PenA, a penicillin-binding protein-type thioesterase specialized for small peptide cyclization

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Abstract: Penicillin-binding protein-type thioesterases (PBP-type TEs) are a recently identified group of peptide cyclases that catalyze head-to-tail macrolactamization of nonribosomal peptides. PenA, a new member of this group, is involved in the biosyntheses of cyclic pentapeptides. In this study, we demonstrated the enzymatic activity of PenA in vitro, and analyzed its substrate scope with a series of synthetic substrates. A comparison of the reaction profiles between PenA and SurE, a representative PBP-type TE, showed that PenA is more specialized for small peptide cyclization. A computational model provided a possible structural rationale for the altered specificity for substrate chain lengths.

Keywords: Cyclopeptide, Biocatalyst, Cyclase

Introduction

Cyclic peptides exhibit improved membrane permeability and resistance against proteolytic enzymes, as compared to their linear counterparts (Tsomaia, 2015). Cyclization confers structural rigidity and constrains the peptide shape, similar to the binding state to biological targets, thus enhancing affinity and specificity. Although macrocyclization is a general strategy of peptide modification when considering pharmaceutical applications, the control of undesired epimerization and oligomerization during the ring-closure remains challenging (White & Yudin, 2011). Cyclization generally becomes more problematic when the chain length is decreased, as the preferable E-geometry of peptide bonds prevents cyclization points from being in proximal space. Several strategies have been invented to tackle this obstacle, such as incorporation of turn-inducing protecting groups (Skropeta et al., 2004), cyclization on solid support (Alcaro et al., 2004) and contraction of the ring size of a larger intermediate (Meutermans et al., 2003). On the other hand, small cyclic peptides such as cyclic tetrapeptides and cyclic pentapeptides are frequently found in nature (Abdalla, 2016; Jing & Jin, 2020), and the chemo- and regio-specific ring-closure during their biosyntheses is achieved by a group of enzymes referred to as peptide cyclases, which could be exploited as alternative catalytic tools for the cyclization of small peptides.

Penicillin-binding protein (PBP)-type TEs are a recently discovered family of peptide cyclases involved in the biosynthesis of a series of nonribosomal macro lactams (Kuranaga et al., 2018, Matsuda, Kuranaga, et al., 2019). In contrast to canonical peptide cyclase domains, such as the type-I thioesterase (TE) and the terminal condensation domain (Ct), the PBP-type TEs are physically discrete from an assembly line and thus act in trans. The PBP-type TE group members examined thus far exclusively catalyze macrolactamation. PBP-type TEs are encoded in the biosynthetic gene clusters of several nonribosomal macro lactams ranging from hexapeptide to decapeptide, including surugamides (Kuranaga et al., 2018) and desotamides (Li et al., 2015; Ding et al., 2020), ullaungmycins (Son et al., 2017), noursamycins (Mudalung et al., 2019), and mannopeptimycins (Magarvey et al., 2006). Among them, SurE, a PBP-type TE for surugamide biosynthesis, is of particular interest because it acts in trans to two distinct nonribosomal peptide synthetases (NRPSs) to cyclize a structurally unrelated set of linear intermediates (i.e., octapeptide for surugamides A–E and decapptide for cyclosurugamide F) (Fig. 1) (Matsuda, Kobayashi, et al., 2020). SurE exhibits broad substrate tolerance in vivo and in vitro, indicating its potential use as a biocatalyst for peptide cyclization and a genetic tool for synthetic biology (Matsuda et al., 2020). A promiscuous peptide cyclase with multiple physiological substrates is a promising candidate for the further development of tools for producing cyclic peptides. However, SurE is currently the only example of a biochemically characterized PBP-type TE that acts on more than one NRPS assembly line.

Pentaminomycins are a growing family of cyclic pentapeptides isolated from several Streptomyces species (Carretero-Molina et al., 2020, Hwang et al., 2020; Jang et al., 2018, Kaweewan et al., 2020). Interestingly, the BE-18257s, a structurally distinct group of cyclic pentapeptides, are concomitantly produced along with pentaminomycins (Carretero-Molina et al., 2020; Hwang et al., 2020, Kaweewan et al., 2020), suggesting their biosynthetic relationship. The biosynthetic gene cluster of pentaminomycins (pen) was proposed (Hwang et al., 2020; Kaweewan et al., 2020), and experimentally validated quite recently by heterologous expression (Fig. 1) (Román-Hurtado et al., 2021). We herein refer to the genes according to the report by Hwang et al. (Hwang et al., 2020). In the pen cluster, the NRPS gene penN2, responsible for pentaminomycin biosynthesis, is encoded in the neighboring region of the NRPS gene penN1, responsible for BE-18257s. Notably, both assembly lines lack canonical termination domains at their C-termini, suggesting the presence of unusual cyclization mechanisms. Analysis of the pen cluster identified the single PBP-like enzyme PenA, encoded in the upstream region of penN1. As no other candidate...
gene for chain-termination and cyclization is encoded in pen, PenA is hypothesized to act on both the penN1 and penN2 assembly lines to catalyze the macrolactamization of two families of cyclic pentapeptides (Hwang et al., 2020; Kaweewan et al., 2020).

In this study, we demonstrated the macrolactamization activity of PenA in vitro, validating it as a new member of the PBP-type TEs. A series of synthetic substrates revealed that the scope of PenA is similar to that of SurE, in terms of the strict requirement for heterochiral residues at both termini and the broad tolerance of residues in the middle. On the other hand, PenA and SurE exhibit distinct preferences for the substrate chain length. The structural model of PenA provides a possible rationale for the difference in the enzymatic properties.

Material and methods

Synthesis of substrate mimics

The detailed procedure for solid phase peptide synthesis (SPPS) and synthesis of substrate mimics are provided in supplementary materials.

Preparation of recombinant PBP-type TEs

The recombinant PenA (GenBank protein ID: WP_158102277) in Streptomyces cacaoi NBRC12748 was prepared by following procedure. DNA fragment coding for PenA was amplified by KOD One (Toyobo) using a set of primer PenA_Fw/PenA_Rv (PenA_Fw: CCGgaattccatatg

| penA             | penN1   | penN2   | surA   | surA    | surB   | surB   | surC   | surC    | surD   | surE   |
|------------------|---------|---------|--------|---------|--------|--------|--------|---------|--------|--------|
| pen              | N-deco  | N-deco  | C-deco | C-deco  | C-deco |
| pen              | N-deco  | M-deco  | C-deco | C-deco  | C-deco |
| pen              | N-deco  | M-deco  | C-deco | C-deco  | C-deco |
| pen              | N-deco  | M-deco  | C-deco | C-deco  | C-deco |
| pen              | N-deco  | M-deco  | C-deco | C-deco  | C-deco |

Fig. 1. Structures of pentaminomycins, BE-18257s, surugamides, and their biosynthetic gene clusters. The residues at N- and C-termini of linear precursors were colored in red and blue, respectively. Genes encoding PBP-type TEs and NRPSs are colored orange and gray, respectively.

In vitro assay

In vitro reaction was performed with following condition; 50 μL reaction mixture containing 20 mM Tri-HCl (pH8.0), 400 μM substrate, 1 μM enzyme. Reaction mixture was incubated at 30°C for 2 hours, then reaction was quenched by adding equal volume of 0.1% TFA. Resultant mixture was analyzed by HPLC equipped with reversed-phase column COSMOSIL-3C18-MS-II 4.6 × 250 mm (nacalai tesque). Sample was eluted with gradient method (30-70% in 20 min for mobile phase B) using H2O + 0.05% TFA and acetonitrile + 0.05% TFA as mobile phases A and B, respectively.

In vitro reaction was performed with following condition; 50 μL reaction mixture containing 20 mM Tri-HCl (pH8.0), 12.5–400 μM substrate were pre-incubated at 30°C for 10 min. Reaction was initiated by adding 1 μM PenA or 50 nM SurE then mixture was incubated for 2 min. Reaction was quenched by adding equal volume of 0.1% TFA, then 20 μL of resultant mixture was loaded onto COSMOSIL 3C18-MS-II 2.0 × 150 mm (nacalai tesque) then eluted with gradient method (30-70% in 5 min for mobile phase B) using H2O + 0.05% TFA and acetonitrile + 0.05% TFA as mobile phases A and B, respectively. Column elutes were monitored with UV absorption at 210 nm. Initial velocity was calculated assuming that e (210 nm) values are equal for SNAC substrate and corresponding enzymatic product. e (220 nm) values for dimerized products 37 and 38 were assumed to be double of that for monomer.

Kinetic assay

The concentrations of proteins were measured using a Bio-Rad protein assay kit. Preparation of recombinant SurE was described in previous reports (Matsuda et al., 2020).

Results and discussion

A series of linear substrates resembling pentaminomycin C (3) was synthesized by conventional solid-phase peptide synthesis with DIC/Oxyma-mediated amide coupling and piperidine-promoted Nα-deprotection. Elongated peptides were cleaved from the resin and coupled with N-acetyl cysteamine (SNAC), to afford thioester substrates as the mimics
In vitro assays of PenA, using 13–22 as substrates. The reaction substrates were described on each chromatograph. Chromatographs of samples with active enzyme (red) and boiled enzyme (black) are overlaid. Samples were eluted with a gradient method (30–70% in 20 min for mobile phase B) with H2O + 0.05% TFA and acetonitrile + 0.05% TFA as mobile phases A and B, respectively. Column eluates were monitored with UV absorption at 220 nm.

Table 1. Synthetic Substrates Used in This Study. Mutated Residues are Written in Bold

| Substrate | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | Reaction product | Cyclic or linear |
|-----------|---|---|---|---|---|---|---|---|-----------------|-----------------|
| deOx-PC (13) | Leu | d-Val | Trp | Arg | d-Phe | – | – | – | 26 | Cyclic |
| L1L1L (14) | d-Leu | d-Val | Trp | Arg | d-Phe | – | – | – | 27 | Linear |
| L1L1F (15) | Phe | d-Ala | Trp | Arg | d-Phe | – | – | – | 28 | Cyclic |
| dV2oA (16) | Leu | d-Ala | Trp | Arg | d-Phe | – | – | – | 29 | Cyclic |
| tW3LA (17) | Leu | d-Val | Ala | Arg | d-Phe | – | – | – | 30 | Cyclic |
| tR4LA (18) | Leu | d-Val | Ala | Arg | d-Phe | – | – | – | 31 (73%) | Cyclic |
| dF5oY (19) | Leu | d-Val | Trp | Arg | d-Val | – | – | – | 32 (9%) | Cyclic |
| dF5oY (20) | Leu | d-Val | Trp | Arg | d-Tyr | – | – | – | 33 | Cyclic |
| dF5oG (21) | Leu | d-Val | Trp | Arg | Gly | – | – | – | 34 (8%) | Cyclic |
| dF5oL (22) | Leu | d-Val | Trp | Arg | Phe | – | – | – | N.D. | – |
| tetrapeptide (23) | Leu | Trp | Arg | d-Phe | – | – | – | – | 35–38 | Cyclic/linear |
| tripeptide (24) | Leu | Arg | d-Phe | – | – | – | – | – | 39–40 | Linear |
| SB-SNAC (25) | Ile | d-Ala | Ile | d-Val | Lys | Ile | d-Phe | d-Leu | 11 | Cyclic |

aChemical structures of reaction products are depicted in Supplementary Fig. S2. Reaction yields are described when a substrate was not completely consumed within 2 hr of incubation. N.D.: not detected.
bThis column indicates whether the reaction products of PBP-type TEs are cyclic or linear.
*cYields are described in Fig. 4.
PenA: cyclized by both enzymes with the following kinetic parameters: $k_{\text{cat}} = 6.1 \pm 0.3 \text{ min}^{-1}$, $K_m = 64.4 \pm 7.5 \mu M$. SurE: $k_{\text{cat}} = 297.7 \pm 53.0 \mu M$, $K_m = 96.4 \pm 17.1 \text{ min}^{-1}$. PenA and SurE exhibited strict requirements for the heterochirality of the tetrapeptide $\text{PenA}$ and $\text{SurE}$, respectively. PenA was capable of accommodating small octapeptidyl substrates, whereas SurE requires a dodecapeptidyl substrate. PenA exhibited a strict requirement for the heterochirality of terminal residues, while SurE accepts tetra- and pentapeptides, as shown in Fig. 4. PenA mediated the monomeric cyclopeptide $\text{PenA}$ as a model substrate to investigate the scope of SurE. These cyclizations were observed when the tetrapeptidyl substrate was concomitantly accumulated, and the dipeptide possibly generated from the peptidase activity of PBP-type TEs was observed. As the peptidase activity of PBP-type TEs is not known, this phenomenon requires further investigation in future work.

PBP-type TEs generally consist of an N-terminal PBP domain with a highly conserved catalytic tetrad and a C-terminal lipocalin domain that participates in substrate binding (Matsuda et al., 2020). The catalytic tetrad is located at the bottom of a large cleft between the PBP and lipocalin domains. To gain insight into the structural features that differentiate the chain length specificities between PenA and SurE, we first compared their primary sequences. Although they share high sequence identity (39.32%), a notable difference was observed in the region between $\beta$-strands 5 and 6 in the lipocalin domain (Matsuda et al., 2020). These strands are connected by 7 amino acid residues in SurE, whereas in PenA, this region is elongated up to 17 residues in PenA (Fig. 5a). This extended loop is located at the side of a $\beta$-barrel that forms the substrate-binding cleft (Fig. 5b). In the model structure of PenA that was generated, the extended loop protrudes toward the PBP domain and substantially narrows the binding cleft (Fig. 5c), which may explain why PenA is incapable of accommodating a large octapeptidyl substrate. The extended loop may also play a role in shielding the tetrapeptide-O-PenA intermediate from a second substrate molecule, thus hampering the intermolecular ligation of peptides, in contrast to the case of SurE.

Enzyme-mediated peptide cyclization has been an area of intense research for many years, because of its high chemoselectivity and mild aqueous reaction conditions (Schmidt et al., 2017). However, the use of enzymes to cyclize small peptides, such as tetra- and pentapeptides, is considerably less characterized (Mandalapu et al., 2018; Sardar et al., 2015) compared to the enzymatic cyclizations of medium and large sized peptides. This is probably because small peptides are beyond the scope of the currently available, well-investigated peptide cyclases, such as sortase A, butelase, and subtilisin derivatives, which all accept substrates with ten or more residues (Schmidt et al., 2017). As both naturally occurring and synthetic small peptides are a fruitful class of compounds with a wide range of biological activities (Abdalia, 2016; Sarojini et al., 2019), keen interest is being focused on the development of versatile biocatalysts that enable rapid access to this chemical space.

PenA exhibits a strict requirement for the heterochirality of terminal residues as well as broad tolerance for the side chains in the middle of the sequence. PenA converted most of the tested substrates into their cyclic counterparts, in a nearly quantitative manner. As compared with SurE, PenA is more specialized to small cyclic peptides, as it accepts tetra- and pentapeptides, but not octapeptides. Notably, PenA generated a cyclic tetrapeptide without a detectable amount of the dimerized product. Taken together, this study highlights the potential use of PBP-type TEs for the cyclization of small peptides, which are usually challenging to cyclize by conventional chemical approaches. The data presented...
Fig. 4. Proposed reaction scheme of PBP-type TEs with the tetrapeptidyl substrate 23. Yields of each enzyme are described.

here provide an important basis for the future development of engineered biocatalysts with broadened substrate scopes.

**Supplementary Material**

Supplementary material is available online at JIMB (www.academic.oup.com/jimb).

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Fig. 5. Structural comparison between PenA and SurE. The model of PenA was generated by SWISS-MODEL (Waterhouse et al., 2018) using crystal structure of SurE (PDB ID: 6KSU) as template. (a) Domain architecture of PBP-type TEs and primary sequence alignment of the lipocalin domains. (b) Comparison between the lipocalin domain of SurE (6KSU, cyan) and that of the PenA model (gray). The extended loop region in PenA is colored red. (c) Overall structure of SurE (6KSU, left) and that of the PenA model (right). The catalytic tetrad is shown in magenta. The color scheme for the lipocalin domains is the same as in (b).

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Conflict of Interest
The authors declare no conflict of interest.

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