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Thiolsubtilisin acts as an acetyltransferase in organic solvents

Dar-Fu Tai[a,*], Wen-Chen Liaw[b]

[a] *Department of Chemistry, National Dong-Hwa University, Hualien, Taiwan*
[b] *Department of Chemical Engineering, National Yunlin University of Science and Technology, Yunlin, Taiwan*

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Abstract The catalytic mechanism of arylamine *N*-acetyltransferase has been proposed to involve Cys-His-Asp as its catalytic triad. Thiolsubtilisin, a chemically modified enzyme that has a catalytic triad of Cys-His-Asp at the active site, mimics the catalysis of arylamine *N*-acetyltransferase, serotonin *N*-acetyltransferase, histone *N*-acetyltransferase and amino acid *N*-acetyltransferase. Thiolsubtilisin not only can catalyze amino acid transacetylation, but is also able to catalyze amine transacetylation. Ethyl acetate was used as the acylating reagent to form *N*-acetyl amino acids and amines in organic solvents with moderate yield. Hence, these findings broaden our understanding of the structural features required for *N*-acetyltransferases activity as well as provide a structural relationship between cysteine protease and other *N*-acyltransferases.

Key words: Thiolsubtilisin; *N*-Acetyltransferase; Catalytic triad; Ethyl acetate; Transacetylation

1. Introduction

Thiolsubtilisin [1–5], an artificial thiol protease, is chemically modified from subtilisin by replacing serine-221 with cysteine at the active site [6]. The modification alters the catalytic triad of the enzyme from Ser-His-Asp to Cys-His-Asp. The kinetic specificity of subtilisin and thiolsubtilisin has been compared in aqueous solutions [7,8] as well as in organic solvents [9]. Thiolsubtilisin catalyzed alcoholysis in a variety of esters [9], active ester [8] or peptide [7] hydrolysis and peptide synthesis [10,11]. The thiol group of thiolsubtilisin at the active center possesses a highly nucleophilic character, not only in the uncomplicated thiolate ion that predominates at high pH, but also in neutral and weakly acidic media [12,13].

Generally, acetyltransferase catalyzes acetylation through the transfer of an acetyl group from acetyl coenzyme A (AcCoA). The crystal structure of arylamine *N*-acetyltransferase reveals a cysteine protease-like mechanism [14,15]. The most important of these is the presence of the Cys-His-Asp catalytic triad. This cysteine residue has previously been shown to be acetylated to form an acyl-enzyme intermediate, followed by transferring the acetyl group to arylamine [16]. However, the catalytic mechanism of the other acetyltransferases is somewhat different and ambiguous. The structure of serotonin *N*-acetyltransferase suggests the formation of an acetyl-protein intermediate with one (His-122) or two (His-120 and -122) histidine residues [17]. On the other hand, histone *N*-acetyltransferase [18] catalyzes the acetylation of the ε-amino groups of specific lysine residues on the amino-terminal tails of the histone proteins. The catalysis is proceeded by forming a ternary complex with acetyl-CoA and histone, from which the ε-amino of Lys-14 subsequently attacks the carbonyl carbon of CoA directly to generate acetyl-histone. GNA1, a novel member of the Gcn5-related *N*-acetyltransferase superfamily, participates in UDP-GlcNAc biosynthesis by catalyzing the formation of GlcNAc6P from AcCoA and GlcN6P. The catalytic mechanism is unclear, a ternary complex with His residue was proposed [19,20].

We report herein a new method of using thiolsubtilisin to catalyze the acetylation of free amino acids and amines using transacetylation. The chemical modified enzyme mimics the catalysis of these acetyltransferases. This finding further highlights the importance of a Cys-His-Asp catalytic triad in the structure of an *N*-acetyltransferase. We also demonstrated the use of ethyl acetate to replace AcCoA as the acetyl donor and provide a simple technique to enzymatically protect the amino group [21]. Compared to chemical methods [22], this method has the advantages of applying the cheap, stable and safe reagent ethyl acetate. In addition, the immobilized thiolsubtilisin is reusable after an easy separation from the substrate and enzyme.

2. Materials and methods

2.1. Materials

Amino acids and their derivatives, 1-methyl-3-phenyl-propylamine, serotonin, methyltryptamine, 1-(1-naphthyl)ethylamine, *Aspergillus oryzae* protease (type XXIII) and most of enzymes were purchased from Sigma. Papain, isopropyl ether (IPE), 2-propanol, methanol, acetone, acetonitrile, ethyl acetate, ethanol and other solvents were obtained from Merck.

2.2. Preparation of thiolsubtilisin [5]

150 mg of subtilisin was dissolved in 4.5 ml of 0.05 M imidazole buffer, pH 7.4, containing 0.05 M $CaCl_2$. To this solution 150 μl of phenylmethanesulfonyl fluoride (20 mg/ml, in acetonitrile) was added and the reaction mixture was incubated at room temperature for 1 h. The phenylmethanesulfonated enzyme was treated with 450 mg sodium thiolacetate and incubated at 40°C for 20 h. The crude thiolsubtilisin was then gel-filtered on a Sephadex G-25 column equilibrated with 0.01 M phosphate buffer, pH 7.0, containing 1 mM EDTA and 0.07 M potassium chloride.

2.3. Preparation of an agarose mercurial column [23]

About 10 g of agarose was treated with 1 g of BrCN at pH 11 for 10 min. At the end of the reaction the agarose was washed with 200 ml of 0.1 M $NaHCO_3$ at pH 9.0 in a Buchner funnel and resuspended in 50 ml of 10% DMSO at 0°C. 0.32 g of *p*-aminophenylmercuric

*Corresponding author. Fax: (886)-3-8661487.
E-mail address: dftai@mail.ndhu.edu.tw (D.-F. Tai).

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Table 1
Transacylation of amines with different reagents[a]

Substrate	Reagent	Product	Conversion (%)
L-Leucinamide	Ethyl acetate	*N*-Acetyl-L-leucinamide	64.3
L-Leucinamide	Ethyl benzoate	*N*-Benzoyl-L-leucinamide	14.3
L-Leucinamide	Benzyl acetate	*N*-Acetyl-L-leucinamide	0
L-Leucinamide	Butyl acetate	*N*-Acetyl-L-leucinamide	0
L-Leucinamide	Isopropyl acetate	*N*-Acetyl-L-leucinamide	0
N^{α}-Cbz-L-lysine	Ethyl acetate	N^{α}-Cbz-N^{ε}-acetyl-L-lysine	72.1
N^{α}-Cbz-L-lysine	Ethyl benzoate	N^{α}-Cbz-N^{ε}benzoyl-L-lysine	13.2

[a]Immobilized thiolsubtilisin, 0.2 mmol of substrate, 1.8 ml of reagent and 0.2 ml of methanol were incubated at 37°C for 2 days.

acetate was dissolved in 5 ml of DMSO and added slowly. The suspension was stirred slowly and stored at 0°C for 20 h. After the period the suspension was warmed to 30°C and filtered. The agarose was resuspended in 20 ml of 20% DMSO solution at 35°C for 5 min and filtered off. The treatment was repeated four times. The material was packed into a column and slowly washed with 500 ml of 20% dimethylsulfoxide. The column was then washed with 0.1 M phosphate buffer pH 8.0.

2.4. Purification on an agarose mercurial column [5]

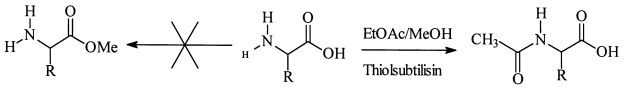
90 mg of thiolsubtilisin obtained by the procedure described above was applied to the mercurial column and washed with 15 ml of 0.01 M phosphate buffer, pH 7.0, containing 1 mM EDTA and 0.07 M potassium chloride. The flow rate through the column was 20 ml/h. The thiolsubtilisin retained by the column was eluted with 0.5 mM $HgCl_2$ in 0.01 M phosphate buffer, pH 7.0, containing 0.07 M potassium chloride. The resulting mercuri-thiolsubtilisin was activated with 0.1 volume of 0.05 M cysteine solution, pH 7.0, for 5 min at room temperature. The thiolenzyme free of mercury was isolated on a Sephadex G-25 column equilibrated with 0.01 M phosphate buffer, pH 7.0, containing 1 mM EDTA and 0.07 M KCl. The purified thiolsubtilisin was lyophilized and kept under nitrogen at 5°C.

2.5. Thiolsubtilisin-catalyzed transacetylation

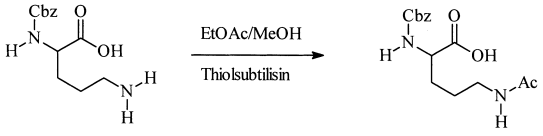
To a screw-cap vial of crude celite (0.1 g), 0.01 g of thiolsubtilisin and 0.1 ml of 3 M potassium citrate buffer, pH 6, were added. The mixture was incubated at 30°C with constant shaking for 30 min. Amino acid (0.2 mmol), 1.8 ml of ethyl acetate and 0.2 ml methanol were added to the vial and incubated at 37°C for 2 days. Adding 2 ml of 1 M HCl solution followed by 1 ml of ethyl acetate terminated the reaction. The analyses were carried out by withdrawing 0.05 ml from the ethyl acetate layer of each sample and subjecting to high-performance liquid chromatography.

3. Results and discussion

We initially used immobilized thiolsubtilisin to catalyze the esterification of amino acids and their derivatives in non-aqueous solvents [24,25], but the result was not promising. However, this reaction generated acetylated amino acid as a side product (Scheme 1). We therefore turned our attention to investigate this phenomenon.



Scheme 1.



Scheme 2.

Several esters were used to examine their potential as a substitute for AcCoA in transacetylation. Ethyl acetate or ethyl benzoate were able to serve as the acylating reagent with methanol at a ratio of 9:1 (e.g. nine parts of ethyl acetate and one part of methanol), while butyl acetate, benzyl acetate and isopropyl acetate failed (Table 1). Ethyl acetate is the best ester to cooperate with thiolsubtilisin. It is worthwhile to note that acetylation of the ε-amino group of a lysine derivative (Scheme 2) was executed with a good yield (Table 1). This catalytic behavior is similar to that of a histone *N*-acetyltransferase [18].

Several solvents were tested for promoting transacetylation, including chloroform, dichloromethane, IPE, acetone, acetonitrile, methanol, ethanol, propanol and butanol. Among these solvents, methanol (at the ratio of 3:7 to ethyl acetate) was able to facilitate dissolving amino acid to afford the best result (Table 2). A higher ratio of methanol ($>30\%$) inhibited the enzyme and lowered the transacetylation yield. The suitable pH for acetylation is 6–6.5. We also examined several proteases, i.e. papain, bromelain, subtilisin, *A. oryzae* protease, and ficin, using the same conditions as tryptophan acetylation, but no acetylation occurred. Subtilisin catalyzed acylation [26] and resolved the amines [27] but it required using an active ester. It appeared that thiolsubtilisin has a unique character among organic solvents. As shown in Table 2, several amino acids were converted to their acylated product.

Although the yield was not high, thiolsubtilisin also catalyzed amine transacetylation in a less polar solvent. The results in Table 3 show that the substrate specificity of thiolsubtilisin is broad upon amine acetylation. Thiolsubtilisin not only catalyzed the transacetylation in ethyl acetate as a serotonin *N*-acetyltransferase [17] to obtain *N*-acetyl-serotonin (Table 3), but also demonstrated the catalytic activity of an

Table 2
Transacetylation of amino acids[a]

Amino Acid	EtOAc/MeOH (v/v)	Conversion (%)
L-Ala	9/1	14
L-Asp	9/1	22
L-Glu	9/1	41
L-His	9/1	5
L-Phe	9/1	22
L-Trp	9/1	17
L-Trp	8/2	24
L-Trp	7/3	37
L-Tyr	9/1	15
L-Val	9/1	0

[a]Immobilized thiolsubtilisin, amino acid (0.2 mmol), 2 ml of a mixed solvent of ethyl acetate and methanol were incubated at 37°C for 2 days.

Table 3
Transacetylation of amines[a]

Amine	Solvent	Solvent ratio (v/v)	Conversion (%)
Glycinamide	EtOAc		28.7
L-Phenylalaninamide	EtOAc		17.0
Serotonin	EtOAc		15.1
Methyltryptamine	EtOAc		10.3
1-(1-Naphthyl)ethylamine	EtOAc/IPE	1:1	10.0
1-Methyl-3-phenyl-propylamine	EtOAc/IPE	1:1	18.3

[a]Immobilized thiolsubtilisin, amine (0.2 mmol), 2 ml of ethyl acetate or a mixed solvent of ethyl acetate and IPE were incubated at 37°C for 2 days.

arylamine *N*-acetyltransferase [14,15] to acetylated 1-(1-naphthyl)ethylamine (Table 3).

In conclusion, we report for the first time an immobilized thiolsubtilisin-catalyzed *N*-transacetylation in organic solvents. It diminished the barrier between a cysteine protease and an acetyltransferase. We demonstrated that a chemical modified protease with a Cys-His-Asp triad could act as an *N*-acetyltransferase. Therefore, it is necessary to reexamine the possibility of this catalytic mechanism for some *N*-acetyltransferases, as well as other *N*-acyltransferases. Generating this kind of catalytic triad might facilitate producing an artificial cysteine protease or an *N*-acetyltransferase.

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