ENZYMATIC BREAKDOWN OF TETANUS TOXIN

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Summary

Treatment of tetanus toxin with papain at 55°C resulted in breakdown of the molecule to yield an atoxic fraction with a molecular weight of approximately 40 000. The highly purified material exhibited partial immunological identity with the parent toxin, showed no toxicity and elicited the formation of neutralizing antibodies against tetanus.

Introduction

Tetanus toxin, a protein with a molecular weight of approximately 140 000, has in the past attracted considerable interest because of its extreme toxicity to several mammalian species (for review articles, see ref. 1-2). Although the purification of the toxin from culture filtrates of Cl. tetani is easily accomplished, structural and functional studies are hampered by the lability of this protein in the purified state (3). In the present investigation, the toxin has been subjected to proteolytic treatment with the aim of obtaining split products which might be more amenable to structural studies. The isolation and some properties of an atoxic fraction after treatment of the toxin with papain, is described.

Materials

Papain (EC. 3.4.4.10.), 30 U/mg, was purchased from Boehringer,

Mannheim, Germany. Sephadex gel was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Tetanus toxin was isolated from culture filtrates of Cl. tetani, grown on Latham medium essentially as described (4,5). The final product contained 2 800 - 3 000 Lf/mg N (6). Horse tetanus antitoxin (3 400 Lf/ml) for determining flocculation values was a product of Behringwerke, Marburg, Germany.

Antisera against split products of tetanus toxin or against papain were raised in rabbits by subcutaneous injection of 1 mg of protein in Freund's complete adjuvans followed by a booster injection after 4 weeks. Ten days later, the animals were exsanguinated and their serum tested for antibodies.

Analytical methods

Protein was determined according to Lowry et al. with serum albumin as standard (7). Polyacrylamide gel electrophoresis in SDS buffer was carried out according to Weber and Osborn (8). Double diffusion analysis in agar gel was performed by the method of Ouchterlony (9). Analytical gel chromatography for the determination of Stokes' radius was performed on a column (1.5 x 85 cm) of Sephadex G-200, equilibrated with 0.05 M phosphate buffer, pH 8.0, containing NaCl (0.25 M) and Na_2EDTA (0.025 M). The data were treated according to Siegel and Monty (10), using the relationship developed by Ackers (r = 215 mμ, ref. 11). Sedimentation velocity measurements were obtained from runs in 0.1 M phosphate buffer and the data were corrected for the concentration of the solute.

Digestion of tetanus toxin with papain

To a sample (0.7 g of protein; 300 000 Lf units) of tetanus toxin in 45 ml of 0.1 M phosphate buffer, pH 6.5, containing 0.001 M Na_2EDTA, were added 6 mg of cysteine-HCl and 4.5 ml of papain (10 mg protein/ml, 30 U/mg). The mixture was kept at 55°C for

4 hours, cooled and applied to a column (5 x 100 cm) of Sephadex G-100, eluted with 0.1 M Tris-HCl, pH 8.0, containing 1 M NaCl. The material corresponding to fraction C (Fig. 1)

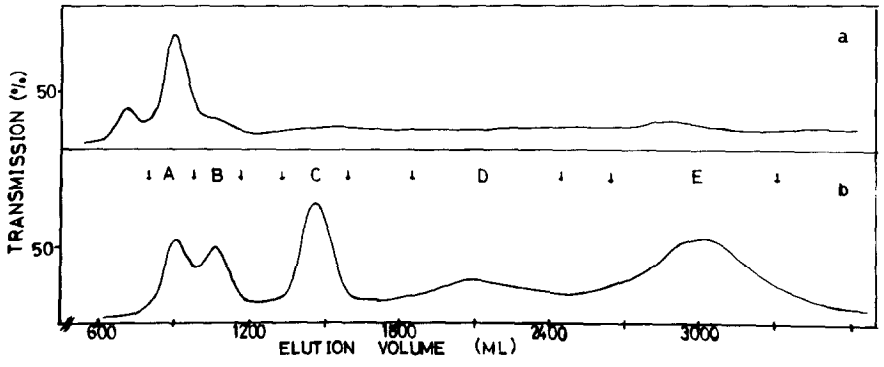

Fig. 1 Gel chromatography of tetanus toxin on Sephadex G-100 (a) before and (b) after digestion with papain. The effluent was continuously monitored at 254 nm and fractions were collected as indicated.

was concentrated by ultrafiltration and rechromatographed twice on the same column in order to remove traces of toxin which would otherwise interfere with the biological assays.

Toxicity and immunogenicity testing

Guinea pigs (250 - 350 g) were given subcutaneous injections of increasing doses of the fractions obtained after Sephadex chromatography. Mortality rates were recorded for 7 days. To test for immunogenicity, toxoids (5 μg N per ml) were adsorbed to 0.2 % $Al(OH)_3$ gel, diluted 1:100 and injected in 2 ml quantities into guinea pigs (10 animals per group). The animals were challenged 4 weeks later with 20 - 30 LD_{50} doses of tetanus toxin. After 10 days, the number of healthy animals was determined.

Results

Treatment of tetanus toxin with papain at 55°C followed by gel chromatography gave the pattern shown in fig. 1. Five fractions, denoted A - E were isolated and analyzed separately. Fractions A and B showed essentially the same toxicity per mg of protein as the parent toxin and were indistinguishable from the latter on polyacrylamide gel electrophoresis. Fraction C is described below. Fraction D lacked material reacting with tetanus antitoxin on double diffusion analysis. This fraction contained papain as determined by (a) proteolytic activity against L-α-benzoyl-arginine ethyl ester (12); (b) reaction with specific rabbit antiserum against papain; and (c) co-elution with ^{14}C-carboxy-methylated papain added to the digestion mixture prior to chromatography. Fraction E represented low molecular weight material and has as yet not been characterized further.

Properties of Fraction C

The material corresponding to fraction C (yield: 156 mg of protein, 22 %) showed partial identity with its parent toxin when tested with the Ouchterlony double diffusion analysis against horse antitoxin. With rabbit antiserum against fraction C, complete identity with tetanus toxin was observed (Fig. 2). The specific flocculation value of this fraction was 10 000 Lf/mg N, which exceeds by far the value obtained for the parent toxin. Hence the yield of fraction C, based on Lf-units, amounted to about 80 % of the starting material.

Fraction C was fairly resistant to further digestion with papain. Thus, redigestion of fraction C, employing five-times more papain per mg protein than for the standard incubation mixture, caused a loss of about half of the starting material. In contrast, recoveries of 20 % or less were recorded if fraction C was hydrolyzed with papain in the presence of dithiothreitol (0.001 M).

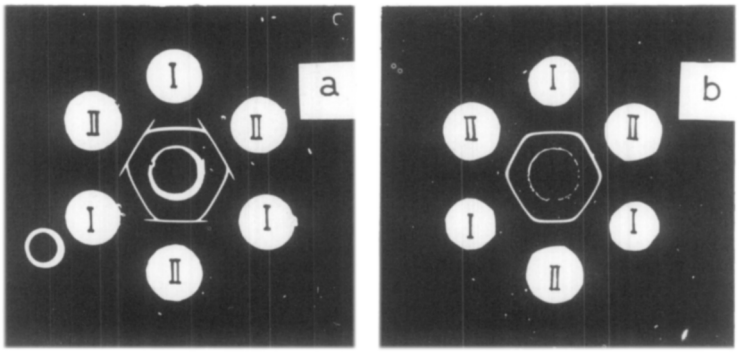

Fig. 2a Ouchterlony diffusion analysis of (I) fraction C (Fig. 1) and (II) tetanus toxin against equine tetanus antitoxin (centre well).

2b Same as a) except that the centre well was filled with rabbit antiserum against fraction C.

It should be noted that inclusion of this sulfhydryl reagent did not affect the activity of papain. The result would therefore seem to suggest that maintenance of disulfide bonds may be important for the stability of fraction C.
Although homogenous in the ultracentrifuge (3.8 S) the material from fraction C showed at least four bands on analysis on polyacrylamide gel electrophoresis (Fig. 3). On electrophoresis in SDS buffer, one zone in the molecular weight range 40 000 - 45 000 was obtained. This value agrees well with the value computed from the ultracentrifuge data presented in table 1.

Biological assays

Fraction C, after removal of traces of toxin by rechromatography was harmless to guinea pigs when injected in doses of 1 000 Lf units per animal. On immunizing guinea pigs with this fraction (immunizing dose, approximately 0.6 µg of protein per animal), the number of survivors ranged from 50 - 100 % after challenge with tetanus toxin. Higher doses of immunogen protected the animals completely.

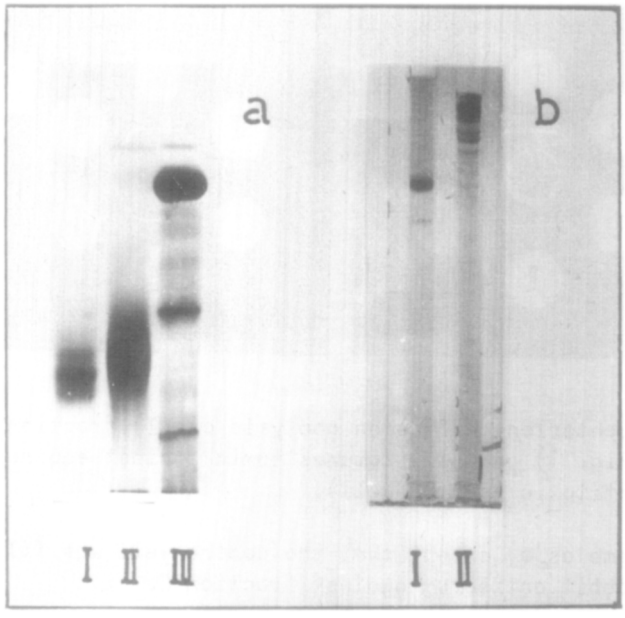

Fig. 3a Analysis of fraction C (I) on polyacrylamide gel electrophoresis. Tetanus toxin (II) and human serum (III) were run as reference compounds.

3b Analysis of fraction C (I) and tetanus toxin (II) on polyacrylamide gel electrophoresis in SDS buffer.

Table 1

Physico-chemical characteristics of fraction C

Sedimentation velocity coefficient	3.8×10^{-13} sec^{-1}
Stokes' molecular radius	25 Å
Diffusion constant	8.6×10^{-7} cm^2 sec^{-1}
Molecular weights	
By sedimentation diffusion analysis	42 500
By SDS gel electrophoresis	44 500

Discussion

On the basis of immunochemical and functional studies, previous work from several laboratories has indicated that separate deter-

minants on the tetanus toxin molecule may be responsible for (a) toxicity, (b) fixing to brain matter, and (c) immunological reactivity of this protein (13-17). Proteolytic fragmentation of tetanus toxin appears to be a worthwhile approach as a first step in attempting to define such receptor sites at the molecular level. In this report, a highly reproducible procedure for the isolation of a fraction from tetanus toxin, exhibiting partial immunological identity with the starting material, is described. It is concluded that the fraction isolated, although apparently lacking toxic determinants, may still induce the formation of neutralizing antibodies against tetanus. At present, it is not known whether a single antigenic determinant of fraction C is responsible for the production of such protective antibodies. Whether or not this fraction has lost the capacity to interact with brain matter also remains to be elucidated.

Tetanus toxin was remarkably stable to proteolysis by papain. Little or no digestion was observed at incubation temperatures below 40°C. The yield of the reaction product was, however, dependent upon maintenance of disulfide bonds and it was shown that the recovery of fraction C was impeded by the presence of dithiothreitol, an agent capable of hydrolyzing such bonds.

Some properties described here concerning fraction C are similar to those reported for "Antigen 2" a spontaneous conversion product of tetanus toxin (sedimentation constant, lack of toxicity and partial reactivity with tetanus antitoxin). The latter product was reported to have a specific flocculation activity of about 300 Lf/mg protein, however and was a poor immunogen (13,14). Fraction C, by contrast, was a potent immunogen and exhibited a specific flocculation activity in excess of 1200 Lf/mg protein. Since the conversion of tetanus toxin to Antigen 2 according to Peetoom and Van der Veer (13) has not been observed with the preparations used in this investigation, a direct comparison has not yet been possible.

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