

# Tetanus toxin: structure and purification



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Tetanus is regarded amongst the most severe and fatal disease since ancient times [1]. Tetanus is termed from a Greek word 'Tetanos' which means- to contract \*. The disease is generally initiated due to deep wounds, cuts, and during catastrophic situations like natural calamities, physical trauma, etc. [a] It was first discovered by Hippocrates in early 19<sup>th</sup> century \*. However it was etiologically described by Carle and Rattone who were first to produce tetanus in animals by injecting pus extracted from an infected human with the same disease in 1884. During this same period Nicolaier also produced tetanus in animals from soil samples. Further research in 1889 by Kitasato revealed that animals were infected by this disease when they were injected with a particular organism isolated from a human patient \*. Nocard in 1897 revealed that this disease can be treated by the use of its antitoxin. Moreover in 1924 the significance of toxoid came into existence during World War I which was formulated by Descombey and this passive immunization against tetanus was majorly used during World War II \*.

#### Structure of tetanus toxin:

The tetanus toxin is of 150kD comprising of three fragments i. e. A, B and C having a molecular weight of 50kD each [n]. Fragments A and B were observed to be non-spastically toxic and also to block the release of catecholamine. It also inhibits the action of synaptic nerves and thus exhibits an important role in the toxicity of the toxin. On the other hand fragment C is regarded as the non-toxic subunit but retaining the required antigenic properties of the toxin. This fragment is seen to bind with gangliosides, motor endplates and synaptic membranes. It also helps in transportation of the toxin from the periphery to the central nervous system [o].

*Krieglestein et al.* in 1990 stated that tetanus toxin is a 151-kD protein. The complete amino acid sequence is known. The mature toxin is made of two peptide and contains 10 half-cystine residues. Treatment with 4-vinylpyridine in the presence of 6M guanidine converted six of them into s-pyridylethyl cysteine residues are determined by amino acid analysis. When alkylation was preceded by mercaptolysis, all 10 halfcystine residues were recovered in the s-pyridylethylated form. It was therefore concluded that the toxin contains six sulfhydryl groups and two disulfide bond [r]

#### Mode of Action:

*Rossetto et al.* in 2001 reported that the neuromuscular syndromes of tetanus is caused by neurotoxins produced by bacteria of the genus *Clostridium* of 150 kDa proteins consisting of three-domains, endowed with different functions: neurospecific binding, membrane translocation and specific proteolysis of three key components of the neuroexocytosis apparatus. After binding to the presynaptic membrane of motoneurons, tetanus neurotoxin (TeNT) is internalized and transported retroaxonally to the spinal cord, where it blocks neurotransmitter release from spinal inhibitory interneurons. TeNT cleave specifically at single but different peptide bonds, VAMP/synaptobrevin, a membrane protein of small synaptic vesicles [s].

*Kegel et al.* in 2002 stated that the 50kD light chain subunit comprises of zinc metalloproteases which cleaves synaptobrevin that is not involved in neuroexocytosis [t].

*Foster* in 2009 Stated that TeNT enters the body via wounds and initially binds and internalizes into the peripheral terminals of motoneurons where it is transported by retrograde axonal transport to the motoneuron in the spinal cord. TeNT is transported to somatodendritic postsynaptic sites and is released into the synaptic cleft where it undergoes receptor mediated uptake into the presynaptic termini of the inhibitory interneurons, from where it translocates into the cytosol and inhibits neurotransmitter release. [u].

Starting material for purification of tetanus toxin:

Raynaud in 1951 developed a technique of using non-autolyzed toxin direct from the organism i. e. *Clostridium tetani* [i] . This technique gave an advantage of obtaining a more concentrated form of toxin as compared to that obtained from the filtrates [i][j]. For this purpose the organism was generally cultured and subcultured using Tarozzi medium\* and modified Tarozzi medium [j] Latham medium was also widely used for the same reason [j] [k]. *M. Matsuda et. al* in 1989 also used modified Latham medium for culturing of *Clostridium tetani* [o] . *Muller and Miller* in 1954 investigated that pancreatic digest of casein contained some inhibitory content which was solved by charcoal treatment [y]. Toxin was also extracted by treating the bacterial cells in hypertonic solution using 0. 1M sodium citrate and 1M sodium chloride as stated by *Bernard Bizzini et. al* [q]

Conventional method to produce tetanus vaccine:

The Harvard strain of *Clostridium tetani* is grown in a fermentor for about a week using a semisynthetic medium. This leads the bacteria to lyse and

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release the toxin obtained in the supernatant. This method yielded about 60-80 Lf/ml. This yield is then filtered and detoxified using formaldehyde. This reacts with the toxin molecule mainly the amino groups of lysine resulting in imine formation, further reacts with the unstable groups of imidazole or phenol ring finally involves a cross-linking reaction between the both the amino groups. Formaldehyde also affects the 3-D structure, therefore making the toxic conformational epitopes [y].

#### Purification by HPLC:

*Kunihiro Ozutsumi et. al.* in 1985 used extracts from the organism for purification of tetanus toxin using High performance liquid chromatographic methods (HPLC) [j]. The toxin extracted from the

previously described method was initially purified using ammonium sulfate precipitation followed by ultracentrifugation in order to get rid of the unwanted particulate matter by filtering it through a 0.2 µm membrane filter. The concentrated sample in the equilibrating buffer at a pH of 7.5 proceeded through a final step of purification by running it on HPLC using a column of a TSK G3000 SW of 0.75 x 60 dimensions. This column was equilibrated using 0.1M sodium-phosphate buffer at a pH of 6.8 and the flow rate was maintained at 0.6 ml/min. The fractions obtained were tested for its protein content at 280nm using a UV spectrophotometer [j]. Further the efficiency of HPLC was compared with another gel filtration method using Ultrogel column [j] [o].

#### Purification using Sephadex G-100:

For large scale production of tetanus toxoid, Alcohol precipitation was used for immunization purpose by Pillemer L. *et. al* [b]. However, *Levine et. al* in 1951 used to purify the tetanus toxoid by ammonium sulfate precipitation [c]. Later, further purification and characterization of the toxoid was achieved by filtering it through Sephadex gels using G-100 columns as stated by Williams C. *et. al* in 1965 [d]. This simplified and low cost method yielded four separable fractions of the toxoid where the first two fractions of 55-65% non dialyzable nitrogen possessed significant antigenic properties. The next fraction obtained was of smaller molecular weight and showed poor antigenicity when injected in animals; however the fourth fraction obtained was not identified but was predicted to be metabolic by-products of the organism and had no significant role [d]. Before running on the column the protein concentration was determined using a UV spectrometer at 280nm. Chromatographic gel filtration was performed using a column of 1.2 x 0.062 m dimension. The column was packed and equilibrated with 0.1M phosphate buffer with a pH of 8.5, additionally 1% formaldehyde can be added to inhibit the bacterial growth. The void volume after equilibration was maintained at 800ml at the flow rate was fixed at 80ml/hr. The sample loading volume was around 50ml and was concentrated to about 100,000 Lf. The four fractions were collected and were further separated by recycling them on the same column [d]. This method gave an efficient insight on how to purify and separate different components of the toxoid.

*M Matsuda* in 1989 carried out the separation of fragment A-B treated with urea by running it on a column packed with Sephadex G-25, equilibrated with 0.02M tris-HCL buffer containing trace amounts of dithiothreitol and

urea [o]. Other gels such as Sepharose 4B and Sephadex G-200 was also used by researchers like *Bernard Bizzini* , Immunodiffusion test was also carried out using *Ouchterlony's* method [o] [p] [q].

#### Papain Digestion of Tetanus toxin:

Further research by *Helting* and *Zwister* in 1974 made possible to obtain fragment C from Tetanus toxin which has significant antigenic properties but lack pathogenicity and thus occupies a major role in immunization [e].

*Helting et. al* stated that Tetanus toxin can be degraded in a specific pattern.

The mild papain digestion cleaves the F(ab) region. The papain enzyme breaks the 150kD toxin into two parts, one comprising of the C-terminal of the heavy chain i. e. of 47kD which corresponds to the Fragment C of the toxin whereas the other part of 95kD consists of N-terminal heavy chain subunit along with the lighter chain polypeptide forming the fragment B (refer to Figure 2). This Fragment B was observed to have a toxic effect on mice when injected with a sufficient dose and also has an adverse effect on the nervous system, thus it was necessary to purify and obtain only Fragment C for immunization and to further study its immune response [f]. The purified Fragment C was separated and obtained by chromatographic methods and by using anti-Fragment C IgG [f].

*Ulrich Weller* in 1989 performed papain digestion for 16 hours of overnight stirring of the toxin at 25°C at a concentration of 40ug/ml. The toxin was suspended in 10mM sodium-phosphate buffer at pH of 6.5 with 1mM EDTA and NaN<sub>3</sub> and 10mM cysteine. After the incubation period 0.5mM of L-I-chloro-3-tosylamido-7-amino-2-heptanone was added as a stop solution in

order to inactivate papain by further incubating it at room temperature for 30 min and was then cooled to 0°C with saturated ammonium sulfate solution at pH 6.5 with further centrifugation. The precipitate was resuspended in the same buffer mentioned. This further proceeded for its separation and purification on Sephadex G-100 column and the fractions were collected at the flow rate of 15ml/hr b\*.

These fractions were further pooled and concentrated using a Centiprep 10 concentrator and the buffer was changed to 0.5 M NaCl with 30mM Tris-HCl at pH 7.5. The fragments B and C showed up homogeneously on SDS-PAGE. The fragment C was further dialyzed against 10mM sodium phosphate buffer at a pH of 7.5. The samples obtained were further checked for its protein content at 285nm and was determined by modified lowry method after trichloroacetic acid precipitation. They also ran an SDS-PAGE using reducing and non-reducing gels and was stained by Coomassie blue-250 and the chains and fragments of the toxin were determined according to their known amino acid sequence b\*

Other methods developed to obtain fragment C:

Fishman *et al.* (1992) pointed out that the non-toxic binding fragment of tetanus toxin (fragment C) binds avidly to neural tissue and has a growing number of neurobiological uses. Its current utility is limited by both its high commercial cost and the complex procedure for its preparation requiring highly purified tetanus toxin. A short procedure was developed which prepares fragments of tetanus toxin from crude *C. tetani* extracts. The resultant proteins are atoxic with molecular sizes and immunological



properties closely resembling fragment C. These proteins undergo retrograde axonal and apparent transneuronal transport in a fashion similar to fragment C [v].

*Ledoux et al.* in 1994 indicated that tetanus toxin once internalized via receptor-mediated endocytosis, form membrane channels in order to traverse the endosomal membrane and enter the cytoplasm of the nerve terminal forming an association between neurotoxin monomers which results in an oligomeric form of the neurotoxin necessary for assembly of a channel through the hydrophobic interior of the endosomal membrane, thereby allowing passage of the neurotoxin or its active fragment through the resulting pore [w].

Technique used to test the specificity of the heavy and light chain subunits:

*Matsuda and Yoneda* in 1975 isolated the heavy and light chain subunits from a toxin reduced by treatment with dithiothreitol-urea[g] [h]. *Kunihiro Ozutsumiet. al.* in 1985 used the technique of electrophoresis using sodium-dodecyl-sulphate polyacrylamide gel i. e. SDS-PAGE as shown in Figure (3). and was further used to put up a western blot in order to check the specificity of the isolated subunits obtained [l] [m] [j]. SDS-PAGE allowed the toxin to stack at 49kD corresponding to the fragment C subunit and 85kD comprising of the 4heavy chain subunit [j]

*Goretzki and Habermann* in 1985 characterized enzymatic fragments of tetanus toxin by immunoblotting using a set of previously characterized antibodies and a set of novel antibodies. The selected antibodies recognized the light chain, fragment C ( $\beta 1$ ) and the complementary piece ( $\beta 2$ ) of the

heavy chain when blotted on nitrocellulose. All toxin preparations contained intrinsic esterase activity which became manifest in the presence of urea. The main product of papain hydrolysis is fragment C, which appears as a double band under non reducing conditions but is homogeneous when reduced. Chymotryptic digestion hydrolyses the heavy chain well but leaves the light chain largely intact. Tetanus toxin is very resistant against trypsin as compared with other proteases, although this enzyme splits numerous different links [x].