Application Note

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Protein Identification and Phosphorylation Site Determination by *de novo* sequencing using PepFrag™ MALDI-Sequencing kit

Introduction

Introduction of sulfonyl group to N-terminal α-amino group of peptide using sulfonylating agent generate simplified peptide fragment spectrum of only the y-ions in appropriate condition of MALDI-PSD or ESI-MS/MS. Resulting de novo sequence of peptides or phosphopeptides enable to identify proteins unambiguously and characterize the phosphorylation site.

Strategy

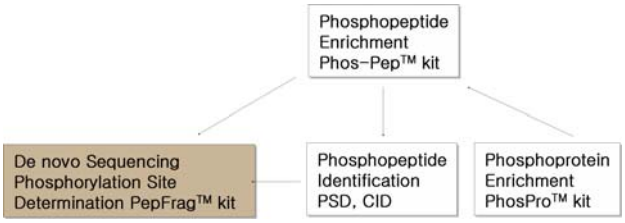

Fig.1.Phosphorylation site determination with MALDI-PSD or ESI-MS/MS using PepFrag™ kit in conjunction with the phosphopeptide enrichment from peptide mixture using PhosPep™ kit.

Materials & Methods

Materials

PepFrag™ MALDI-Sequencing Kit

Guanidination Reagent
(O-methylisoureahydrogen sulfate salt solution)
Guanidination Buffer
(Ammonium hydroxide solution)
Sulfonation Reagent
(4-Sulfophenyl isothiocyanate)
Sulfonation Buffer

Procedure Summary

1. In gel guanidination(optional)
2. Trypsin digestion
3. Sulfonation
4. Enrichment (for phosphopeptide)
5. Peptide cleaning and MS analysis

Results and Discussion

In-gel guanidination and trypsin digestion

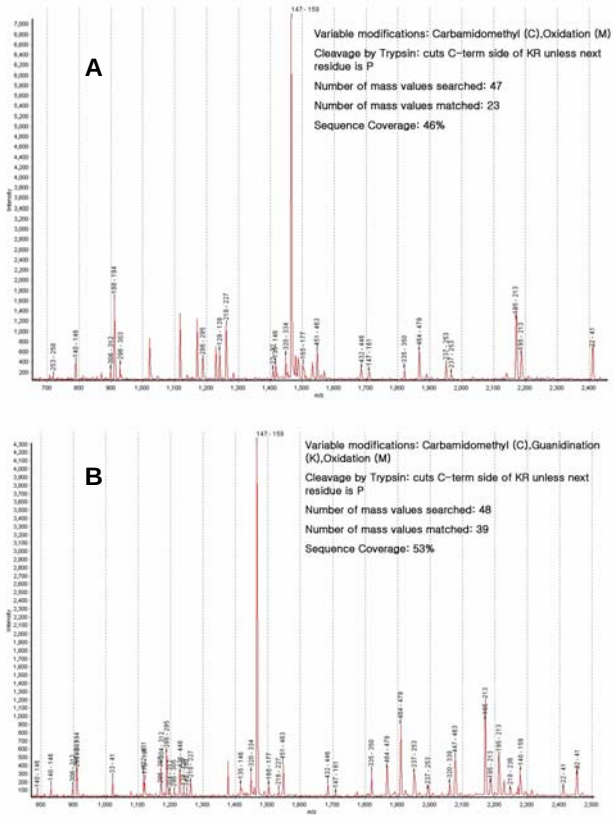

Fig.2. MALDI-TOF mass spectrum of Rubisco separated by 2-DE gel from total protein extract of *Arabidopsis Thaliana* seedlings. Each spectrum represents the sequence-matched peptides from trypsin digest of unmodified Rubisco (panel A), and modified Rubisco by in-gel guanidination prior to in-gel trypsin digestion (panel B).

Guanidination reaction modify the ε-amino group of lysine side chains into guanidinium groups and this reaction enhance the peptide mass fingerprinting. This reaction is also a protection of lysine side chains to prevent unnecessary derivatization upon sulfonation reaction for *de novo* sequencing. In-gel guanidination for whole protein fixed in gel plug prior to trypsin digestion gives advantages excluding additional peptide-cleaning steps which are inevitable in in-solution or solid phase guanidination reaction for tryptic digest of proteins.

Figure 2 shows the more increased sequence coverage of peptide mass fingerprinting using MALDI-TOF in guanidinated trypsin digest (panel B) than normal digest (Panel A). This implicate that guanidination reaction at ε-amino group of lysine side chains do not prevent proteolytic reaction of trypsin on lysine amino acid residues. This reaction process set-up guarantees the selective derivatization by sulfonylation reagent(SPITC) on α-amino group of peptides and facilitate the entire guanidination and sulfonylation reaction for de novo sequencing by eliminating additional peptide-cleaning-up process. Figure 3 shows the spectrum containing Lys-terminated peptides guanidinated by in-gel guanidination (panel A) and sulfonated Arg-or Lys-terminated peptides by subsequent sulfonation reaction.

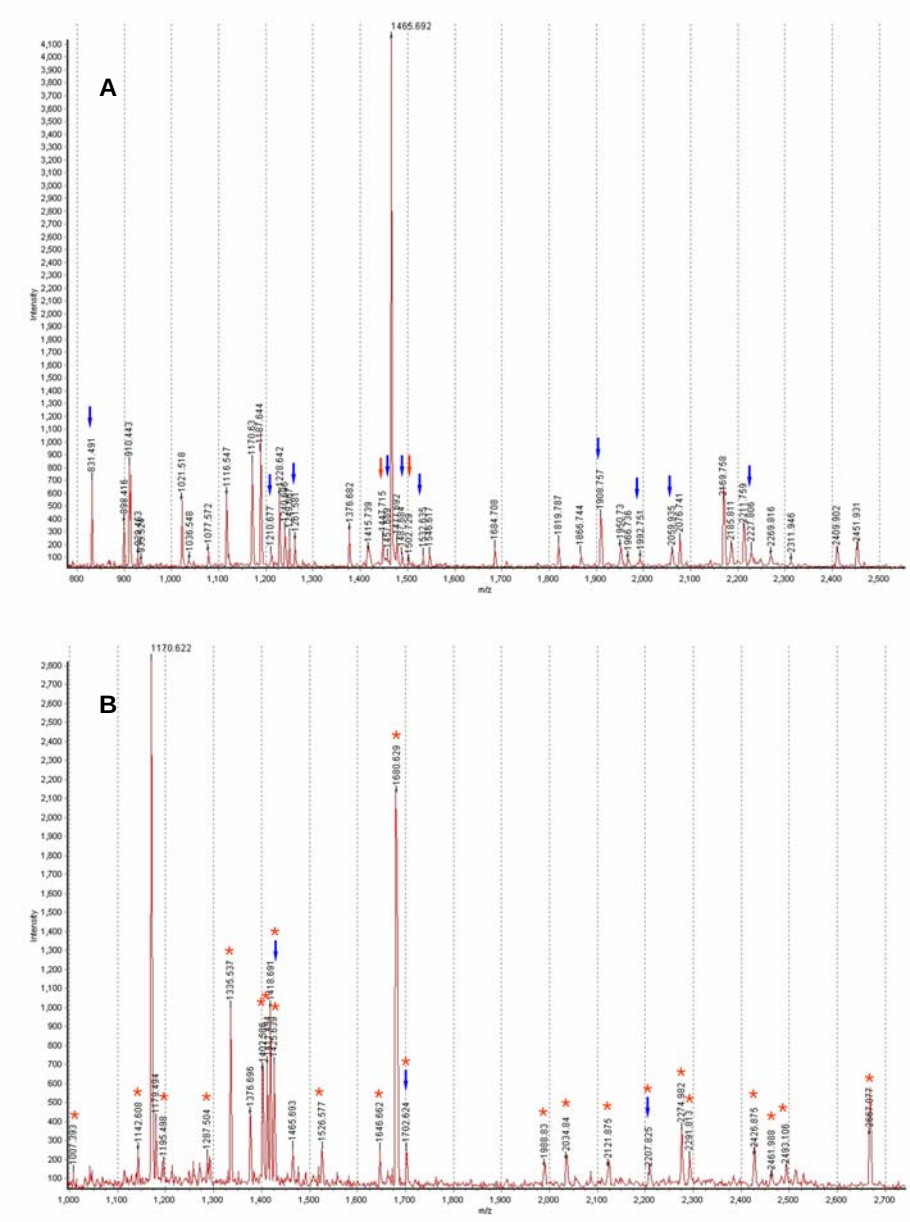

Fig.3. In-gel guanidination and SPITC labeling of Rubisco. In panel A and B, blue and red arrows denote the guanidinated peptide and remaining unmodified peptides respectively. In panel B, red asterisk denotes the N-terminal sulfonated peptides.

Protein identification by de novo sequencing

Some of N-terminal sulfonated peptides shown in Figure 3 were sequenced using MALDI-PSD fragmentation and proteins were identified by database searching. Two Arg-terminated peptide derivatized by sulfonation agent (SPITC), which is shown as 1402 and 1680 (m/z) in Figure 3 B, was analysed.

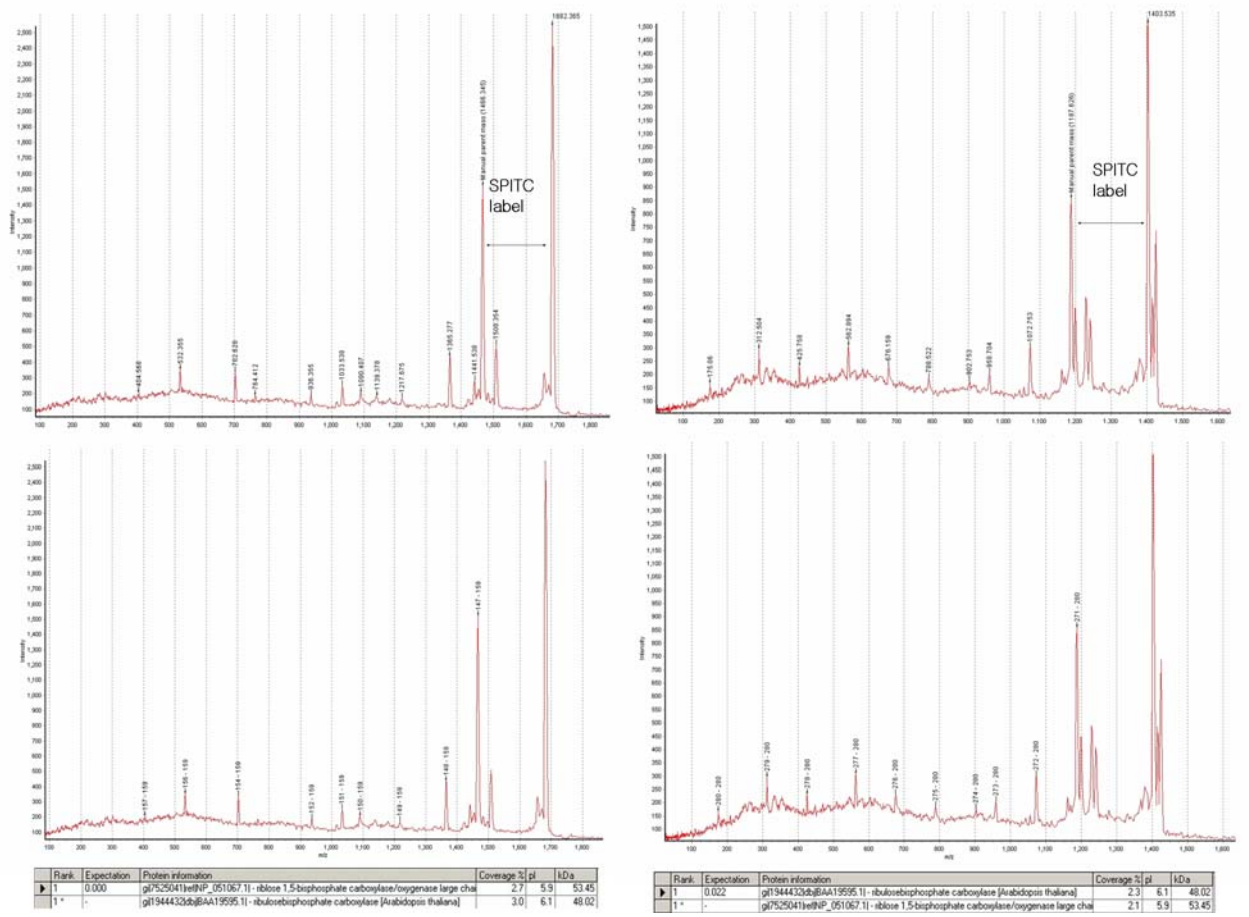

Fig.4. Peptide fragmentation and identification by MALDI PSD analysis of N-terminal sulfonated peptides. Upper panels represent peptide fragment mass of derivatized peptide and lower panels show the sequence covered-fragment of identified proteins.

Phosphopeptide enrichment of derivatized phosphopeptides

Ionization suppression of phosphopeptides in mass spectrometry hampers the identification of phosphopeptide and characterization of phosphorylation site. Phosphopeptide enrichment from complex mixture of trypsin digest facilitates the identification and characterization of phosphoproteins. Using PhosPep^{TM} kit, phosphopeptide enrichment was examined on the peptide solutions of various reaction conditions for guanidination and sulfonation (Fig.5.).

Guanidinated phosphopeptide and its sulfonated phosphopeptide were enriched with no detectable difference in comparison with the untreated normal phosphopeptide (Fig.5 A, C and D). The pH controlled sulfonation by SPITC without pretreatment for protection of ε-amine group of lysine side chain selectively sulfonated the α-amino group of phosphopeptide (Fig.5. B)

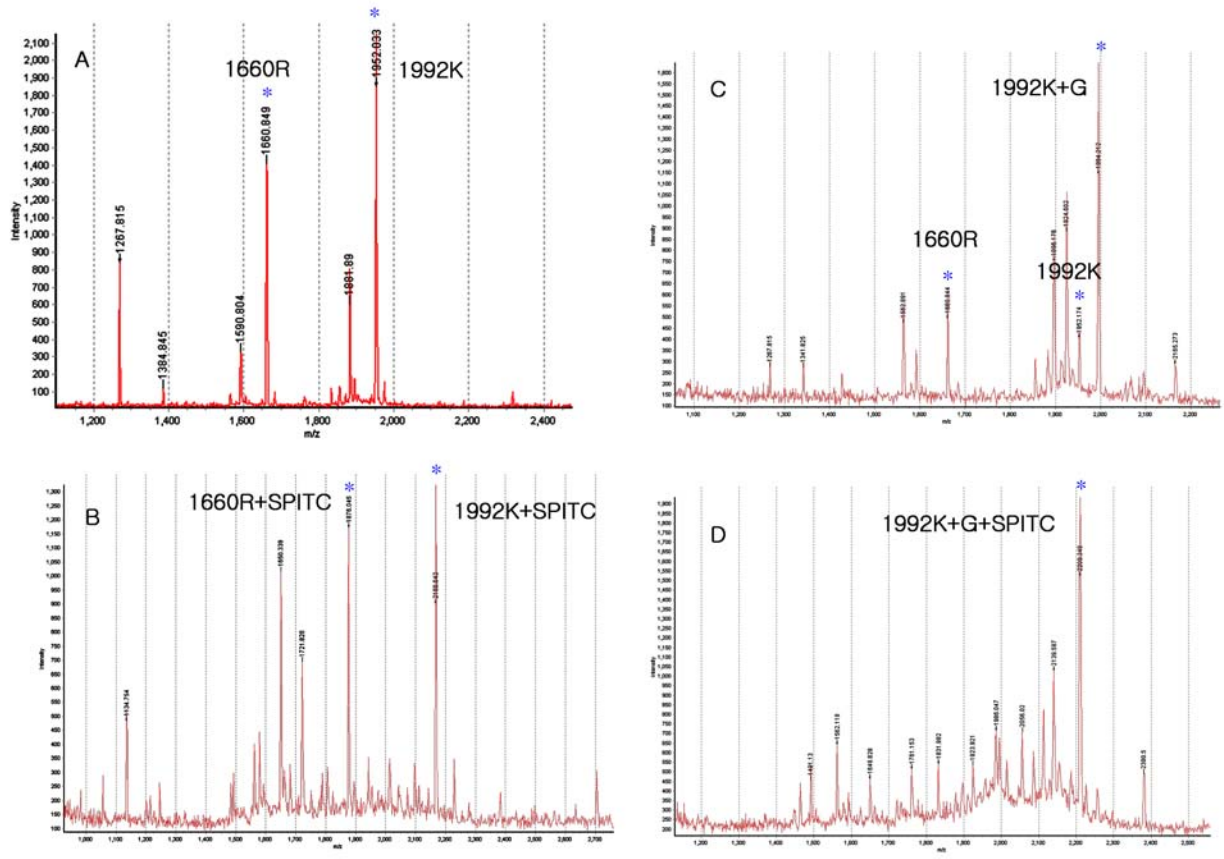

Fig.5. Phosphopeptide enrichment from trypsin digest of α-s1-casein. Phosphopeptides were enriched using PhosPep™ from underivatized (Panel A), pH controlled sulfonated (panel B), in-gel guanidinated (panel C), and in-gel guanidinated prior to trypsin digestion and subsequent sulfonated trypsin digest (panel D) of α-s1 casein. Blue asterisks denote enriched phosphopeptides. Label R or K on mass denote the Arg- or Lys-terminated peptide, respectively.

Phosphorylation site determination

Characterization of phosphorylation site was performed. PepFrag™ MALDI-Sequencing kit was used for guanidination and sulfonation of phosphopeptide. PhosPep™ was used for enrichment of phosphopeptide from complex mixture of phosphoprotein trypsin digest. Some example was shown in Figure 6, 7, 8 and 9.

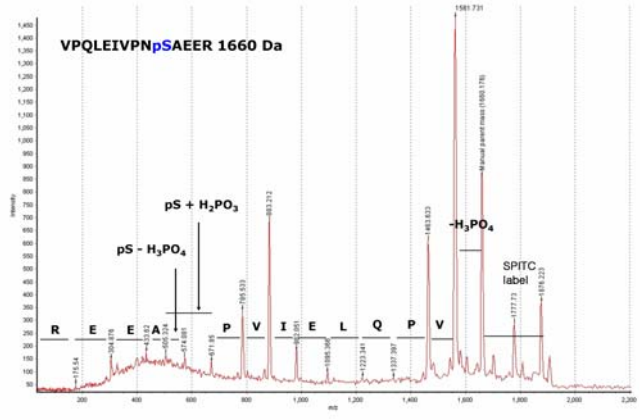

Fig.6. Sequencing and identification of phosphorylation site of Arg-terminated phosphopeptide. Trypsin digest of α-s1-casein was SPITC labeled and enriched using PhosPep™ prior to MALDI-based sequencing.

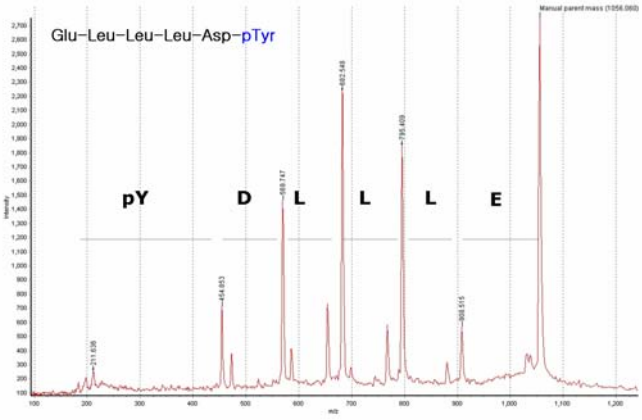

Fig.7. Sequencing and identification of phosphotyrosin from synthetic phosphopeptide.

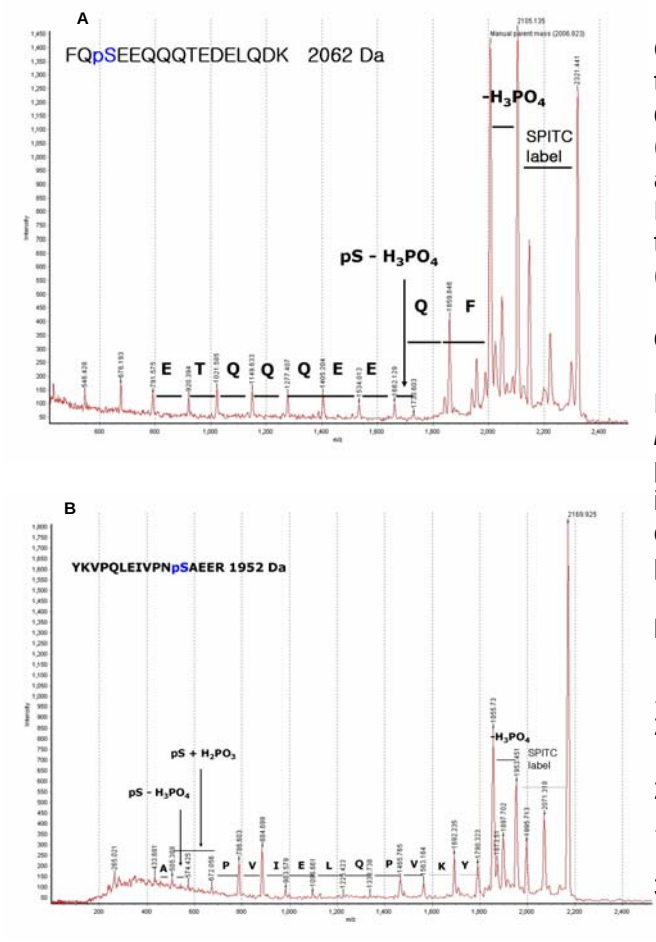

Fig.8. Sequencing and identification of phosphorylation site from Lys-terminated or Lys-containing phosphopeptide.

One phosphoserine was determined from each three peptides. This phosphorylated serine was determined by the mass difference of 69 amu (phosphoserine - H_3PO_4) or 167(serine + HPO_3) as shown in Figure 6 and Figure 8. Phosphotyrosine was determined by measuring the mass difference of 243 amu(tyrosine + HPO_3) (Fig.7.)

Conclusion

MALDI-Sequencing kit is a powerful tool for *de novo* sequencing of peptide and identification of proteins. And also MALDI-Sequencing kit, using in conjunction with PhosPep™, efficiently characterize the phosphorylation site of phosphorylated proteins.

References

1. Thomas, K. et al, Electrophoresis,21,2252-2265 (2000)

2. Gevaert K. et al, Electrophoresis, 22, 1645-1651 (2001)

3. Yong Ho,K. et al, Proteomics, 4,1684-1694