28], TEM-1 comprises the central β-sheet domain and two α-helical domains, one of which includes the catalytic residues Ser70, Lys73, Ser130, and Glu166. Thr160 is located in this domain, and distant from the catalytic site (Fig. 3). The neighboring region of position 160 includes the terminal parts of three α-helices (Helix 2, Met69—Ala86; Helix 6, Lys146—Asn154; and Helix 8, Ala184—Leu194), while Thr160 is part of an unstructured strand (Asp157—Asp163) extending along Helix 6. Nine positions were then chosen for saturation mutagenesis (Met68, Thr71, Phe72 from Helix 2; Leu75, Leu148, Leu152 from Helix 6; Met186 from Helix 8; and Arg161, Leu162 from the extended strand), and the 171 resulting variants were examined for the cell-based activity (Fig. S2 and Table S1). Most of the variants did not confer the antibiotic resistance at either pH5.8 or 7.0, whereas 17 variants were found to confer the resistance at pH 5.8 and not at pH 7.0. However, only TEM-1(L162E) exhibited an actual preference for the acidic pH in *in vitro* assay (Fig. 2C), whereas the 16 other variants did not show the desired property *in vitro* (Fig. S3).

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