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Macrolactonization catalyzed by the terminal thioesterase domain of the nonribosomal peptide synthetase responsible for lichenysin biosynthesis

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Abstract—The excised terminal thioesterase of the lichenysin nonribosomal peptide synthetase was found to be a highly efficient and versatile enzyme. Its activity strictly requires the *R* configuration of the β-hydroxy fatty acid and the side chains of aspartate-5 and isoleucine-7, but tolerates changes in five other residues of the substrate. Characterization of this enzyme facilitates future effort to engineer the lichenysin synthetase for biotechnological applications.

Natural cyclic peptide products are a pharmaceutically important class of natural products, such as the well-known immunosuppressant cyclosporin A[1] and the potent antibiotic vancomycin.[2] They are biosynthesized by modular nonribosomal peptide synthetases (NRPS) through a common thiotemplate mechanism.[3] The last domain of these NRPS is a thioesterase (TE) that cyclizes a linear precursor synthesized by preceding domains of the synthetases. Recently, Walsh and co-workers found that the thioesterases of both tyrocidine and gramicidin NRPS possess an intrinsic broad substrate spectrum that allows for chemoenzymatic synthesis of a diverse array of analogues of the natural products and peptide/ketide hybrids.[4] This chemoenzymatic method has successfully been applied to optimize the therapeutic index of tyrocidine A.[5] More important, characterization of these versatile thioesterases greatly facilitates the efforts to bioengineer the modular NRPS to generate novel structures.

Naturally occurring lipopeptides, such as iturins, surfactins, and lichenysins, are a unique sub-category of nonribosomal peptide products.[6] They differ from other natural peptide products in that a β-hydroxy fatty acid is incorporated into the cyclic backbone. The terminal thioesterase in the NRPS catalyzes a lactonization between the hydroxyl group of the fatty acid moiety and the activated carboxy terminus in a head-to-tail cyclization of the linear precursor. Such a thioesterase from the NRPS for the surfactin biosynthesis has been tested active toward the *N*-acetylcysteamine (NAC) thioester of the linear lipopeptide. The active site residues were identified by site-directed mutagenesis on the basis of its crystallographic structure in complex with the substrate.[7,8] However, this thioesterase was found to have a particularly high K_M constant in the millimolar range, an unfavorable property for an enzyme. In this study, we characterized the carboxy terminal thioesterase of the NRPS of lichenysin D, a cyclic lipopeptide resembling the surfactins, and found that it possesses a favorable kinetic profile more suitable for biotechnological applications.

The NRPS for the biosynthesis of lichenysin D was cloned and sequenced by Marahiel and co-workers in 1999.[9] It has a modular structure as shown in Figure 1a, which is closely similar to the surfactin synthetase. The thioesterase gene licC TE was amplified from the genomic DNA of *Bacillus licheniformis* ATCC 10716 and its product was expressed in *E. coli*. The overproduced functional domain was purified to homogeneity with a hexahistidine tag at the carboxy terminus. Its substrate (LPwt, Fig. 2) was chemically synthesized with (*R*)-3-hydroxyl butyric acid and *N*-acetylcysteaminyl (NAC) thioester as the surrogates of the β-hydroxyl fatty acid and phosphopantetheinyl linker, respectively, in the native substrate (Fig. 1b) of the enzyme.

Keywords: Thioesterase; Nonribosomal peptide synthetase; Lichenysin; Biosurfactin.
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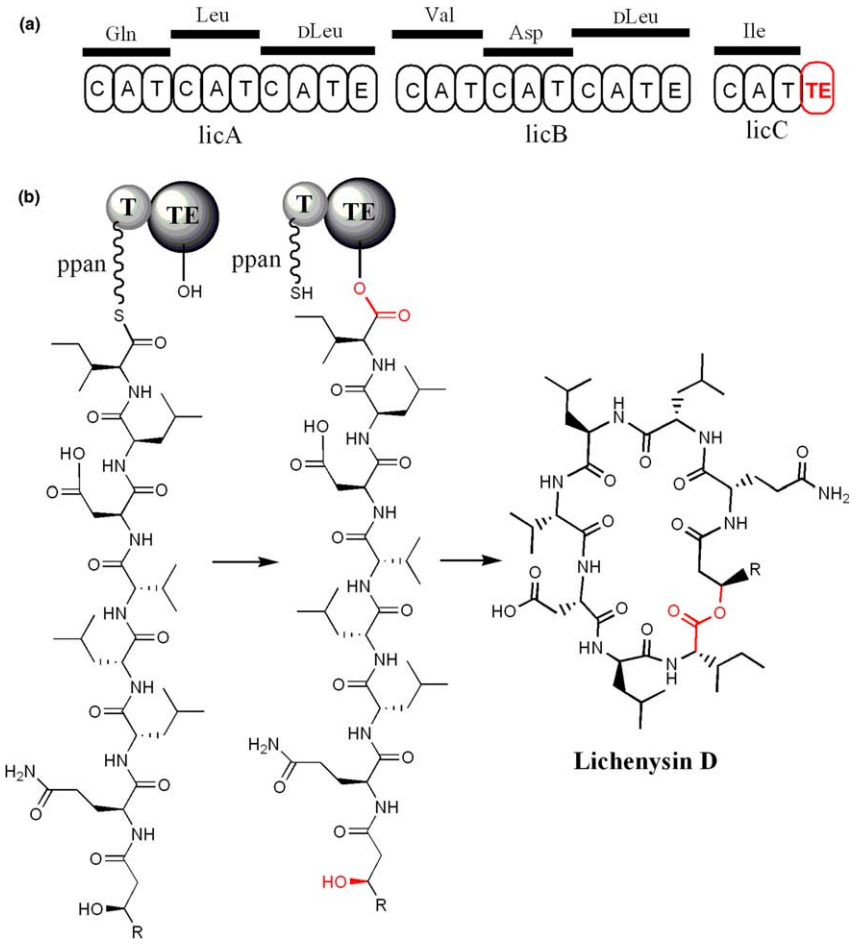
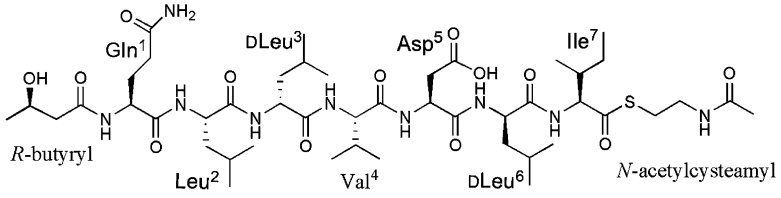
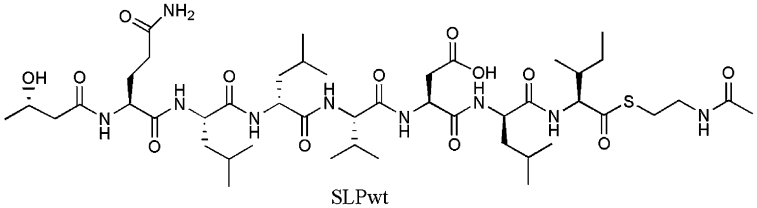


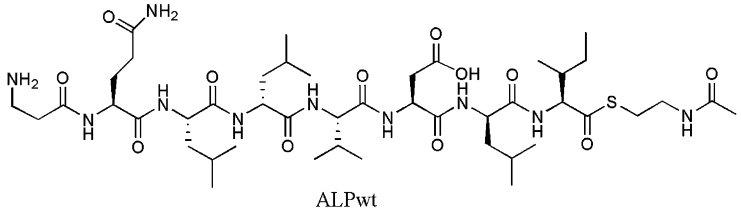
Figure 1. Modular nonribosomal peptide synthetase of lichenysin D (a) and the reaction catalyzed by the last thioesterase (licC TE) functional domain (b). Each module is comprised of condensation (C), adenylation (A), thiolation (T), and epimerization (E) domains and responsible for activation, attachment, and modification of one constitutive amino acid residue. ppan = phosphopantetheinyl linker.



LPwt: no substitution; LP1: Gln^1 = Ala; LP2: Leu^2 = Ala; LP3: $DLeu^3$ = DAla;
LP4: Val^4 = Ala; LP5: Asp^5 = Ala; LP6: $DLeu^6$ = DAla; LP7: Ile^7 = Ala



SLPwt



ALPwt

Figure 2. The structures of the lipopeptide-SNAC with a native substrate sequence and its analogues.

Initial activity test of licC TE toward the substrate showed that the enzyme is active, forming two products as shown in Figure 3A, whereas the substrate was recovered in a control reaction without the enzyme. LC–ESIMS analysis of the enzymatic reaction mixture showed that the two product peaks at 25.17 min and 27.30 min are the hydrolysis and correct cyclization products, respectively. Ratio of the cyclic to hydrolytic product was determined to be 1.07. Using a one-point HPLC kinetic assay similar to the previous studies,[4b,c] a typical Michaelis–Menton kinetic behavior was found for both the enzymatic hydrolysis (K_M of 8.13 ± 0.61 μM and a k_{cat} of 94.49 ± 5.15 min^{-1}) and macrolactonization (K_M of 6.83 ± 0.37 μM and a k_{cat} of 106.19 ± 5.29 min^{-1}). In comparison to the cyclization reaction catalyzed by the surfactin thioesterase (Srf TE) toward substrate as an NAC thioester (k_{cat}/K_M = 0.86 mM^{-1} min^{-1}, cyclic to linear product ratio = 2.5)[8] or a thiophenol ester (k_{cat}/K_M = 90 mM^{-1} min^{-1}, cyclic to hydrolytic product ratio = 0.78),[10] licC TE has a much lower K_M value and a signficantly higher k_{cat} for the cyclization reaction, with at least 346-fold increase in catalytic efficiency (k_{cat}/K_M = 15.56 ± 0.21 μM^{-1} min^{-1}). This high catalytic efficiency for licC TE is comparable to that of the tyrocidine and gramicidin thioesterases (k_{cat}/K_M = 20 μM^{-1} min^{-1} and 2.4 μM^{-1} min^{-1}, respectively).[4a] These results indicate that the chain length of the β-hydroxy fatty acid (R group, Fig. 1b) might has a little effect on the substrate recognition and catalytic activities of the thioesterase and that NAC is a sufficient mimic of the phosphopantetheinyl tether linking the lipopeptide to the peptidyl carrier protein (or thiolation domain, Fig. 1b).

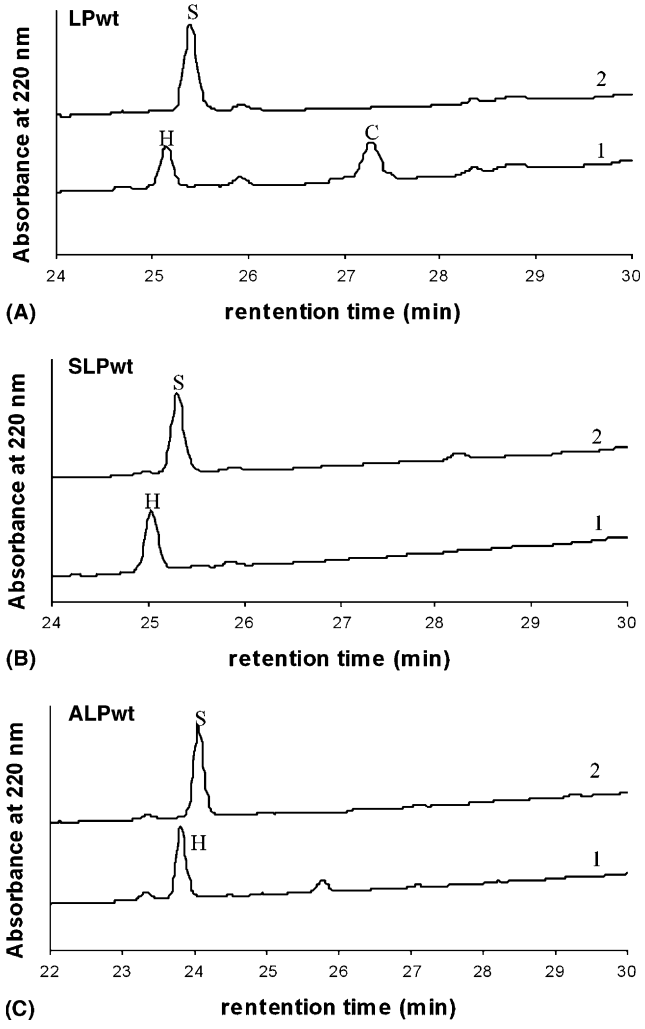


Figure 3. HPLC chromatograms of turnover products of the substrates LPwt (A), SLPwt (B), and ALPwt (C) by the thioesterase licC TE. Trace 1 was obtained from incubation of the thioesterase with the substrates for 2 h at room temperature, whereas trace 2 was obtained from incubation of the substrates without the enzyme. S = substrate; C = cyclic product; H = hydrolytic product.

To explore the structural requirement on the β-hydroxy fatty acid of the linear substrate for the thioesterase activity, the configuration on the fatty acid was changed from *R* to *S* and the hydroxyl group was substituted with an amine group in the β-alanine substitution of the β-hydroxy fatty acid. The substrate analogues, SLPwt and ALPwt (Fig. 2), were similarly synthesized by a solid-phase method. Characterization data of the analogues and their turnover products by licC TE are summarized in Table 1 along with that for the wild type substrate LPwt and other analogues. As shown in Figure 3, HPLC analysis revealed that cyclization products of the linear precursors SLPwt and ALPwt were not formed in incubation with the thioesterase licC TE but the hydrolytic products were readily formed in both cases, whereas no reaction was detected in control experiments without the enzyme.

The enzymatic kinetic parameters were similarly determined for the hydrolysis of both SLPwt (K_M = 11.95 ± 0.20 μM, k_{cat} = 233.17 ± 5.02 min^{-1}, k_{cat}/K_M = 19.49 ± 0.09 μM^{-1} min^{-1}) and ALPwt (K_M = 9.41 ± 0.41 μM, k_{cat} = 162.65 ± 5.22 min^{-1}, k_{cat}/K_M = 17.29 ± 0.22 μM^{-1} min^{-1}). In comparison to the enzymatic hydrolysis of the wild type substrate LPwt, the K_M values for hydrolysis of both analogues were little changed whereas the k_{cat} values were significantly increased. These results show that the enzymatic cyclization of the linear precursors has a highly stringent requirement on the stereochemistry and the functional hydroxyl group in the β-hydroxy fatty acid, whereas both structural elements in the fatty acid contribute little to binding of the linear precursors and formation of acyl-*O*-TE intermediate (Fig. 1b) by the enzyme. The failure of the thioesterase to cyclize the lipopeptide SLPwt in the formed acyl-*O*-TE intermediates may be due to the mis-orientation of the hydroxyl nucleophile caused by the *R* to *S* configuration change. On the other hand, no enzymatic cyclization of the ALPwt analogue may stem from the absence of a general base to deprotonate the positively charged *N*-terminal amine group or the probable misplacement of the *N*-terminal amine from an appropriate attacking position on the carboxy terminal thioester because of its positive charge, which is absent in the native substrate.

To find the influence of the side chains on the linear substrate on the catalytic efficiency of licC TE, the

Table 1. Characterization of the linear lichenysin D substrate NAC thioesters and their turnover products by the thioesterase licC TE

Entry	Substrates			Cyclic product			Hydrolytic product			C/H ratio[a]
	t_R (min)[b]	Calcd mass	$[M+H]^+$	t_R (min)	Calcd mass	$[M+H]^+$	t_R (min)	Calcd mass	$[M+H]^+$	
LPwt	25.40	999.58	1000.24	27.30	880.53	881.35	25.17	898.54	899.28	1.07
SLPwt	25.32	999.58	1000.29	nd	880.53	nd	25.03	898.54	899.22	0
ALPwt	24.07	984.58	985.38	nd	865.53	nd	23.82	883.54	884.13	0
LP1	25.90	942.55	943.17	28.05	823.51	824.30	25.65	841.52	842.13	0.24
LP2	23.88	957.53	958.21	24.75	838.48	839.26	23.43	856.49	857.32	0.70
LP3	23.15	957.53	958.18	25.05	838.48	839.26	22.78	856.49	857.03	0.68
LP4	24.43	971.54	972.26	25.98	852.50	853.28	24.15	870.51	871.20	0.43
LP5	24.98	955.59	956.16	nd	836.54	nd	24.70	854.55	855.18	0
LP6	23.18	957.53	958.18	24.25	838.48	839.29	22.80	856.49	857.17	1.96
LP7	22.95	957.53	958.12	nd	838.48	nd	22.57	856.49	857.11	0

[a] Ratio of the cyclic product to the hydrolytic product (nd = not detected).

[b] Retention time of the compounds in HPLC analysis with a reverse-phase XTerra™ MS C_{18} column (5 μm, 3.9 × 150 mm) under the following conditions: 0% acetonitrile in water over 5 min, 0–80% acetonitrile in water over 20 min, and then 80–100% acetonitrile in water over 10 min at a flow rate of 1 mL min^{-1}.

constituent amino acids were sequentially substituted with alanine, without changing the configuration of the α-carbon, to obtain a series of substrate analogues as shown in Figure 2. HPLC and LC–MS characterization data of the synthetic analogues and their turnover products by licC TE are summarized in Table 1. The kinetic parameters (k_{cat} and K_M) for these alanine analogues were determined using the same method for the wild type substrate LPwt and plotted in Figure 4a and b for the hydrolytic and cyclization activities of licC

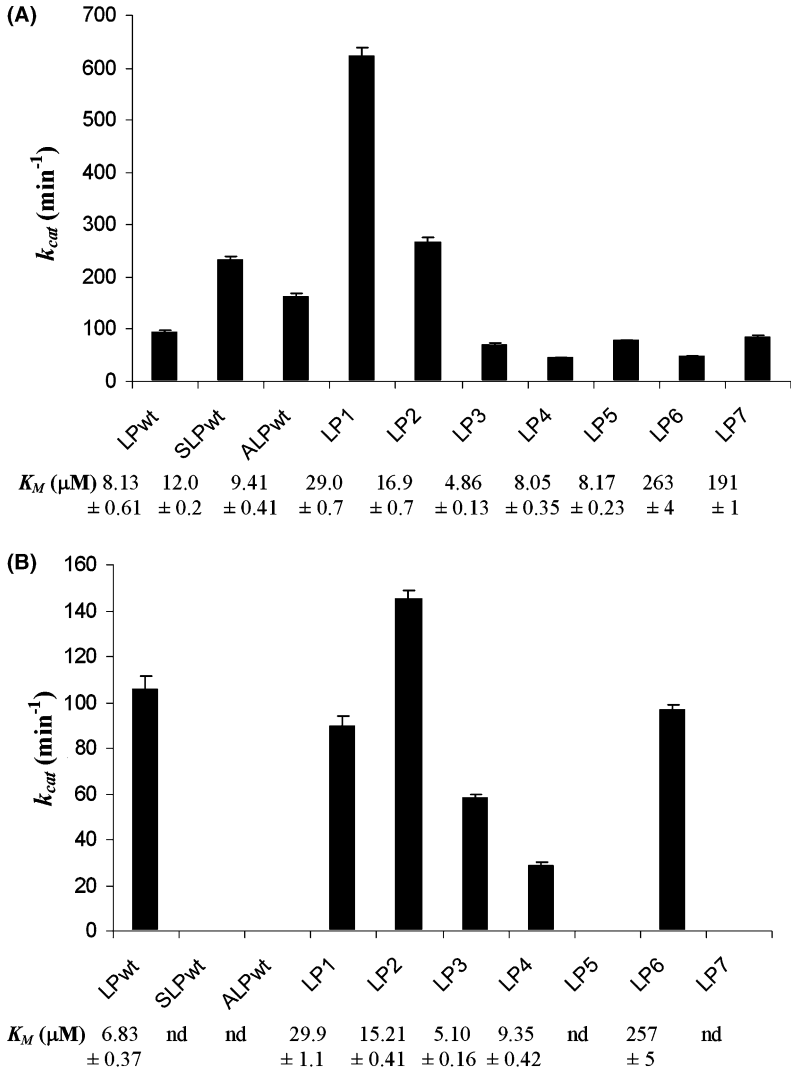


Figure 4. Kinetic parameters for the hydrolysis (A) and cyclization (B) of the lipopeptide-SNAC by the licC TE thioesterase. nd = not determined.

TE, respectively. From this figure, it is obvious that change of Ile[7] or Asp[5] into an alanine results in no cyclic products and the side chains of both residues are essential for the cyclization activity of the thioesterase. However, substitution of other residues by an alanine has a limited effect on the k_{cat} for the cyclization reaction, with a moderate reduction of up to 3.7-fold (for the substitution of Val[4]). In contrast, the turnover rate constant (k_{cat}) for the hydrolytic reaction is little affected or even increased by the alanine substitutions, ranging from a reduction of up to 2.1-fold (for the substitution of Val[4]) to an increase of up to 6.6-fold (for the substitution of Gln[1]). These results for the hydrolytic reactions show that the acyl-*O*-TE intermediate is readily formed in all cases and its formation is little affected by the side chains of the amino acid residues on the linear substrate precursors. Examining the K_M values for both the enzymatic hydrolysis and cyclization reactions (where appropriate) of the alanine analogues (Fig. 4), it can be found that the binding constant of substrate by licC TE, which is approximately equal to K_M, is little affected by alanine substitutions of Leu[2], D-Leu[3], Val[4], and Asp[5], whereas the binding is significantly weakened by alanine substitution at Gln[1], D-Leu[6], and Ile[7] with an increase of K_M of 3.6-, 32.3-, and 23.4-fold, respectively, in comparison to that of the native substrate LPwt.

From the characterization of the undesired hydrolysis, it can be concluded that formation of enzyme–substrate complex is little affected by most structural elements of the substrate except the side chains of Gln[1], D-Leu[6], and Ile[7], in the licC TE-catalyzed reactions. The reactive acyl-*O*-TE intermediate is readily formed for all substrate analogues, independent of all the tested structural elements. However, the enzymatic macrolactonization has more stringent requirements on the substrate. The configuration of the β-hydroxy fatty acid, its hydroxyl nucleophile, and the side chains of Ile[7] and Asp[5] are essential to the cyclization reaction. This indicates that the orientation of the nucleophile is important to the cyclolactonization and significantly influenced by the nature and absolute configuration of the hydroxyl nucleophile. The side chains of Ile[7] and Asp[5] may facilitate the nucleophile to achieve the appropriate attacking position through interactions with the licC TE active site residues. Change of these structural features on the substrate will result in no cyclization reaction. On the other hand, the licC TE-catalyzed cyclization shows considerable tolerance to changes in the side chains of the remaining residues, namely D-Leu[6], Val[4], D-Leu[3], and Gln[1]. Considering both the high catalytic efficiency and flexibility on a large portion of the substrate, the excised licC TE may be utilized to access novel structures through a chemoenzymatic method developed for other NRPS thioesterases.[4]

In summary, we found that the lichenysin NRPS thioesterase licC TE is a highly efficient enzyme. The essential structural elements of the substrate were determined for the catalytic efficiency, substrate binding, and partition between hydrolytic and cyclization reactions of this thioesterase. The high catalytic efficiency and substrate flexibility of licC TE will allow its utilization in generation of cyclic lipopeptides, which are a distinct class of cyclodepsipeptides with exceptional biosurfactant activities and industrial significance. In addition, the defined substrate specificities of the terminal thioesterase will facilitate future modification of the lichenysin NRPS to produce altered lipopeptide structures of industrial interests.

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