# Introduction

The main stream in current protein analysis is proteome analysis utilizing a mass spectrometer and a search engine based on genomic databases to identify proteins. Proteins form specific 3-dimensional structures and display the functions of the protein concerned by having that 3-dimensional structure. The 3-dimensional structures of proteins consist of four levels, one of which is the tertiary structure formed by pairs of the side chains of the amino acids that comprise the protein. One of these 3-dimensional structures is created by intramolecular crosslinking by disulfide (S-S) bonds of cysteines in a molecule. Disulfide bonds have the function of stabilizing the 3-dimensional structure of the protein and normally form S-S bonds in many cases. For this reason, it is sometimes necessary to identify cysteines when conducting an amino acid sequence analysis. When using a protein sequencer, even if the half-cystine residue on N-terminal side is converted to ATZ-half-cystine by Edman degradation, the half-cystine residue will form a disulfide bond with the half-cystine on C-terminal side, and elution and detection by HPLC will be impossible. Therefore, sequence analysis is generally carried out after reductive alkylation of the protein to sever the disulfide bonds, and the cysteine is then identified as a PTH-modified cysteine.

This article introduces an example of identification of a PTH-modified cysteine (phenylthiohydantoin derivative of reductively-alkylated cysteine) by analyzing reductively-alkylated peptides/proteins with PPSQ™-50A series protein sequencer systems (isocratic system and gradient system).

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# Reductive Alkylation Protocol

Several reductive alkylation methods for samples containing disulfide bonds are available. Here, diithiothreitol (DTT) was used as the reductant, and alkylation was conducted by carboxymethylation using monooiodoacetic acid, carbamidomethylation using iodoacetamide, and pyridylethylation using 4-vinylpyridine. Fig. 1 shows the reaction protocol of reductive alkylation. In order to apply the treatment samples directly to the PPSQ-50A series without purification, N-ethylmorpholine was used in the reaction solution, as it has little effect on Edman degradation. Because the optimum pH differs depending on the alkylation agent, pH was adjusted by using an appropriate concentration of N-ethylmorpholine.

The sample used here was 100 pmol synthetic oxytocin containing one intramolecular disulfide bond (Peptide Institute, Inc., Code: 4084-v).

Table 1 and Table 2 show the conditions of the analysis by the protein sequencer when using the isocratic system and the gradient system, respectively.

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**Next, 3 μL (30 pmol) of the reductively-alkylated sample solution was added to a PVDF membrane and dried. After drying, a further polybrene solution was added to the PVDF membrane and the sequence analysis was started. Fig. 2 and Fig. 3 show the sequence analysis results for oxytocin. Fig. 3 is an enlarged view of part of Fig. 2.**
Fig. 2 Amino Acid Sequence of Reductively-Alkylated Oxytocin and Its Chromatograms
Orange: Carboxymethylation (CM), Brown: Carbamidomethylation (CAM), Blue: Pyridylethylation (PE);
1st Cycle : Raw Chromatogram, 6th Cycle : Subtraction Chromatogram

Fig. 3 Amino Acid Sequence of Reductively-Alkylated Oxytocin and Its Chromatograms (Partial Enlargement of Fig. 2)
Orange: Carboxymethylation (CM), Brown: Carbamidomethylation (CAM), Blue: Pyridylethylation (PE);
1st Cycle : Raw Chromatogram, 6th Cycle : Subtraction Chromatogram
From these analysis results, when using the isocratic system, elution of the cysteine occurred in the vicinity of 2.4 min with PTH-carboxymethylation, 4.3 min with PTH-carbamidomethylation, and 8.9 min with PTH-pyridylethylolation. However, because the cysteine was detected near the elution position of PTH-Asp with PTH-carboxymethylation and near that of PTH-Thr with PTH-carbamidomethylation, care is necessary in identification. In contrast, identification is easy with pyridylethylolation, as elution of the PTH-pyridylethylatology cysteine occurred at 8.9 min and did not overlap with the other PTH-standard amino acids. On the other hand, when using the gradient system, elution of the cysteine occurred in the vicinity of 6.5 min with PTH-carboxymethylation, 14.3 min with PTH-carbamidomethylation, and 19.7 min with PTH-pyridylethylolation. With PTH-carbamidomethylation cysteine, detection occurred near the elution position of PTH-His, indicating that care is required in identification. Essentially, preparation of protein sequencer samples by desalination is recommended in order to avoid salt contamination of the samples as far as possible. In this report, it was possible to conduct the sequence analyses without desalination by reducing the concentrations of salts that affect Edman degradation. However, in sample preparation by carbamidomethylation, peaks originating from reductive alkylation reagents were detected in the vicinity of 11.2 min and 16.6 min (Figs. 2 and 3). Desalination treatment of the samples is necessary in order to remove these peaks.

Reductive Alkylation of Cysteine Residue in Protein Sequencer

To facilitate identification of cysteine residues, Shimadzu PPSQ-50A series protein sequencers include an analysis program that conducts reductive alkylation in the instrument, followed directly by Edman degradation. The isocratic system uses a reaction program in which pyridylethylolation of the sample is conducted in the reactor, while the gradient system uses a reaction program for sample carbamidomethylation in the reactor. Fig. 4 shows the respective reaction schemes. Fig. 5 shows the chromatograms for the 6th cycle of a sequence analysis of 30 pmol of lysozyme (Sigma-Aldrich Co. LLC, product No. L6876). Cysteine residue which has formed a disulfide bond with C-terminal cysteine residue exists in the 6th cycle, demonstrating that cysteine residues which have been stably modified by the dedicated reaction program can be detected easily.

(a) Pyridylethylolation, (b) Carbamidomethylation

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