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**RESEARCH PAPER**

# Mass Spectrometric Analysis of S-palmitoylation of Hemagglutinin from Influenza A Virus

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**Abstract:** S-palmitoylation in protein is one of the most important kinds of lipid modification and plays a vital role in cell signal transduction, metabolism and other processes. S-palmitoylation is formed by covalently binding of palmitic acid with sulfhydryl group of cysteine residue in protein through thioester bond. In this study, acyl-biotin exchange reaction was firstly used to convert S-palmitic acid on the hemagglutinin protein from influenza A virus into biotin-labeled tag. The biotin-labeled protein was then enriched by streptavidin-linked beads and further purified by electrophoresis, followed by in-gel digestion. The results showed that the ratio of biotin concentration of the sample with hydroxylamine treatment (+HA) to that of the sample without hydroxylamine treatment (–HA) was larger than 3. Mass spectrometric analysis of the digestion mixture of the enriched hemagglutinin protein from influenza A virus identified two S-palmitoylation modification sites that were located on carboxyl terminal region of hemagglutinin protein such as Cys562 and Cys565, respectively. This research offers a specific and effective method for large-scale analysis of S-palmitoylated proteins.

**Key Words:** Influenza virus; Hemagglutinin protein; Acyl-biotin exchange; S-palmitoylation

## 1 Introduction

S-palmitoylation in protein refers to covalent attachment of saturated 16-carbon palmitic acid on the sulfhydryl group of cysteine residue in protein through thioester bond[1], which is one of the most important kinds of lipid modification. S-palmitoylation is a reversible process and plays a vital role in a variety of processes such as cell signal transduction, cancer, nerve transmission and transportation in cell membrane[2–4]. Although the importance of lipid modification especially S-palmitoylation has widely been recognized, many aspects of this protein modification are not well understood. The progress in this field is slow, in which the research method faces a huge challenge[5]. Early studies used mass spectrometry (MS) to directly analyze the peptide fragments that was modified with S-palmitoylation[6,7]. This method requires purification of the target protein, which is usually difficult to analyze strong hydrophobic S-palmitoylation-modified protein with high sensitivity and throughput. In recent years, many methods for characterization of S-palmitoylation modified protein were developed on the basis of specific labeling of S-palmitoylation[8]. These methods are divided into *in vivo* metabolic labeling method[9–11] and *in vitro* chemical labeling method[12–14]. The *in vivo* metabolic labeling refers to adding analogues of palmitic acid containing either an azido or an alkyne moiety to culture medium of cells, which can be incorporated into cellular proteins via metabolic pathways. The azido or alkyne moiety in lipid-modified protein can be selectively linked to biotin-containing tag via click chemistry reaction, which can then be enriched and detected. In 2004, Drisdel *et al*[15] put forward *in vitro* chemical labeling, which included the

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blockage of all free sulfhydryl moieties in cysteines of proteins, the cleavage of thioester bond between fatty acid and the modified cysteine by hydroxylamine, and the biotin labeling of the newly generated free thiol moieties. As shown in Fig.1, the biotin-labeled proteins are enriched and purified, followed by MS analysis. This workflow involves acyl-biotin exchange reaction (Acyl-biotinyl exchange, ABE), which means the exchange of acyl and biotin groups.

In this study, acyl-biotin exchange reaction, affinity chromatography and MS techniques were used to analyze S-palmitoylation modification on hemagglutinin protein of influenza A virus. Two S-palmitoylation sites were identified. This study provides a specific and effective method for large-scale analysis of S-palmitoylated proteins.

## 2 Experimental

### 2.1 Instruments and reagents

The following instruments were used: Q Exactive mass spectrometry, Easy-nLC 1000 nL high performance liquid chromatography (HPLC) system (Thermo Scientific, USA), PepMap100 $C_{18}$ trap column (20 mm × 75 μm, 3 μm), PepMap100 $C_{18}$ reversed-phase capillary chromatographic column (150 mm × 0.075 mm, 3 μm, Thermo fisher Dionex, USA), Mini-PROTEAN Tetra Cell vertical electrophoresis (Bio-Rad, USA), Allegra TM X-22R centrifuge (Beckman Coulter, USA), Power Look 2100XL-USB with UTA & MagicScan Gel imaging (UMAX, USA), Varioskan microplate reader (Thermo, USA), and Vertical Rotators (Qilin Medical Instrument Factory, China).

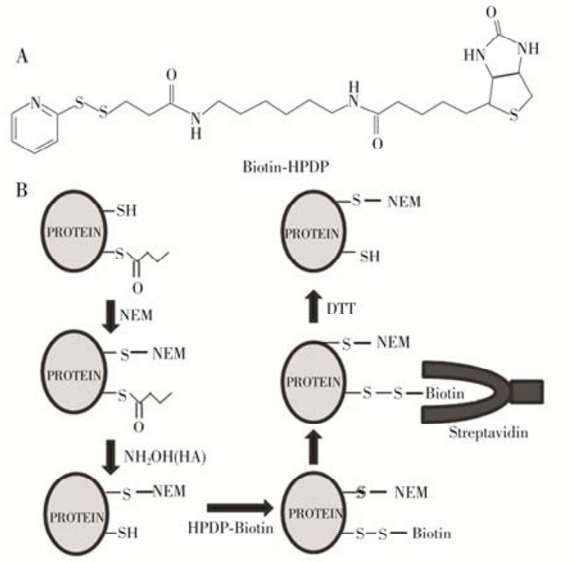


Fig.1 (A) Structure of *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio) propionamide (HPDP-Biotin), (B) Workflow of acyl-biotinyl exchange (ABE) method

HPDP-biotin (HPDP-Biotin (N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio) propionamide)) and streptavidin-agarose were purchased from Pierce. Sequencing-grade TPCK-modified trypsin was obtained from Promega. Ammonium bicarbonate, dithiothreitol, and iodoacetamide were purchased from Bio-Rad. 10% Triton X-100, *N*-ethylmaleimide (NEM), hydroxylamine (HA), formic acid, chloroform, acetonitrile, and phenylmethane- sμlfonyl fluoride (PMSF) were purchased from Sigma-Aldrich. Bovine serum albumin (BSA) and polyvinyl fluoride (PVDF) were from Millipore. Enhanced chemiluminescence substrate reagents were from GE Healthcare. All other chemicals were of analytical grade and were made by manufacturers in China. Human influenza virus strain H3N2 was kept in our laboratory. Ultra-pure water was prepared with a Milli-Q water purification system (Millipore, USA).

### 2.2 Virus cultivation and purification

Embryonated chicken eggs were inoculated with influenza A virus H3N2. After incubated for 72 h at 35 °C, the allantoic fluid was collected from the eggs, cleared by centrifugation at 5000 rpm for 15 min at 4 °C. A 20%–60% (*w/w*) sucrose gradient was used to isolate virus from the supernatant with SW40Ti rotor (Beckman-Coulter, USA) centrifugal at 35000 rpm for 1 h at 4 °C. The band containing the virus was collected and diluted with four volumes of buffer containing 100 mM Tris, 10 mM $MgCl_2$ buffer (pH 7.4), and applied onto the discontinuous sucrose gradient (30% and 60%, *w/w*) solution. Centrifugation was performed at 38000 rpm for 1 h at 4 °C, the above step was repeated at least 3 times to insure the purity of the virus particles. Finally, the band containing the virus was carefully collected and suspended in the buffer, then stored at –80 °C.

### 2.3 Acyl-biotin exchange reaction

Sufficient amount of lysis buffer (LB) (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EDTA) was prepared. Then, 1 mL virus sample was added into 2 mL blocking buffer (LB containing 50 mM NEM, 1 mM PMSF, 2% (*V/V*) Triton X-100), followed by incubation at 4 °C for 1 h with continuous agitation. Five volumes of acetone were added into the mixture to precipitate the proteins at –20 °C for 3 h. The precipitate was collected by centrifugal sedimentation (3500 rpm, 3 min), then the precipitate was dissolved in 400 μL LB buffer containing 1% (*w/V*) sodium dodecyl sulfate (SDS), 10 mM NEM, and kept at 37 °C for 10 min. 2 mL buffer (LB containing 50 mM NEM, 1 × PI, 1 mM PMSF, 0.2% (*V/V*) Triton X-100) was added into the mixture, followed by incubation at 4 °C overnight with continuous agitation. Five volumes of acetone was used to precipitate the proteins at –20 °C for 3 h and separated by centrifugal sedimentation (3500 rpm, 3 min), the precipitate was then collected and dissolved in 400 μL LB buffer containing 1% SDS and kept at