# 論文の内容の要旨

# Development of chemical post-translational modification reactions on azoline-containing peptides expressed in a reconstituted cyclodehydratase-coupled translation system (試験管内生合成されたアゾリン含有ペプチドの化学的翻訳後修飾反応の開発)

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**Introduction**

Backbone modifications such as azoles, azolines and azolidines are often seen in bioactive peptidic natural products (**Figure 1**). Since these heterocyclic structures would provide peptides with several characteristics such as rigid global conformation, hydrogen bonding sites with fixed orientations, improved proteolytic resistance, and cell membrane permeability, they are important motifs of the bioactive peptides and the backbone modification would be the general strategy for organisms to develop a wide variety of bioactive peptides.

Our laboratory has previously developed an *in vitro* biosynthesis system for azoline-containing peptides by integrating the Flexible *In vitro* Translation (FIT) system and a

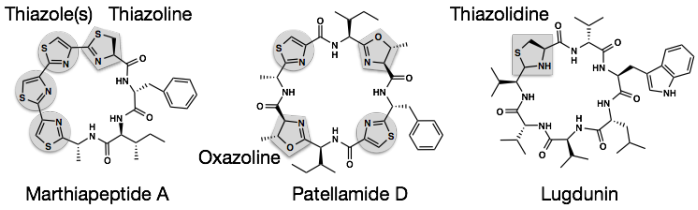


**Figure 1.** Peptidic natural products with backbone modifications such as thiazoles, thiazolines, oxazolines, and thiazolidines.

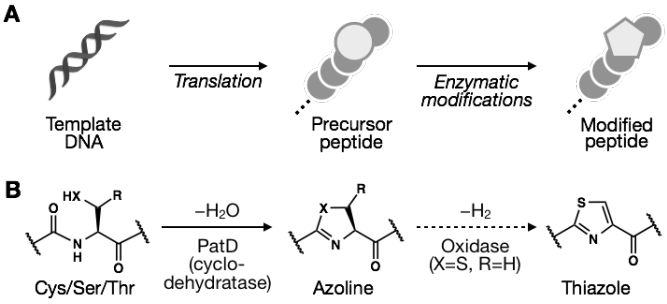


**Figure 2.** (A) Schematic view of FIT–PatD system: *in vitro* biosynthesis system of diverse azoline-containing peptides. (B) Reaction scheme of the post-translational modifications.

post-translational cyclodehydratase, PatD, which is involved in the biosynthesis of patellamides. In the *in vitro* biosynthesis system, referred to as FIT–PatD system, Cys/Ser/Thr residues involved in translated peptides are modified by PatD to the corresponding azoline moieties (**Figure 2**), allowing one-pot synthesis of azoline-containing peptides. We have revealed an unprecedented substrate tolerance of PatD and demonstrated that this system can be applied for diverse azoline-containing peptides.

In natural biosynthesis systems *in vivo*, azolines are often converted to the corresponding aromatic azoles and saturated azolidines by enzymatic oxidation and reduction, respectively. However, during my master degree studies, attempts to isolate active PatG (the oxidation enzyme in patellamide biosynthesis) failed and enzymatic oxidation of azolines generated by the FIT–PatD system could not be achieved. In my doctoral studies, I have attempted to devise alternative chemical methodologies to generate peptides containing a wide range of backbone modifications by combining the enzymatic cyclodehydration by PatD and chemical modifications of the azoline moiety.

**Synthesis of thiazole-containing peptides by chemical modification of thiazolines (Chapter 2)**

The FIT–PatD system could introduce consecutive thiazolines into a precursor peptide containing consecutive cysteines. When a model peptide containing two consecutive cysteines with N-terminal truncated leader peptide (**Figure 3A**) was modified by PatD, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) of the modification product detected a major peak (**Figure 3B, peak a**) accompanied by a side peak (**Figure 3B, peak b**). The major peak was corresponding to the desired peptide containing two consecutive thiazolines, and the side peak had molecular mass smaller by two mass units than the desired product. From several experiments, this "byproduct" was suggested to be a peptide containing a thiazole-thiazoline structure, which was formed by laser-induced oxidation during the MALDI-TOF-MS measurement (**Figure 3C**). From these results, it was hypothesized that chemical modification of the consecutive thiazolines would enable *in vitro* synthesis of thiazole-containing peptides.

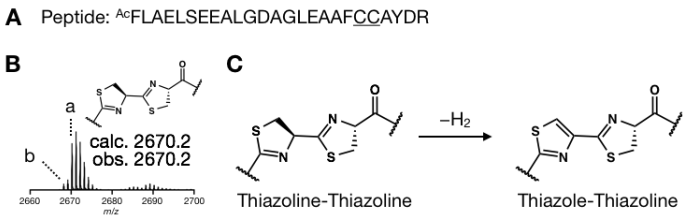


**Figure 3.** (A) Sequence of a model peptide. Consecutive cysteines to be modified are underlined. N-terminal $^{Ac}$F stands for *N*-acetyl phenylalanine. (B) MALDI-TOF-MS spectra of the PatD modification product of model peptide containing consecutive cysteines. The peak a is corresponding to the desired product with consecutive thiazolines and the peak b is the byproduct to be discussed in the main text. (C) Proposed reaction for the byproduct formation.

*(For the reasons of patent applications and future publications, details are closed in the following parts.)* After optimization of several modification conditions, MALDI-TOF-MS detected efficient conversion of the consecutive thiazolines into the thiazole-thiazoline structure. In addition, MS/MS analysis and hydrolysis in acidic conditions supported the formation of the thiazole-thiazoline structure. To further confirm the structure of the modification product, the precursor peptide was labeled site-specifically by a 2,3,3-$d_3$-cysteine as well as a canonical cysteine by means of genetic code reprogramming method using an aminoacylating ribozyme, flexizyme. The deuterium labeling assay enabled the determination of the modification site as well as mechanistic investigation of the modification reaction.

In summary, an artificial post-translational modification reaction has been developed for the synthesis of

thiazole(s)-containing peptides by the integration of the enzymatic cyclodehydration by PatD with the chemical modification, enabling for oxidase-free synthesis of thiazole(s)-containing peptides.

**Synthesis of Ψ[$CH_2NH$]-containing peptides by chemical modification of thiazolines (Chapter 3)**

In several biosynthesis systems of natural thiazolidine-containing peptides, thiazolines are enzymaticaly modified for the introduction of thiazolidines. Inspired by this biosynthesis reaction in nature, in this research, chemical modification of the thiazoline after PatD modification was attempted in order to achieve *in vitro* synthesis of thiazolidine-containing peptides.

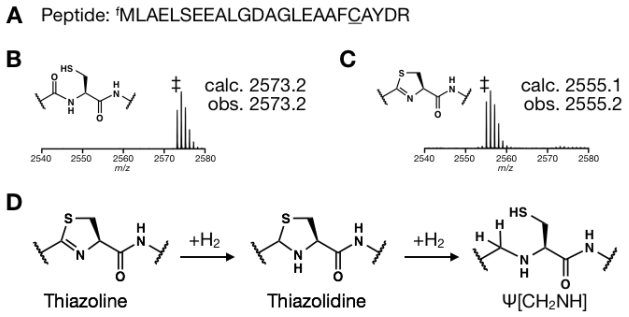


**Figure 4.** (A) Sequence of a model peptide. The cysteine residue to be modified is underlined. N-terminal $^{f}$M stands for *N*-formyl methionine. (B, C) MALDI-TOF-MS spectra of reaction products. (B) Translation product and (C) PatD modification product. In the both spectra, the monoisotopic peaks are labeled with double dagger (‡). (D) Reaction scheme for the Ψ[$CH_2NH$] formation.

*(For the reasons of patent applications and future publications, details are closed in the following parts.)* A model peptide containing a single cysteine residue to be modified by PatD was designed (**Figure 4A**), and after ribosomal synthesis of the model peptide and subsequent PatD modification, MALDI-TOF-MS detected a major peak corresponding to the desired peptide containing a single thiazoline (**Figure 4B and C**). Then, the resulting peptide was further modified. MALDI-TOF-MS detected a peak shift corresponding to the formation of the Ψ[$CH_2NH$] structure via thiazolidine (**Figure 4D**) and the formation of the Ψ[$CH_2NH$] structure was supported by MS/MS analysis as well as alkylation of the free thiol by 2-iodoacetamide. The mechanism for Ψ[$CH_2NH$] structure formation was proposed and model reactions, in which model compounds were synthesized and chemically modified as well, supported the formation of Ψ[$CH_2NH$] structure and the proposed mechanism for Ψ[$CH_2NH$] structure formation. Finally, the modification reaction was applied for a variety of peptide sequences, demostrating the wide substrate tolerance of this modification method.

In summary, an artificial post-translational modification reaction has been developed for the synthesis of thiazolidine- and Ψ[$CH_2NH$]-containing peptides by the combination of the cyclodehydration by PatD and the chemical modification. This method adds a new synthetic path to access backbone-modified peptides, which could not be synthesized by only enzymatic reactions.

**Conclusion**

In conclusion, in this research I have developed novel methods involving the post-translational enzymatic cyclodehydration by PatD and chemical modifications on ribosomally synthesized peptides to yield peptides with various backbone modifications. The installation of thiazoles, which are prevailing in bioactive peptidic natural products as well as Ψ[$CH_2NH$] structures, which is one of the well known peptidomimetic structures, would provide unique structural scaffolds into peptides, which possibly leads to the development of novel bioactive peptides.