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RESEARCH PAPER

Chemical Modifications of Peptides and Proteins with Low Concentration Formaldehyde Studied by Mass Spectrometry

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Abstract: Formaldehyde has been widely employed to immobilize clinical tissue specimens, inactivate toxins and viruses in biomedical fields. Formaldehyde can react with active groups in bio-molecules such as proteins, resulting in protein cross-linking, inactivation and immobilization. By using several standard peptides and tryptic peptides from the matrix protein of influenza virus as experimental models, we studied the chemical modifications of peptides and proteins with formaldehyde by matrix-assisted laser desorption ionization time-of-flight mass spectrometry and nano-electrospray quadruple time-of-flight tandem mass spectrometry. The reaction between formaldehyde and peptides was performed under the conditions consistent with those during inactivation of virus (4 °C, 0.025% Formalin (37% (*w*/*w*) formaldehyde solution), and 72 h). The results indicated that under the above conditions, formaldehyde reacted with amino groups of *N*-termini of standard peptides to generate methylol adducts, which was further condensed into imines to generate +12 Da products. Furthermore, formaldehyde could react with side chain of two amino acids such as arginine and lysine, yielding +12 Da product each. The analysis of the reaction between formaldehyde and tryptic peptides from matrix protein of influenza virus showed that +24 Da products could be detected in most peptides due to the combinational contribution of *N*-terminus of peptide (+12 Da) and side chain of *C*-terminal arginine or lysine (+12 Da). Moreover, a +36 Da product was detected for a peptide with an internal miss-cut site. The results indicated that low-concentration formaldehyde primarily reacted with amino groups on *N*-termini of peptides and proteins, as well as the side chains of arginine and lysine residues. The present study provided an effective mass spectrometry-based method for analyzing the reaction between low-concentration formaldehyde and peptides and proteins, as well as strategies for interpretation for the mass spectra of reaction products.

Key Words: Formaldehyde; Peptide; Matrix protein; Chemical modification; Mass spectrometry

1 Introduction

In the biomedical field, formaldehyde has been employed in variety of applications such as fixation of clinical tissue samples and inactivation of toxin and virus, *etc.* Formaldehyde can react with the active groups in biological macromolecules such as proteins, resulting in protein crosslinking, inactivation, and even fixation. In the research area of pathogenic microorganism, especially the highly pathogenic microorganisms such as avian influenza virus, formaldehyde is often used for treatment of sample with high pathogenicity, so that the microbial genetic materials such as

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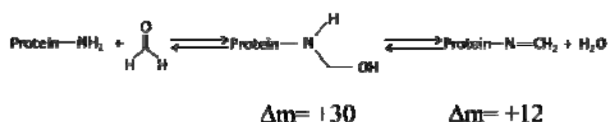
ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) cannot be effectively replicated due to chemical modification and crosslinking.

In recent years, the emerging influenza viruses mutate rapidly, resulting in regional and even global flu pandemic[1,2]. Therefore, the researches on the structure and function of viral proteins, as well as on the interaction between virus and its host become one of the hot areas in viral research[3–6]. To study the primary structure of viral proteins, molecular biology-derived methods are routinely used for viral gene sequencing, in which the corresponding amino acid sequence can be indirectly deduced. By using modern soft ionization mass spectrometry, primary structure sequence of protein could be directly obtained [7,8]. In addition, post-translational modifications on the protein were easily characterized by mass spectrometry [9–11], which were hard to be interpreted by gene sequencing. However, due to the high pathogenicity of emerging influenza A virus, the virus samples must be strictly inactivated by chemical modification of the viral substances such as nucleic acid or protein prior to further analysis in common laboratory.

Early studies[12,13] indicated that formaldehyde could readily react with amino group in amino acids to generate methylol adduct, then lose a molecule of water via condensation reaction as follows.



The chemical modification of protein by formaldehyde was influenced by many factors, such as reaction time, temperature, concentration of formaldehyde, *etc*[14]. In most of the published literatures, high concentration formaldehyde was often used to react with peptide and protein[15], which resulted in strong modification on peptide and protein. Therefore, high concentration formaldehyde was widely used in tanning industry and medical fields.

However, in the studies of vaccine preparation, antigen analysis and mass spectrometric analysis of pathogenic microorganisms, the treatment with high concentration formaldehyde would pose adverse effects on both the antigenicity and yield of the vaccine due to excess modification and crosslinking of bio-molecules such as proteins. Therefore, in order to reduce the adverse effect of chemical modification on both viral antigen titer and mass spectrometric analysis, low concentration formaldehyde was used to treat samples containing viruses. For example, during the inactivation of influenza virus for sample preparation of influenza vaccine, the samples were treated with 0.025% (*V*/*V*) Formalin (37% formaldehyde solution) at 4°C for 72 h[16]. In the present study, we investigated the chemical modification of formaldehyde on a set of synthetic peptides under the same conditions as those used in actual inactivation of influenza virus (final concentration of formaldehyde was 3.33 mM). By using matrix-assisted laser desorption ionization time-of-flight mass spectrometry and nano-electrospray quadruple time-of-flight tandem mass spectrometry, we studied the chemical modification of low concentration formaldehyde on a set of standard peptides and a tryptic peptide mixture from the matrix protein of influenza virus.

2 Experimental

2.1 Instruments and reagents

The main instruments used in the experiment included QSTAR nano-electrospray quadruple time of flight mass spectrometer (Applied Biosystems), Voyager DE STR matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Applied Biosystems), Optima L-100K ultracentrifuge (Beckman Coulter), ZipTip C_{18} Pipette Tips (Millipore) and Nano-electrospray ionization source (Protana).

Standard peptides including Bradykinin (1-7, RPPGFSP), Angiotensin II (DRVYIHPF), Angiotensin I (DRVYIHPFHL), and Substance P (RPKPQQFFGLM-NH2) were purchased from Sigma (USA). Sequencing-grade TPCK-modified trypsin was from Promega. α-Cyano-4-hydroxycinnamic acid (CHCA), formalin (37% formaldehyde solution), iodoacetamide and dithiothreitol were purchased from Sigma (USA). Methonal and acetonitrile (ACN) were from Fisher. Ultrapure water prepared by a MilliQ water purification system (Millipore) was used throughout the experiment. Human influenza virus H1N1 strain was provided by Jilin Yatai Biological Pharmaceutical Inc.

2.2 Mass spectrometric analysis of chemical modification of formaldehyde on standard peptides

Peptide sample solution (66 μM) was prepared by dissolving a certain amount of peptide samples in 50 mM phosphate buffer (pH 7.5). Approximately 2.5 μL of Formalin solution (prepared by diluting Formalin solution (37% *w*/*w* formaldehyde aqueous solution) 200 times) was added into 50 μL of peptide sample solution. The solution were then vortexed immediately, kept at 4 °C for a certain period of time, desalted using C18 ZipTips, mixed with the matrix solution, and dried at room temperature for the further MALDI-TOF-MS analysis. Meanwhile aliquots of desalted samples were employed for the ESI-MS analysis.

2.3 Isolation and purification of viral matrix protein

The H1N1 influenza A virus was inoculated into 9–10 days SPF chicken embryos. After 72-hour incubation at 35 °C, the allantoic fluid was collected and centrifuged at 5000 rpm for 15 min to remove sediment. The supernatant was again

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