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The combined use of analytical tools for exploring tetanus toxin and tetanus toxoid structures

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ABSTRACT

Aldehyde detoxification is a process used to convert toxin into toxoid for vaccine applications. In the case of tetanus toxin (TT), formaldehyde is used to obtain the tetanus toxoid (TTd), which is used either for the tetanus vaccine or as carrier protein in conjugate vaccines. Several studies have already been conducted to better understand the exact mechanism of this detoxification. Those studies led to the identification of a number of formaldehyde-induced modifications on lab scale TTd samples. To obtain greater insights of the changes induced by formaldehyde, we used three industrial TTd batches to identify repeatable modifications in the detoxification process. Our strategy was to combine seven analytical tools to map these changes. Mass spectrometry (MS), colorimetric test and amino acid analysis (AAA) were used to study modifications on amino acids. SDS-PAGE, asymmetric flow field flow fractionation (AF4), fluorescence spectroscopy and circular dichroism (CD) were used to study formaldehyde modifications on the whole protein structure. We identified 41 formaldehyde-induced modifications across the 1315 amino acid primary sequence of TT. Of these, five modifications on lysine residues were repeatable across TTd batches. Changes in protein conformation were also observed using SDS-PAGE, AF4 and CD techniques. Each analytical tool brought a piece of information regarding formaldehyde induced-modifications, and all together, these methods provided a comprehensive overview of the structural changes that occurred with detoxification. These results could be the first step leading to site-directed TT mutagenesis studies that may enable the production of a non-toxic equivalent protein without using formaldehyde.

1. Introduction

Tetanus toxin (TT[1]) is a potent neurotoxin produced by *Clostridium tetani* bacteria, and in 2013 was the cause of over 58,000 deaths worldwide [1]. TT binds to motor neurons using specific receptors [2], and is then internalized and transported into the cell body using axonal retrograde transport. In the spinal cord, TT blocks the release of inhibitory neurotransmitters by cleaving synaptobrevin-2, leading to hyperactivity of the motor neurons and consequently spastic paralysis [3].

TT is a 150.7 kDa protein composed of a 52.4 kDa light chain, responsible for synaptobrevin cleavage, and a 98.3 kDa heavy chain [4,5]. The heavy chain can be further subdivided into two domains, the N-terminal domain (46.7 kDa), responsible for cell penetration [6], and the C-terminal domain (51.6 kDa), also called the tetanus toxin fragment C (TTFC), which governs TT neuronal specific binding [6,7]. TT has yet to be fully crystallized, and as such its entire structure remains unknown. Currently, only the light chain and the TTFC 3D structures have been resolved [2,8].

Vaccination against tetanus disease caused by *Clostridium tetani* has been used since the 1930s. The vaccine is composed of the detoxified TT protein, tetanus toxoid (TTd); which is obtained by formaldehyde

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[1] The abbreviations used are: AAA, amino acid analysis; AF4, asymmetrical flow field-flow fractionation; AGC, automatic gain control; CID, collision induced dissociation; dRI, differential refractive index; DLS, dynamic light scattering; ESI, electrospray ionization; FTICR, Fourier transformed ion cyclotron resonance; FWHM, full width at half maximal resolution; HC, heavy chain; LC, light chain; MALS, multi-angle light scattering; MS/MS, tandem mass spectrometry; Mw, molecular weight; NCE, normalized collision energy; QELS, quasi elastic light scattering; Rg, gyration radii; Rh, hydrodynamic radius; SEC, size exclusion chromatography; TIC, total ions currents; TNBS, trinitrobenzene sulfonate; TT, tetanus toxin; TTd, tetanus toxoid; TTFC, tetanus toxin fragment C; UPLC, ultra-performance liquid chromatography.

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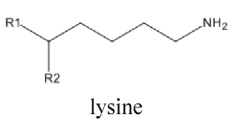
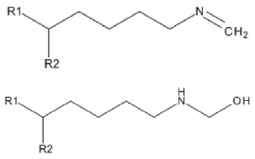
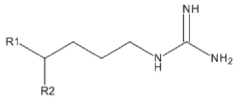
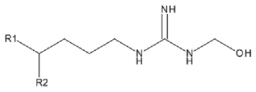
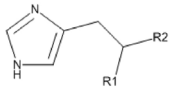
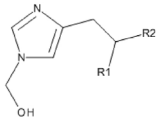
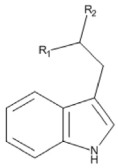
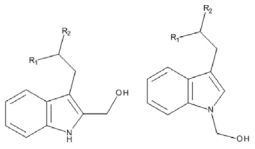
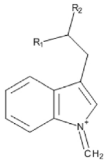
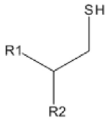
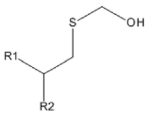
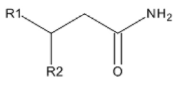
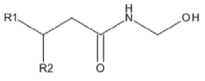
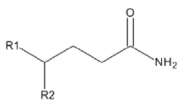
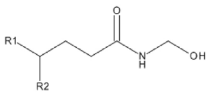
treatment of TT. TTd protein is also widely used as carrier protein in conjugate vaccines. Due to TTd immunological properties, the T-cell independent response is converted to a T-cell dependent one, which boosts the immunity response directed against the conjugated bacterial polysaccharide [9,10].

The formaldehyde detoxification process induces several TT amino

Table 1
Formaldehyde-induced modifications on amino acids and their corresponding mass changes. Groups R1 and R2 represent N and C-terminal functions.

Amino acid	Modified amino acid	Mass modification	Ref.
 lysine		Schiff base +12.000 Da Methylol adduct: +30.011 Da	[12,13]
 arginine		Methylol adduct: +30.011 Da	[14]
 histidine		Methylol adduct: +30.011 Da	[15]
 tryptophan		Methylol adduct: +30.011 Da	[16]
		Imine adduct: +13.008 Da	[12]
 cysteine		Methylol adduct: +30.011 Da	[17]
 asparagine		Methylol adduct: +30.011 Da	[12]
 glutamine		Methylol adduct: +30.011 Da	[12]