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Determining and characterizing hapten loads for carrier proteins by MALDI-TOF MS and MALDI-TOF/RTOF MS

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ABSTRACT

The increasing number of bioconjugates used for bioanalytical purposes and in pharmaceutical industries has led to an increasing demand for robust quality control of products derived from covalently linking small molecules to proteins. Here we report, for the first time, a matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF)-based method to determine the quantity and location of the hapten zearalenone (ZEN) introduced to the carrier protein conalbumin (Con). This bioconjugate is of special interest because of its application in lateral flow immunoassays commercially available for fast testing of food and feed for the presence of ZEN, a common contaminant of all major cereal grains worldwide. Mass spectrometry (MS) analysis of the intact protein turned out to be highly reproducible allowing for the determination of the average hapten load of the carrier protein. In that way an easy and fast method to screen for changes in ZEN load after bioconjugate synthesis was established. For a more detailed hapten load characterization, measurements at the peptide level were of importance. Systematic studies, implementing post-source decay (PSD) and high- and low-energy collision-induced dissociation (CID), showed characteristic fragmentation pattern for three model peptides carrying between one and three lysines (the primary target for the ZEN modification) besides other, less obvious modification sites (serine, arginine and the N-terminus). By this, indicative reporter ions (m/z 203 and 316) and neutral losses ($\Delta m/z$ 373 and 317) for the ZEN modification in general, plus immonium ions (m/z 87, 142 and 159) for the lysine modification in particular were identified. Based on these findings, proteolytic peptides, tentatively assigned to be modified, were unequivocally confirmed to be affected by bioconjugation. For a protein carrying on average only 2–3 modifications per molecule 29 Lys out of 59 potential modifications sites were actually modified. Considerations taking the protein structure into account showed that the affected Lys were predominantly located on the protein's surface.

1. Introduction

Bioconjugates are widely used reagents, finding applications in biomedical fields, bioanalytics, artificial enzymes, immunogenes, biomimetics or nanoelectronics. The conjugate can be formed completely synthetically, though its use is directed towards life science application, or the conjugate is formed by covalently linking a biomolecule (or a fragment thereof) to another molecule, which does not necessarily have to be of biological origin. Fields of application for bioconjugates can be grouped into six major areas: (1) assay and quantification, (2) detection, tracking, and imaging, (3) purification, capture, and scavenging, (4) catalysis and chemical modification, (5) therapeutics and *in vivo* diagnostics and (6) vaccines and immune modulation [1].

Many conjugates can be used in more than one area with equivalent success and proteins are by far the most common targets for modification. The most frequently used carrier proteins for conjugation are bovine serum albumin (BSA), ovalbumin, conalbumin (Con), thyroglobulin, different forms of immunoglobulin, fibrinogen, and keyhole limpet hemocyanin. Every conjugation process involves the formation of a covalent bond between two functional

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groups, and reactive groups coupling to amine-containing molecules (proteins) are the most common present on modification reagents. The primary coupling reactions for modification of the protein's amines proceed by acylation or alkylation after activation of the modifying reaction partner. An *N*-hydroxysuccinimide (NHS) ester formed from a carboxylate in the presence of carbodiimide is the most common activation chemistry for acylating agents [2,3].

Low molecular mass antigens (haptens) like mycotoxins, small molecule drugs or pesticides are usually non-immunogenic and therefore do not cause an immune response. However, it is possible to elicit antibodies with affinity to such haptens by conjugating to a carrier protein forming an immunogen [4]. Thus, hapten-protein conjugates can be used to produce monoclonal antibodies, but can also be utilized for immunoassays. Yet, chemical linkage of haptens to proteins can bring changes in their biological functionality [5] and a high degree of substitution can adversely affect the activity and specificity of antibodies produced or protein conjugates employed in analytical strategies that are technically based on a competitive immunoassays, such as lateral flow immunoassay (LFIA).

Structural and immunogenic effects of hapten loading were shown for BSA [6], highlighting the critical effect of reproducible bioconjugate synthesis. Many different analytical tools can be used to check for bioconjugate quality, yet during the last decades mass spectrometry (MS) became the most important one. Matrix-assisted laser desorption/ionization (MALDI) MS has been used in comparison with UV spectroscopy and titration to characterize BSA-hapten conjugates already very early [7], but limited mass resolution of the time-of-flight (TOF) instrumentation and poor overall sensitivity led to broad and weak signals and it was found at this time that liquid chromatography (LC) coupled to electrospray ionization (ESI) iontrap MS gave a clearer representation of the hapten-protein distribution [8]. However, since then MS instrumentation has improved significantly and this study will show the capabilities of modern TOF and TOF/TOF instruments for robust protein-hapten analysis. MALDI-based MS analysis was chosen due to the fact that not all bioconjugates are accessible by LC-ESI MS approaches. The presented study will show that high hapten load can lead to increasing hydrophobicities of the bioconjugate leading finally to protein precipitation. For LC approaches this also implies a very likely irreversible binding of the modified protein/peptide to the stationary phase of the chromatographic column.

A MALDI-TOF MS approach can be chosen to circumvent these limitations. Moreover, sample preparation is much faster because micro-purification using ZipTip technology is the only purification step needed. Although hapten-protein distribution is not directly accessible, the high absolute sensitivity allows ascertaining the average hapten load by determining mass differences for the protein species. A simple division of the determined mass difference by the relative molecular mass (M_r) of the hapten gives the number of interest – the hapten load. Here a MALDI-TOF-MS analysis is much straighter forward than an ESI approach, where accurate deconvolution of the multiply charged molecules for each protein species has to be determined. This usually asks for high resolution mass analysis because of protein heterogeneity (isoforms, post-translational modifications, multiple conjugations).

In this study the mycotoxin zearalenone (ZEN) was used as hapten and Con as the carrier protein. ZEN is a secondary metabolite of a crop infesting fungus, has low acute toxicity, but exhibits estrogenic effects in some species, and the onsets of several sexual disorders and alteration in the development of the sexual apparatus have been described [9,10]. ZEN therefore threatens food supplies for animals and humans on a daily basis. Consequently an easy-to-use quick test applicable in the field is of high and urgent interest. For ZEN testing LFIAs are already used today, *i.e.* AgraStrip® (Romer Labs Division Holding GmbH, Tulln, Austria).

It was the aim of this study to develop a robust MALDI-linear TOF and TOF/RTOF MS-based method to check mycotoxin-protein conjugates (ZEN/Con conjugates) in terms of hapten load and localization of hapten attachment. The average hapten load was finally derived from intact protein and mycotoxin-protein conjugates measurements. Hapten localization was deduced from peptide analysis after in solution digestion with trypsin.

2. Materials and methods

2.1. Reagents

All chemicals were of analytical grade if not stated otherwise. ProteoMass Albumin MALDI MS standard (Alb; 66,429 Da for $[M+H]^+_{avg}$), conalbumin from chicken egg white (Con, iron-free, C0755), adrenocorticotropic hormone fragment 1–17 (ACTH; $[M+H]^+_{mono}$ at m/z 2093.8), trifluoroacetate salt of Lys-Arg-Thr-Leu-Arg-Arg ($[M+H]^+_{mono}$ at m/z 829.54), Met-Lys-[Ser2, Arg3, Pro5, Arg8]-bradykinin (bradykinin; $[M+H]^+_{mono}$ at m/z 1327.74), α-cyano-4-hydroxycinnamic acid (CHCA), D,L-dithiothreitol (DTT), iodoacetamide (IAA), β-mercaptoethanol (β-MC), trifluoroacetic acid (TFA), phosphate buffered saline pH 7.4 (PBS), guanidine hydrochloride, N,N′-dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) were provided by Sigma-Aldrich (St. Louis, MO, USA). Ammonium and sodium bicarbonate (NH_4HCO_3 and $NaHCO_3$) as well as sinapic acid (SA) were from Fluka (Buchs, Switzerland). Acetonitrile (ACN) was obtained from Merck (Darmstadt, Germany) and water was of Millipore grade (18.2 MΩ cm resistivity at 25 °C). Phosphorylase b (Phosb; 97,159 Da for $[M+H]^+_{avg}$), from rabbit muscle was obtained from Protea (Morgantown, WV, USA). Trypsin (proteomics grade) was purchased at Roche (Penzberg, Germany). Zearaleon (ZEN) and its activated form carrying an O-(carboxymethyl)-hydroxylamine group (ZEN-CMO) were prepared in-house by Romer Labs Holding for research purposes (Fig. 1).

2.2. Hapten-peptide/protein synthesis and purification

To attach the hapten (ZEN-CMO) to a carrier protein or peptide, NHS/DCC chemistry was applied [2]. Hapten conjugates were synthesized by Romer Labs Division Holding as previously described [11]. Briefly, the protein/peptide was weighed in a glass vial, dissolved in 0.1 M $NaHCO_3$ and activated for different durations at different temperatures (for details see results). ZEN-CMO, NHS and DCC were dissolved in DMF separately. ZEN-CMO activation was carried out by mixing DCC, NHS and ZEN-CMO and leaving the solution at rest. The activated ZEN-CMO was added to the protein solution on ice whilst gently stirred at 4 °C. All reaction partners were mixed at certain molar ratios to achieve a defined coupling ratio (cr) according to n(protein/peptide) = cr × n(ZEN-CMO) with n(NHS) = n(DCC) = 1.5 × n(ZEN-CMO). For the final product purification, 1.5 mL of the conjugate was diluted with 1 mL of 0.01 M

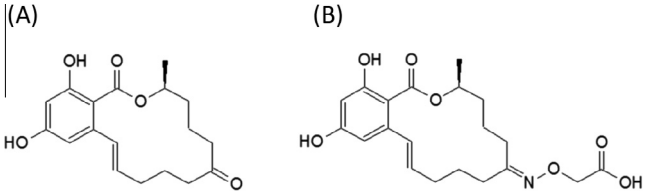

Fig. 1. (A) Zearalenone (ZEN) with an elemental composition of $C_{18}H_{22}O_5$ corresponding to an average molecular mass of 318.37 Da (monoisotopic mass of 318.15 Da). (B) ZEN-CMO with an elemental composition of $C_{20}H_{25}NO_7$ corresponding to an average molecular mass of 391.42 Da (monoisotopic mass of 391.16 Da).