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Subtiligase as a hydrothiolase for the synthesis of peptide thioacids

Xiao-Hong Tan, Renliang Yang, Andre Wirjo, Chuan-Fa Liu *

Division of Chemical Biology and Biotechnology, School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551, Singapore

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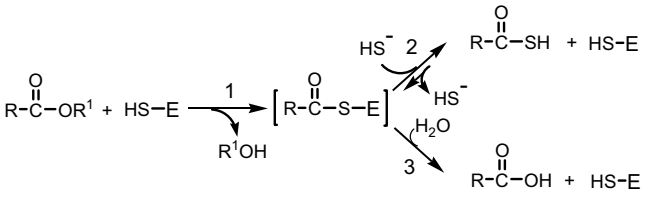
Abstract

Subtiligase catalyzes the hydrolysis or the aminolysis of a peptide glycolate ester substrate via an acyl-enzyme thioester intermediate. We show that this intermediate can be intercepted by a hydrosulfide ion to generate a peptide thioacid as the hydrothiolysis product. Also shown is the use of the so-prepared peptide thioacids in mini thiol capture ligation.

Enzyme engineering is a powerful approach to the generation of new biocatalysts for organic synthesis. Through site-directed mutagenesis, enzyme structure can be changed in a rational way to confer a desirable property to the engineered enzyme, such as altered substrate specificity, improved catalytic efficiency, enhanced stability, or even the activity to catalyze a new chemical reaction.[1] The design of subtiligase by Wells et al. is a true demonstration of how rational protein engineering can be employed to convert a normally destructive protease to a synthetically useful peptide ligase.[2,3] Subtiligase is a double mutant of subtilisin BPN', in which the active site residue Ser221 is mutated to Cys and the Pro225 residue to Ala. This double mutation reduces effectively the amidase activity of subtiligase while maintaining a significant level of esterase activity. More importantly, the aminolysis/hydrolysis ratio is improved dramatically presumably because the soft electrophilic thioester acyl-enzyme (acyl-E) intermediate prefers a softer amine nucleophile over water in the deacylation step, making it an effective catalyst for peptide bond formation.[2] The synthetic value of subtiligase was highlighted in the total synthesis of a functionally active ribonuclease A analog.[4] Interestingly, it was shown recently that subtiligase could also catalyze the thiolysis of peptide esters for the formation of peptide thioesters in an ester-to-thioester transesterification reaction.[5] By performing the reaction under a kinetically-controlled mechanism, a number of peptide thioesters of various length and amino acid composition were prepared from their glycolate ester substrates in reasonably good yield.[5] In this Letter, we show that subtiligase can also function as a hydrothiolase to catalyze the hydrothiolysis of a suitable peptide ester with hydrosulfide anions. This reaction is based on the similar rationale that being a soft base, the hydrosulfide ion would react effectively with and accept the acyl group from the soft-acidic acyl-E thioester intermediate, leading to direct formation of a peptide thioacid (Scheme 1).

We first investigated the peptide thioacid formation in a model system with the glycolate ester peptide substrate, Ac-His-Ala-Ala-Pro-Phe-glcFG-NH_2. Several sulfide compounds were tested, including H_2S dissolved in water,



Scheme 1. Thiolysis catalyzed by subtiligase converting a peptide C^{α}-ester to thioacid. R = peptide chain. HS-E = subtiligase. R^1 = –CH_2CO–Phe–NH_2.

* Corresponding author. Tel.: +65 63162867; fax: +65 6791 3856.
E-mail address: cfliu@ntu.edu.sg (C.-F. Liu).

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Na_2S, NaSH, and $(NH_4)_2S$. Of these, $(NH_4)_2S$ was found to be the most convenient to use and gave the best results. It was possible to prepare a $(NH_4)_2S$ solution with high enough effective [HS^-] at pH 7–9 without too much escape of H_2S from the solution. Figure 1 shows catalytic thioacid formation at pH 8 through the hydrothiolysis of Ac-His-Ala-Ala-Pro-Phe-glcFG-NH_2 by $(NH_4)_2S$ as compared to the thioester formation of the same substrate at the same pH with mercaptoacetic acid (MAA).

As one can see from Figure 1, the reaction rates are comparable for thioacid and thioester formation reactions. For example, after 20 min, the starting material, Ac-His-Ala-Ala-Pro-Phe-glcFG-NH_2, was completely consumed in both the reactions. However, there was more hydrolysis in the thioacid formation reaction than in the thioester formation reaction. It was further found that the hydrolysis product mainly resulted from the direct hydrolysis of the ester substrate Ac-His-Ala-Ala-Pro-Phe-glcFG-NH_2, and not from the secondary hydrolysis of the thioacid or thioester product. We made this observation by conducting a separate subtiligase-catalyzed hydrolysis assay on the ester substrate Ac-His-Ala-Ala-Pro-Phe-glcFG-NH_2, the thioacid Ac-His-Ala-Ala-Pro-Phe-SH, and thioester Ac-His-Ala-Ala-Pro-Phe-SCH_2COOH, respectively, in 0.1 M sodium phosphate buffer (pH 8) without any thiol compound but containing 20 mM TCEP, and we found that the latter two were quite stable with less than 10% hydrolysis after 20 min, while the glycolate ester peptide was completely hydrolyzed (data not shown). It is worth noting that the MAA thioester was more stable toward enzymatic hydrolysis than a similar thioester product formed with *N*-acetylcysteamine (ACA) and that more hydrolysis product was formed during the thioester formation reaction with MAA than with ACA.[5] This may be due to the presence in MAA of a negatively charged α-carboxylate. The increased hydrolysis during hydrothiolysis with the HS^- ion may be due to its poorer ability to enter the active site of the enzyme than other alkyl thiols. Still, it is remarkable that, at a very low molar ratio to water molecules, the hydrosulfide anion is rather effective in engaging the acyl-E intermediate at the deacylation step (Scheme 1, step 2).

Subsequently, we examined the influence of pH on the thioacid formation reaction. Three different pH values, pH 7.2, 8.2 and 8.8, were chosen and the results are shown in Figure 2. Having a pH below neutral was found impractical for maintaining a high enough thiol SH^- concentration owing to escape of H_2S. A pH that was too high (>9) would be deleterious to the enzyme and also lead to more hydrolysis.

As seen from Figure 2, of the three pH values, pH 7.2 gave the slowest reaction. Reactions at pH 8.2 and 8.8 were much faster, and pH 8.8 gave a slightly faster reaction as judged by the consumption rate of the substrate. However, a slightly higher hydrolysis-to-hydrothiolysis ratio was observed at pH 8.8. For this reason, pH 8.2 was used in subsequent experiments. One should also note that a substantial amount of hydrolysis product was formed at all three pHs and that lowering the pH to 7 did not improve the thiolysis/hydrolysis ratio. This may be due to the fact that, at low pH, not only is it difficult to achieve a high overall thiol concentration, but the effective concentration of the hydrosulfide ion is also greatly reduced. Again, the peptide acid (peak a) appears to be the result of direct hydrolysis of the peptide ester as the ratio of thiolysis/hydrolysis remained almost unchanged over the reaction course in each case. From these results, one may conclude that the effective concentration of the hydrosulfide nucleophile is crucial in determining the rate and hydrothiolysis/hydrolysis ratio of the enzymatic reaction.

Mechanistically, thioacid formation is similar to thioester formation. However, some profound differences are expected between the two, mainly because of the unique properties of H_2S which, as a gas, has limited aqueous solubility. Its dissociation constants (pK_{a1} 6.9 and pK_{a2}

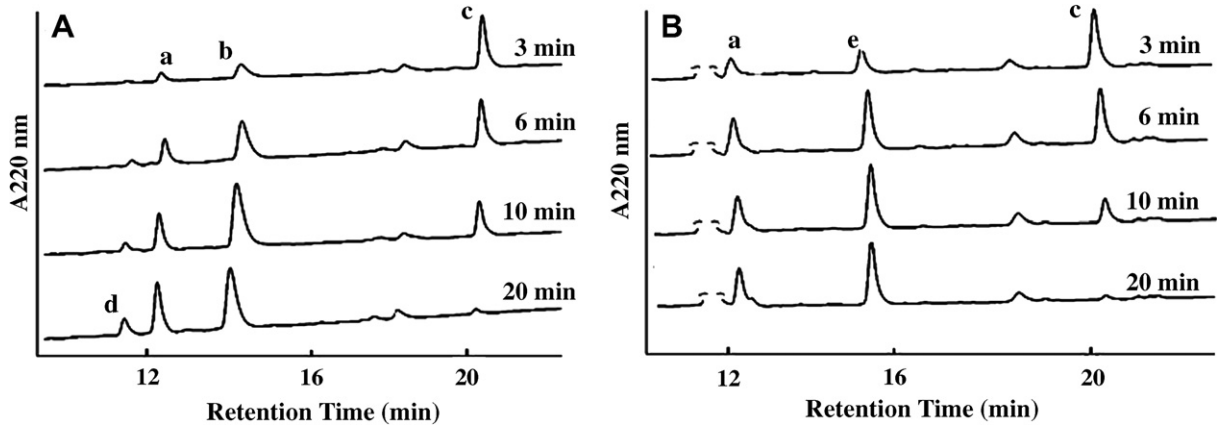

Fig. 1. HPLC monitoring of subtiligase-catalyzed hydrothiolysis of Ac-**HAAPF**-glcFG-NH_2 by 0.2 M $(NH_4)_2S$ (panel A) and thiolysis by 0.2 M MAA (panel B). Peak a: hydrolysis product, Ac-**HAAPF**-OH (m/z $[M+H]^+$ found: 584.5, MW calcd: 583.6); peak b: the newly formed thiol-acid product, Ac-**HAAPF**-SH, (m/z $[M+H]^+$ found: 600.4, MW calcd: 599.6); peak c: the ester starting material, (m/z $[M+H]^+$ found: 845.1, MW calcd: 844.4) and peak e: the newly formed thioester product, Ac-**HAAPF**-SCH_2COOH, (m/z $[M+Na]^+$ found: 680.0 found, MW calcd: 657.0). Peak d: identity unknown (m/z found: 680.4). See Supplementary data for HPLC conditions and relative peak integrations of the hydrothiolysis product.

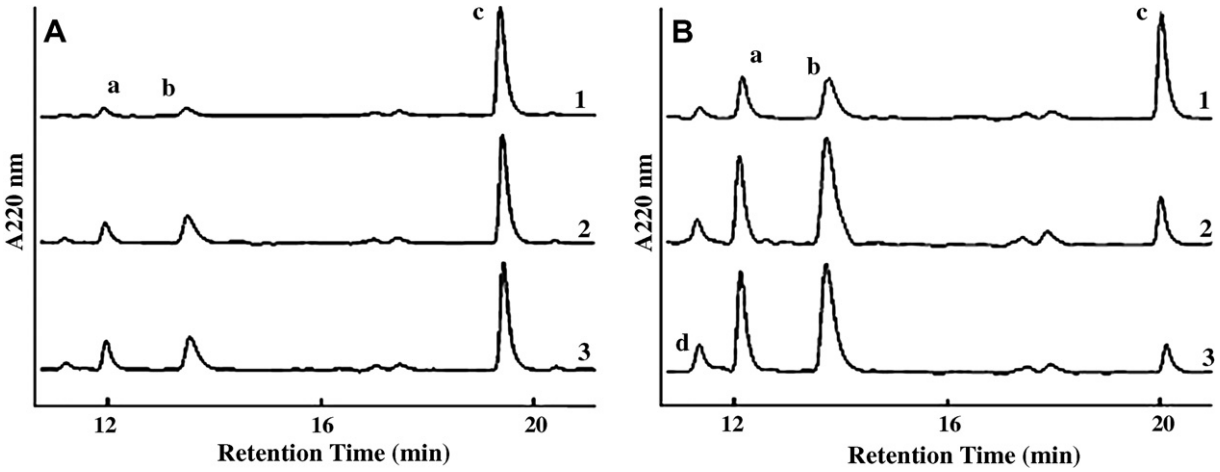

Fig. 2. HPLC monitoring of subtiligase-catalyzed thiolysis of Ac-**HAAPF**-glcFG-NH_2 using *ca.* 0.36 M of $(NH_4)_2S$ for 2 min (panel A) or 8 min (panel B) at different pH values: trace 1 for pH 7.2; trace 2 for pH 8.2 and trace 3 for pH 8.8. Peak a: hydrolysis product; Peak b: Ac-**HAAPF**-SH; and peak c: starting material. Peak d: identity unknown (*m/z* found: 680.4). See Supplementary data for HPLC conditions and relative peak integrations of the hydrothiolysis and hydrolysis products.

$\sim$17.1)[6] also dictates that it would exist mostly in the H_2S form at neutral to acidic pH and as HS^- ions under alkaline pH. Previously, we had found that the optimal pH for thioester formation with simple alkyl thiols was weakly acidic, which minimized the competing hydrolysis reaction.[5] These conditions did not seem to be applicable to thioacid formation due to unavoidable escape of H_2S gas from the reaction medium at low pH. Therefore, the hydrothiolysis reaction must be performed under alkaline pH. Also, the thioacid product, once formed, seemed to be rather stable toward enzymatic hydrolysis, probably because the thioacid group, deprotonated at the pH of the reaction milieu and without the P′ components, would not be recognized by the enzyme as a substrate.

We also studied the effect of thiol concentration on thioacid formation conducted with $(NH_4)_2S$ at pH 8.2. Within the range of the effective thiol concentration tested, which ranged from 0.05 to 0.3 M, we found that the reaction rate and yield of thioacid increased with the increase of effective $[HS^-]$.[7] For example, at $[HS^-] = 0.15$ M, $\sim$15% of thioacid was obtained after 5 min of reaction, whereas at $[HS^-] = 0.3$ M, nearly 40% of the thioacid was formed (see Supplementary data). However, it became difficult to achieve and maintain an effective thiol concentration above 0.4 M without increasing the pH and/or buffer capacity, because of the rather basic nature of $(NH_4)_2S$ and the loss of H_2S that would result from pH adjustment.

To further demonstrate the synthetic value of subtiligase-catalyzed hydrothiolysis for thioacid synthesis, we prepared three other peptide substrates with the glc ester linkage at the C-termini and tested them for thioacid formation (Table 1).

The peptides listed in Table 1, plus the model peptide ester Ac-**HAAPF**-glcFG-NH_2, contain a diverse set of amino acid residues in the sequences. All these peptide esters were substrates of subtiligase, which gave their corresponding thioacids in moderate yields. No intermolecular or intramolecular self-ligation was detected even though the N-terminus of one peptide (peptide **2**) was not protected. Except for the competing hydrolysis reaction, the ester-to-thioacid conversion proceeded smoothly and cleanly without other detectable side reactions. A representative HPLC trace showing the conversion of the glc ester peptide **2** to its thioacid at pH 8.2 is presented in the Supplementary data.

We also showed that the thioacid products prepared by this enzymatic method were useful building blocks for mini thiol capture ligation.[8] This ligation scheme, so named

Table 1
Thioacid formation of other glycolate ester peptides

N	Sequence	Yield (%)	T/H[a]	MW[b] calcd	*m/z*[b] Found
1	Ac-KPGTVA	57	3:1 (76%)	629.7	630.4
2	TKGSAYSGKLEEFVQ	44	1:1 (87%)	1659.8	1660.6
3	Ac-KVLPNIQ	56	2.6:1 (77%)	869.0	869.6

All peptides were C-terminal esters of glcFG-NH_2.

[a] T/H: thiolysis/hydrolysis ratio; values in brackets indicate the amount of ester peptide consumed.

[b] Calculated molecular weight and found ($[M+H]^+$) *m/z* value of the corresponding thioacid products. The thiol solution contained 0.2 M $(NH_4)_2S$ and 0.2 M TCEP in 0.58 M sodium phosphate buffer, pH 8.2. Reaction conditions: peptide **1**—1 μl of 2.5 mM substrate, 0.6 μl of 45 μm subtiligase, 0.4 μl of H_2O and 18 μl of reaction solution, 15 min; peptide **2**—0.5 μl of 5 mM substrate, 1.5 μl of 45 μm subtiligase, 18.0 μl of reaction solution, 3 h; peptide **3**—1 μl of 2.5 mM substrate, 0.3 μl of 45 μm subtiligase, 0.7 μl of H_2O, 18 μl of reaction solution, 11 min.

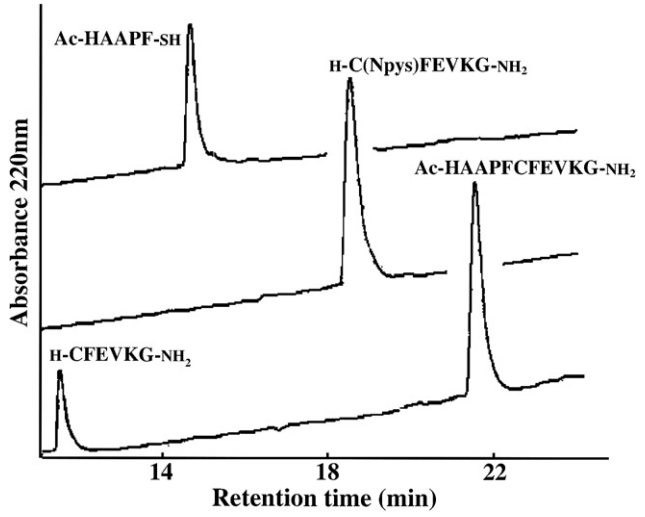

Fig. 3. HPLC monitoring of the ligation reaction between Ac-**HAAPF**-SH and H-**C**(Npys)**FEVKG**-NH_2. Upper trace: thiol-acid peptide; Middle trace: H-**C**(Npys)**FEVKG**-NH_2 (*m*/*z* $[M+H]^+$ found: 835.3, MW calcd: 835.1 calcd); Lower trace: TCEP-treated ligation mixture after 5 min. The peak at 21.7 min is the ligation product, Ac-**HAAPFCFEVKG**-NH_2 (*m*/*z* $[M+H]^+$ found: 1246.5, MW calcd: 1246.2) while the 12.3 min peak is H-**CFEVKG**-NH_2 resulting from the TCEP reduction of remaining H-**C**(Npys)**FEVKG**-NH_2.

after Kemp's prior thiol-capture strategy,[9] relies on specific capture of a thioacid sulfhydryl from the acyl peptide component by an Npys-modified N-terminal Cys residue of the amine peptide component to bring the two peptide components together through a covalent acyl disulfide linkage which undergoes rapid intramolecular *S*,*N*-acyl transfer via a 6-membered ring intermediate, leading to the formation of an amide bond.[8] Therefore, Ac-His-Ala-Ala-Pro-Phe-SH, the thioacid product of Ac-His-Ala-Ala-Pro-Phe-glcFG-NH_2, was tested for ligation with H-Cys(Npys)-Phe-Glu-Val-Lys-Gly-NH_2 (in 3 times molar excess) by using the mini thiol capture ligation method, which gave an almost quantitative yield of ligation product after 5 min of reaction (Fig. 3).

We have demonstrated herein that subtiligase is a viable catalyst to convert a peptide ester to a thioacid in aqueous media under conventional enzyme catalysis conditions. Chemically, preparing peptide thioacids remains a challenging task, and the methods that have proven practically useful are all based on the original benzhydryl linker approach developed by Blake et al.,[10,11] which requires the use of Boc-chemistry SPPS, and therefore hazardous hydrogen fluoride for final deprotection and cleavage. Since the glycolate ester peptides can be easily synthesized using Fmoc-chemistry SPPS[12] and subtiligase has a relatively broad substrate specificity,[2] this enzymatic hydrothiolysis reaction represents a useful alternative for the synthesis of peptide thioacids. To the best of our knowledge, no such enzymatic methods have ever been reported for the preparation of any thioacid compounds. Subtiligase was originally designed as a peptide ligase for peptide bond formation; the results presented here and published previously[5] show that it can also function as a hydrothiolase or thiolase for thioacid or thioester formation. Although many enzyme redesign examples have been reported wherein the active site of an enzyme is reengineered to catalyze a new chemical reaction,[1] it is uncommon that the same reengineered enzyme functions to catalyze multiple reactions. The products of the enzymatic hydrothiolysis reaction reported herein are the difficult to synthesize peptide thioacids, which again illustrates the power and versatility of enzymes as biocatalysts for organic transformations.

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Supplementary data

Instrumentation, materials, peptide synthesis data and detailed experimental procedures. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2008.03.028.

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