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Tetanus toxoid purification: Chromatographic procedures as an alternative to ammonium-sulphate precipitation

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ABSTRACT

Given an existing demand to establish a process of tetanus vaccine production in a way that allows its complete validation and standardization, this paper focuses on tetanus toxoid purification step. More precisely, we were looking at a possibility to replace the widely used ammonium-sulphate precipitation by a chromatographic method. Based on the tetanus toxin's biochemical characteristics, we have decided to examine the possibility of tetanus toxoid purification by hydrophobic chromatography, and by chromatographic techniques based on interaction with immobilized metal ions, i.e. chelating chromatography and immobilized metal affinity chromatography. We used samples obtained from differently fragmented crude tetanus toxins by formaldehyde treatment (assigned as TTd-A and TTd-B) as starting material for tetanus toxoid purification. Obtained results imply that purification of tetanus toxoid by hydrophobic chromatography represents a good alternative to ammonium-sulphate precipitation. Tetanus toxoid preparations obtained by hydrophobic chromatography were similar to those obtained by ammonium-sulphate precipitation in respect to yield, purity and immunogenicity. In addition, their immunogenicity was similar to standard tetanus toxoid preparation (NIBSC, Potters Bar, UK). Furthermore, the characteristics of crude tetanus toxin preparations had the lowest impact on the final purification product when hydrophobic chromatography was the applied method of tetanus toxoid purification. On the other hand, purifications of tetanus toxoid by chelating chromatography or immobilized metal affinity chromatography generally resulted in a very low yield due to not satisfactory tetanus toxoid binding to the column, and immunogenicity of the obtained tetanus toxoid-containing preparations was poor.

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1. Introduction

Tetanus toxin (TTn) is an enormously potent neurotoxin secreted by the anaerobic soil bacterium *Clostridium tetani* [1]. As TTn induces death before an adaptive immunity could be generated, active vaccine immunization is crucial for the prevention of death caused by tetanus [2]. Nowadays, vaccination against tetanus is obligatory during childhood, as it is a part of regular vaccination schedule [3].

Tetanus vaccine is produced by a multi-step process comprising *C. tetani* cultivation, inactivation of secreted TTn by formaldehyde treatment, and purification of the obtained TTn derivatives. Formaldehyde-treated TTn, termed as tetanus toxoid (TTd), is devoid of toxicity, but is still highly immunogenic with a stabilized native conformation and it represents the main constituent of tetanus vaccine [4]. TTn-specific protective immune response can be induced by application of vaccines composed of alum-based adjuvant and TTd as a sole antigen (monovalent vaccine), or within a mixture with additional bacterial/viral antigens (polyvalent vaccine).

More recently, we have been witnessing a raising demand to establish a process of vaccine production in a way that allows its complete validation and standardization. Therefore, several steps in traditional TTd production have to be modified. In this paper we focus on TTd purification. Specifically, we were looking at a possibility to replace the ammonium-sulphate precipitation with the chromatographic method. Although experiments performed in the laboratory imply better characteristics of TTd obtained by formaldehyde treatment of previously isolated TTn, this procedure is not widely implemented in large-scale production, primarily due to safety reasons but also because initial intensive purification is still performed at the level of TTd.

Large-scale purification of proteins by ammonium-sulphate precipitation is a time consuming process and requires centrifugation. In contrast, liquid chromatography-based purification would allow faster and fully controlled TTd purification process. Further-

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more, since proteins separation by salt precipitation is dependant on their solubility within aqueous solution of anti-chaotropic salts there is a high probability of protein co-precipitation and, theoretically, it is impossible to purify a protein to a high degree using only salt fractionations. Using suitable chromatographic techniques or their combination, any protein, irrespective of its abundance within a sample, could be purified.

According to the published papers describing the usage of gel-filtration in TTd purification, it is evident that, in spite of the possibility to obtain TTd preparations of acceptable purity by gel-filtration, the obtained results imply its limited effectiveness, primarily due to aggregation of TTd itself, or with other proteins found in formalized *C. tetani* culture filtrates [5,6]. Based on the TTn's biochemical characteristics, we decided to examine the possibility of TTd purification by hydrophobic chromatography (HIC), chelating chromatography (CC) and immobilized metal affinity chromatography (IMAC).

TTn is heterodimeric glycoprotein comprised of heavy (100 kDa) and light (50 kDa) chains interconnected by disulphide bonds. It has been shown that in its native conformation heavy chain of TTn forms a hydrophobic surface area [7], implying the possibility of TTn purification by HIC. Analysis of TTn amino-acid sequence revealed numerous shorter hydrophobic regions involving up to 10 uncharged amino acids randomly distributed over the entire molecule, and two longer hydrophobic sequences - the first located on the light chain (from Tyr(223) to Ile(253)) and the second within the N-terminal moiety of heavy chain (from Asn(660) to Ala(691)) [8]. Measurement of TTn's Triton X-100 binding capacities and ability to form channel in phospholipid vesicles also implied on hydrophobicity of heavy chain's N-terminal moiety that is enhanced by pH lowering [9]. Furthermore, there are seven His residues within C-terminal part of TTn's heavy chain, arranged in a way that allows TTn purification by methods based on the specific interaction with immobilized metal ions (CC and IMAC) [10].

What occurs to these critical parts of TTn molecule during inactivation process is not clarified. Namely, due to formaldehyde treatment, peptides undergo a great diversity of chemical modifications which nature has not been fully explained yet. It is generally accepted that formaldehyde predominantly reacts with primary amino groups, especially with the functional groups of Lys. Modified lysine residues then react with Gln, Asn, Arg, and Tyr residues, resulting in intra- and intermolecular cross-linking [4,11,12]. It is also observed that more or less pronounced fragmentation of TTn heavy and light chains may occur prior to formaldehyde treatment [4,8,13,14].

Herein, we present the results of the experiments performed in order to evaluate: (1) whether some of the above-mentioned chromatographic techniques could present an alternative to ammonium-sulphate precipitation, and (2) to which extent the characteristics of crude TTn preparation could influence the quality of final TTd purificate. As a starting material, we used TTd samples obtained from differently fragmented crude TTns. Yield, purity, and antigenic and immunogenic characteristics of final purificates were compared among preparations obtained by HIC, CC and IMAC and to preparations obtained by ammonium-sulphate precipitation.

2. Experimental

2.1. Starting material for TTd purification

Starting material for TTd purification were preparations routinely designated as native TTd. Native TTd was obtained by formaldehyde treatment of crude TTn preparations, i.e. supernatants of *C. tetani* culture obtained by filtration (0.1 μm pore size). This is the part of routinely applied procedure in TTd production at the Institute of Virology, Vaccines and Sera – Torlak, Belgrade, Serbia. Native TTd preparations used in our experiments were assigned as TTd-A and TTd-B. They were prepared by formaldehyde treatment of corresponding crude TTn preparations, TTn-A and TTn-B, respectively, and they all passed in-process control tests.

2.2. Tangential ultrafiltration

Prior to any herein described purification process, formaldehyde and small peptides were removed from both TTd-A and TTd-B samples by tangential ultrafiltration against 10 volumes of 0.9% (w/v) NaCl. Starting material for each purification process was 50 ml of corresponding native TTd solution. The apparatus for tangential ultrafiltration consisted of two pumps (one for sample recirculation and the other for 0.9% (w/v) NaCl solution supply), hollow fiber cartridge (GE Healthcare; nominal molecular weight cut-off 30 kDa, membrane area 41 cm^2, fiber internal diameter 0.5 mm, nominal flowpath length 110 cm), feed reservoir having one outlet and 2 inlets (for retentate and for 0.9% (w/v) NaCl solution) and connecting tubings. Total (feed) flow rate trough the cartridge was set to be ~20 ml/min, where permeate flow rate was about 1/10th of retentate flow rate. Native TTd solution (50 ml) was placed within a feed reservoir, then 30 ml of 0.9% (w/v) NaCl was added and sample volume was held constant (~80 ml) during the whole process. Tangential ultrafiltration was carried out until 500 ml have been permeated. Subsequently, a supply of 0.9% (w/v) NaCl was closed and sample was concentrated up to ~20 ml.

2.3. Determination of protein concentration

Protein concentration was determined by Bradford assay [15], using solutions containing increasing concentration of bovine serum albumin (BSA) as reference. The concentration of the protein nitrogen (PN) was determined directly by Kjeldahl method [16], or indirectly by dividing protein concentrations determined by Bradford assay with factor 6.25 [17].

2.4. Precipitation of TTd with ammonium-sulphate

TTd was precipitated by addition of ammonium-sulphate ($(NH_4)_2SO_4$) to a 40% saturation at 25 °C. The solution was allowed to stand overnight at 4 °C to permit precipitation of proteins. After that, samples were centrifuged at 8700 × *g* for 15 min. The supernatant was discarded; the precipitate was dissolved in phosphate buffered saline (PBS) and dialyzed over night against PBS.

2.5. Hydrophobic chromatography

TTd purification by HIC was performed on HiTrap Phenyl HP column (GE Healthcare, Uppsala, Sweden) packed with 5 ml of Phenyl Sepharose (internal diameter × height: 1.6 cm × 2.5 cm). Column was previously equilibrated in 0.5 M $(NH_4)_2SO_4$/3 M NaCl/0.02 M Na-phosphate buffer, pH 7.6. Prior to loading the column, $(NH_4)_2SO_4$ (final concentration 0.5 M) and NaCl (final concentration 3 M) were added to samples. After sample loading, the column was washed out with 5 column volumes (CV) of 3 M NaCl/0.02 M Na-phosphate buffer, pH 7.6 in order to remove unbound/weakly bound proteins. TTd was eluted by 0.02 M Na-phosphate buffer, pH 7.6. The concentration of proteins within collected fractions (1.0 ml) was determined by Bradford assay. Protein fractions were dialyzed over night against PBS and were kept at −20 °C for further analysis.

2.6. Immobilized metal-affinity chromatography and chelating chromatography

TTd purifications based on the interaction with immobilized metal ions were performed by: (1) CC using HiTrap™ chelating HP columns (GE Healthcare, Uppsala, Sweden) packed with 5 ml (internal diameter × height: 1.6 cm × 2.5 cm) of Chelating Sepharose™ High Performance or (2) IMAC using HiTrap™ IMAC HP columns (GE Healthcare, Uppsala, Sweden) packed with 5 ml (internal diameter × height: 1.6 cm × 2.5 cm) IMAC Sepharose™ High Performances. After washing the columns with distilled water, 10 ml of water solutions containing copper(II)sulphate (Cu^{2+}-CC, Cu^{2+}-IMAC), zinc(II)sulphate (Zn^{2+}-CC) and cobalt(II)chloride (Co^{2+}-CC, Co^{2+}-IMAC) at final concentration of 0.02 M were applied in order to saturate the column with specific metal ions. Unbound metal ions were washed out with 5 CV of distilled water. After that, columns were equilibrated with starting buffer (5 mM imidazole/3 M NaCl/0.02 M Na-phosphate buffer, pH 7.5). Concentrations of imidazol and NaCl within sample and its pH were also adjusted to the starting buffer values. After sample loading, the unbound material was washed out with 5 CV of starting buffer. The bound proteins were eluted using 0.5 M imidazole/3 M NaCl/0.02 M Na-phosphate buffer, pH 7.5. The concentration of proteins within collected fractions (1.0 ml) was determined by Bradford assay. Protein fractions were dialyzed over night against PBS and were kept at −20 °C until further analysis. The columns were regenerated by washing with 10 CV of distilled water.

2.7. Flocculation test

Limes flocculation (Lf) values for samples containing TTn or TTd were determined by flocculation test [16] using an in-house-anti-tetanus serum calibrated against the 2nd International Standard for tetanus antitoxin (NIBSC, Potters Bar, UK) as reference. The concentration of reference anti-tetanus serum is given as Lf-equivalent/ml [18]. The determination of sample Lf content was based on fact that formation and subsequent precipitation of antigen–antibody complexes is the most prominent when their concentrations are equal. Briefly, equal volumes of reference antiserum in increasing concentration and TTn/TTd containing sample were mixed and placed in water bath preheated at 50 °C. The tubes were observed continuously, and the tube where the flocculation was the fastest was selected. The Lf value of the sample was calculated from the concentration of antiserum in this tube. The time required for flocculation (Kf) was also registered.

In case of low sample protein concentrations, Lf content was determined by blend flocculation [16]. The only difference between blend flocculation and standard procedure is in the "spiking" of analyzed sample by the addition of TTd with known Lf content.

2.8. Evaluation of antigenic characteristics of isolated TTd

Antigenic characteristics of isolated TTd molecules were analyzed by direct ELISA, using monoclonal antibodies (MoAbs) assigned as MoAb 26 [19], MoAb 51 and MoAb 71 [20] specific for different epitopes on TTd/TTn molecules. ELISA plates (MaxiSorp; Nunc, Roskilde, Denmark) were covered (50 μl/well) with TTd (2 μg/ml TTd dissolved in PBS) by overnight adsorption at 4 °C. The plates were blocked with 1% BSA/PBS (w/v) for 2 h at room temperature. The blocking, as well as all subsequent ELISA steps, were followed by washing (4 × 200 μl/well) with 0.05% Tween 20/PBS (v/v). Biotin-labeled MoAbs (2.5 μg/ml dissolved in 1% (w/v) BSA/PBS, 50 μl/well) were incubated for 1 h at room temperature. The system ExtrAvidin®-peroxidase/OPD was used for detection of binding. The absorbance was read at 492/620 nm ($A_{492/620}$).

2.9. Inhibition of anti-tetanus sera binding to TTn by TTd purificates

Evaluation of immunogenic characteristics of TTd purificates was performed using competitive ELISA. Microtiter plates (MaxiSorp; Nunc, Roskilde, Denmark) were covered (50 μl/well) with TTn (2 μg/ml TTn dissolved in PBS) by overnight adsorption at 4 °C. The plates were blocked with 1% BSA/PBS (w/v) for 2 h at room temperature. The blocking, as well as all subsequent ELISA steps, were followed by washing (4 × 200 μl/well) with 0.05% Tween 20/PBS (v/v). After that, samples (preincubated for 1 h at room temperature) containing purified TTds in increasing concentrations mixed with 0.1 IU/ml of standard human anti-tetanus immunoglobulins (1st International Standard for Tetanus Immunoglobulin, NIBSC code TE-3; NIBSC, Potters Bar, UK) prepared in 1% BSA/PBS (w/v), were added to plates (50 μl/well). Standard serum IgG binding was detected with biotin-labeled anti-human IgG antibodies (Sigma). The system ExtrAvidin®-peroxidase/OPD was used for "visualization" of bound TTn-specific human IgG. The absorbance was read at 492/620 nm.

Solutions containing only standard human anti-tetanus serum in increasing concentrations (up to 0.1 IU/ml) were treated the same way and were used as standards. The percentage of inhibition for each sample was calculated from standard curve $A_{492/620}$ versus standard human anti-tetanus serum concentrations.

2.10. Sodium dodecyl sulphate - polyacrylamide gel electrophoresis

TTn or TTd containing samples were analyzed by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) under reducing (samples contained 5% β-mercaptoethanol) or non-reducing conditions. When analysis was performed on Phast System (Pharmacia, Uppsala, Sweden), commercially available gradient polyacrylamide gels (4–15%) were used. Samples were also analyzed by SDS-PAGE under reducing conditions on 0.75 mm-thick 9% or 7% separating polyacrylamide gels, with 4% stacking gel (Mini Protean II System, Bio-Rad, USA). The gels were stained with Coomassie brilliant blue R-250 stain.

2.11. Determination of free $-NH_2$ group content

The concentration of free $-NH_2$ groups was determined by a colorimetric method using 2,4,6-trinitrobenzene sulfonic acid (TNBSA) [21]. Briefly, concentration of proteins in analyzed samples was adjusted to 100 μg/ml by dilution in 0.1 M sodium-bicarbonate buffer, pH 8.5. The working TNBSA solution (0.01% TNBSA dissolved in 0.1 M Na-bicarbonate buffer, pH 8.5; 0.5 ml) was mixed with 1 ml of protein solution and incubated at 37 °C for 2 h. After that, 0.5 ml of 10% SDS and 0.25 ml of 1 M HCl were added to each sample in order to stop reaction and stabilize the obtained product. Absorbance was measured at 335 nm (A_{335}).

Solutions containing Gly or Lys in increasing concentrations (ranging up to 20 μg/ml) were treated the same way and were used as standards. Free $-NH_2$ group content of analyzed protein samples was determined from the standard curve obtained for Gly or Lys (A_{335} versus free $-NH_2$ group concentration).

3. Results and discussion

3.1. Native TTn

Native TTd preparations, assigned as TTd-A and TTd-B, were prepared by formaldehyde treatment of crude TTn preparations, TTn-A and TTn-B, respectively, and their basic characteristics are listed in

Table 1
Characteristics of crude TTn preparations and corresponding native TTd preparations used as starting material for chromatographic purification of TTd.

	TTn - A	TTn - B	TTd - A	TTd - B
Total nitrogen (TN) (mg/ml)[a]	3.65	3.8	3.36	3.75
Protein nitrogen (PN) (mg/ml)[a]	0.062	0.069	0.061	0.07
Proteins[b] (mg/ml)	0.264	0.300	0.363 (0.381)	0.425 (0.438)
Antigen content (Lf/ml)	75	75	65	70
Time of flocculation (Kf) (min)	14	11	18	14
TTn/TTd purity (Lf/mg PN)	1209	1087	1066	1000

[a] Determined by Kjeldahl method.
[b] Determined by Bradford method; values within parenthesis represent protein concentrations calculated by multiplying corresponding PN concentration with factor 6.25.

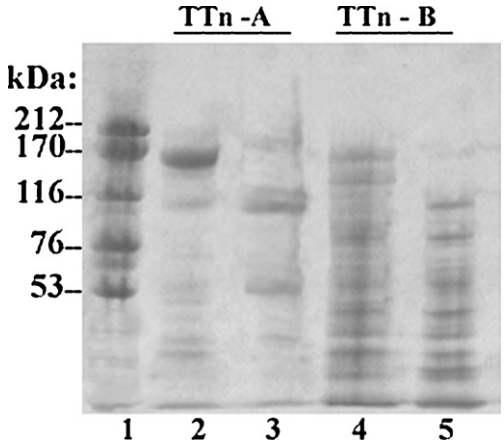

Fig. 1. SDS-PAGE (4–15% polyacrylamide gel, PhastSystem, Pharmacia) of crude TTn-A and TTn-B under non-reducing (lane 2 and lane 4, respectively) and reducing (lane 3 and lane 5, respectively) conditions. High molecular weight molecular markers (Pharmacia) were prepared according to the manufacturer's instructions (lane 1).

Table 1. According to the data presented, the used TTn preparations were very similar. However, their electrophoretical analysis implied a more pronounced fragmentation within TTn-B sample (Fig. 1). Results of free $-NH_2$ content measurement performed on TTns from crude preparations isolated by HIC for the purpose of this measurement, showed significant difference in free $-NH_2$ content. Namely, free $-NH_2$ contents of TTn isolated from TTn-A and TTn-B were 0.588 μmol/mg of protein and 1.515 μmol/mg of protein, respectively. Theoretically, according to the amino acid sequence, i.e. counting both the amino acids located at TTn surface and those within native molecule, there is a 1.4 ± 0.3 μmol $-NH_2$/mg of protein in TTn molecule [8,22,23].

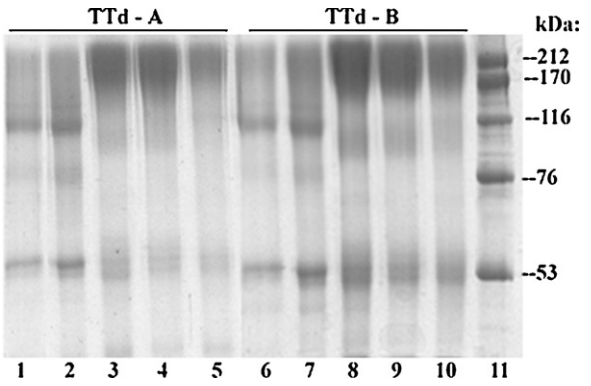

Fig. 2. SDS-PAGE under reducing conditions (9% polyacrylamide gel, Mini Protean II System, Bio-Rad) of native TTd-A (lane 1) and TTd-B (lane 6) and purificates obtained out of them by tangential ultrafiltration (lane 2 and lane 7, respectively), hydrophobic chromatography (lane 3 and lane 8, respectively), ammonium-sulphate precipitation (lane 4 and lane 9, respectively) and Cu^{2+} - chelating chromatography (lane 5 and lane 10, respectively). High molecular weight markers (Pharmacia) were prepared according to the manufacturer's instructions (lane 11).

3.2. Purification of TTd

According to parameters described in Table 1, there were no significant differences between TTd-A and TTd-B samples, which were used as starting material for TTd purification process. Although crude TTn-A and TTn-B preparations differed in respect of fragmentation degree, the electrophoretical analysis of final purificates obtained out of their corresponding native TTd preparations revealed the same pattern of protein bands mobility (Fig. 2, lane 1 and lane 6).

This implies a possibility that, irrespective of the intensive polipeptide chain fragmentation in TTn-B, all its parts were held together by, most probably, non-covalent interactions or disulphide bonds, and then merged succesively in TTd molecule, having the same molecular weight as the one obtained from TTn-A. In order to reveal the extent of native TTn structure preservation in both cases, the purification of TTd was performed by chromatographic techniques based primarily on native TTn characteristics. Namely, TTd was purified from TTd-A and TTd-B preparations by CC and IMAC charged with various metal ions, as well as by HIC. The size of TTd-containig peaks eluted from various chromatographic media after non-TTd proteins elution is presented on Fig. 3. The presence of TTd in eluted fractions was checked by ELISA using MoAbs specific for epitopes located on heavy and light chains of TTn/TTd.

Attending to design a HIC procedure that will allow optimal TTd loading and its elution within one single peak in high yield and purity, several experiments were performed where composition

Table 2
Characteristics of TTd containing fractions purified by tangential ultrafiltration and further by assigned chromatographic procedures.

Samples	TTd-A			TTd-B		
	Proteins (mg)	Yield[a] (%)	TTd purity (Lf/mgPN[b])	Proteins (mg)	Yield[a] (%)	TTd purity (Lf/mgPN[b])
Tangential ultrafiltration	15.63	100	1294	17.85	92.8	1138
$(NH_2)_4SO_4$ precipitated	4.56	57.4	2056	8.56	73.3	1769
HIC	4.10	53.8	2194	8.30	81.5	2000
Cu^{2+}-CC	1.30	8	1250	3.30	5.4	356
Zn^{2+}-CC	0.14	2.8	3156	0.43	3.2	1625
Co^{2+}-CC	nd	nd	nd	nd	nd	nd
Cu^{2+}-IMAC	0.05	6.9	1587	0.95	2.3	525
Co^{2+}-IMAC	0.75	5.4	1475	1.45	3.2	488

nd, not done.
[a] Calculated as Lf content recovery using corresponding native TTd preparation as reference.
[b] PN concentrations were calculated by dividing protein concentrations determined by Bradford assay with factor 6.25.

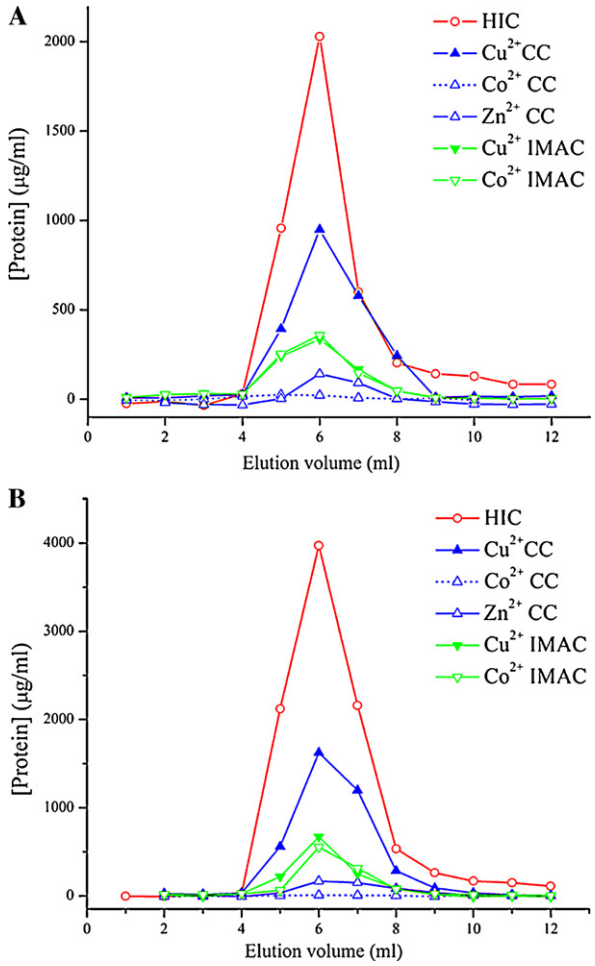

Fig. 3. TTd purificates obtained out of native TTd-A (A) and TTd-B (B) preparations by chromatography performed on HiTrap™ Phenyl Sepharose column (HIC), HiTrap™ chelating HP columns loaded with Cu^{2+}(Cu^{2+}-CC), Co^{2+} (Co^{2+}-CC) or Zn^{2+} (Zn^{2+}-CC) and on HiTrap™ IMAC HP columns loaded with Cu^{2+}(Cu^{2+}-IMAC) or Co^{2+} (Co^{2+}-IMAC). Volume of all used columns was 5 ml. Concentration of proteins within eluted fractions (V = 1 ml) was determined by Bradford assay.

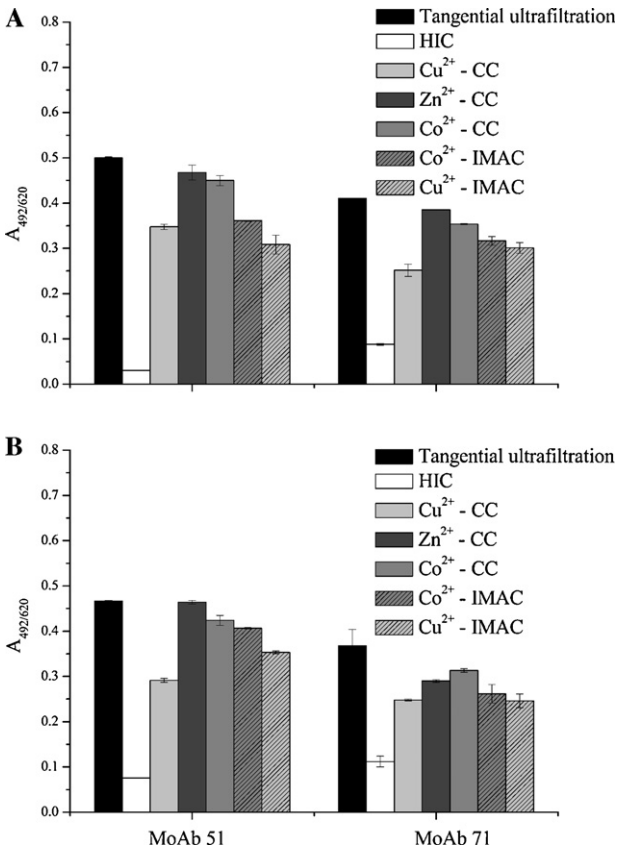

Fig. 5. Evaluation of TTd binding to the specific chromatographic column. Appropriately prepared TTd-A (A) or TTd-B (B) were loaded onto specific matrix. The presence of TTd in protein fractions collected during loading was evaluated by direct ELISA using MoAb 51 and MoAb71. The protein concentration of samples was adjusted to 2 μg/ml prior to adsorption onto microtiter plate. Samples were assayed in triplicate and results are presented as mean $A_{492/620}$ ± standard error.

of loading buffer and elution regime were varied (Fig. 4). Actually, concentration of NaCl and $(NH_4)_2SO_4$ in loading buffer and concentration of NaCl in eluting buffers were varied. In short, it was shown that concentration of $(NH_4)_2SO_4$ within sample loaded on the column must not be higher than 0.5 M in order to prevent TTd precipitation in it. Furthermore, the majority of TTd was eluted with low ionic strength phosphate buffer (20 mM Na-phosphate buffer, pH 7.6), but the amount of TTd detected in fractions eluted with 1.5 M NaCl/20 mM Na-phosphate buffer, pH 7.6, was not negligible either. Taking into account all obtained results, we decided to make the loading conditions more strict by an addition of 3 M NaCl plus 0.5 M $(NH_4)_2SO_4$ into the sample, and to elute TTd with 20 mM Na-phosphate buffer, pH 7.6, after elution of non-TTd containig fractions with 3 M NaCl/20 mM Na-phosphate buffer, pH 7.6.

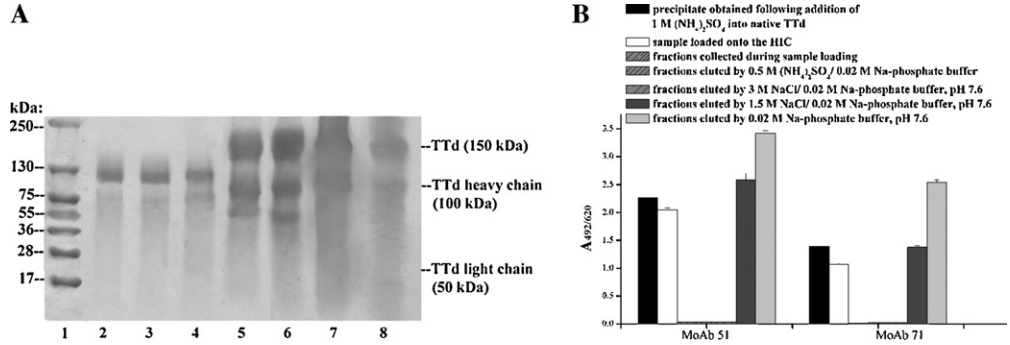

Fig. 4. Summary of optimization of TTd purification by hydrophobic chromatography. (A) SDS PAGE (7% polyacrylamide gel) of samples collected during process optimization: lane 1 – high molecular weight markers (Pharmacia), lane 2 – fractions collected during sample loading (loading buffer 0.5 M $(NH_4)_2SO_4$/3 M NaCl/0.02 M Na-phosphate buffer, pH 7.6), lane 3 – fractions eluted with 0.5 M $(NH_4)_2SO_4$/0.02 M Na-phosphate buffer, lane 4 – fractions eluted with 3 M NaCl/0.02 M Na-phosphate buffer, lane 5 – fractions eluted with 1.5 M Nacl/0.02 M Na-phosphate buffer, lane 6 – fractions eluted with 0.02 M Na-phosphate buffer pH 7.6, lane 7 – sample loaded onto the HIC, lane 8 – precipitate obtained following addition of 1 M $(NH_4)_2SO_4$ into native TTd; (B) evaluation of TTd presence (2 μg/ml protein) by direct ELISA using MoAb 51 and MoAb 71 in samples collected during process optimization. All samples were assayed in triplicate and results are presented as mean $A_{492/620}$ ± standard error.

The principle of protein purification by IMAC and CC are the same and, consequently, many authors do not make the strict distinction between these two methods. Although some differences could be noticed in matrices' structure and the type of covalently immobilized chelating groups on them, both methods are based on specific interaction between immobilized metal ions and certain amino acid side chains exposed on the surface of proteins (mainly His and to a lesser extent Cys and Trp) [24,25]. The strength of interaction with immobilized metal ions is dependent on the type, number and spatial distribution of the amino acid side chains, and on the nature of the metal ion used [24,25]. As it is not always possible to predict which metal ion is the most appropriate, the purification of TTd was assessed on IMAC and CC columns loaded with various transition metals ions. Namely, for purification of untagged proteins, Cu^{2+} ions are frequently used, but when the binding characteristics of an untagged target protein are not known, it is advisable to also test other metal ions (e.g. Zn^{2+}, Ni^{2+}, Co^{2+}) in order to establish the most suitable metal ion to use [24,25].

General characteristic of purifications based on TTd interaction with immobilized metal ions was a very low yield due to unsatisfactory TTd binding to the column in the presence of 5 mM imidazole (Fig. 5). Additionally performed experiments showed that the omission of imidazole from the loading buffer or its use in lower concentration makes these purification procedures less specific, as purity of the obtained TTd preparations was not improved at all (data not shown). The possible explanations for poor interaction of TTd with metal ions-loaded matrices could be the following: (1) already captured Zn^{2+} ions interfere (TTn is Zn^{2+}-dependent endopeptidase), with interactions of TTd and metal ions immobilized on matrix; (2) treatment with formaldehyde affects the mutual orientation of amino acid residues responsible for interactions with immobilized metal ions. The presumption about the necessity of the appropriate three-dimensional arrangement of interacting amino acids is supported by the following facts: (1) significantly higher TTd purity was achieved in preparations purified from TTd-A compared to corresponding preparations obtained from TTd-B; (2) comparing purifications performed on the same matrix (CC or IMAC) from the aspect of yield, the best, but not satisfactory, results were obtained when Cu^{2+} was used; this is in agreement with literature data showing that the single exposed His residue may result in adsorption of the protein to Cu^{2+} [25].

Electrophoretic analysis of all obtained TTd preparations did not show any significant differences among them (Fig. 2). Apart from more or less intensive bands corresponding by molecular weight to heavy (~100 kDa) and light (~50 kDa) chains, protein bands of ≥150 kDa dominated in purified TTd samples. They represent TTd molecules in which formaldehyde-treatment resulted in formation of additional, non-disulphide, covalent bonds between light and heavy chains or their polymers raised due to formation of intermolecular covalent bonds [11]. However, comparison of TTd preparations obtained by chromatography from the aspects of both yield and purity implies on HIC as a method of choice. Furthermore, HIC was the method where the structural characteristics of TTn subjected to formaldehyde treatment had the lowest influence on the purity of the final purification product. Specifically, it seems that fragmentation of TTn molecule, routinely observed by many authors [4,13,14], allows generation of TTd molecules more suitable for purification by HIC, most probably due to the higher exposition of hydrophobic areas.

3.3. Antigenic and immunogenic characteristics of purified TTd

The antigenic profile of TTd preparations obtained by specific purification process was evaluated by direct ELISA, using MoAbs specific for various TTn/TTd epitopes (Fig. 6). MoAb 51 is specific for the epitope positioned on light TTn/TTd chain, while the target

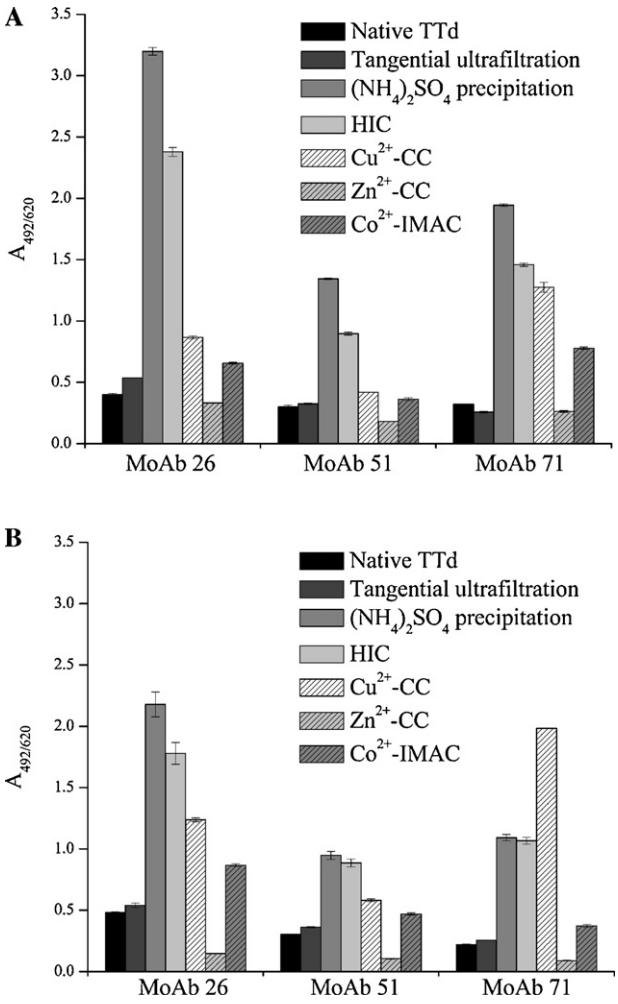

Fig. 6. Binding of tetanus-specific monoclonal antibodies MoAb 26, MoAb 51 and MoAb 71 to TTd purificates obtained out of native TTd-A (A) and TTd-B (B) preparations by assigned chromatographic techniques. Reactivity of MoAbs with TTd-containing preparations (2 μg/ml proteins) was measured by direct ELISA. All samples were assayed in triplicate and results are presented as mean $A_{492/620}$ ± standard error.

epitope of MoAb 71 is located on heavy chain [20]. MoAb 26 specifically recognizes the epitope located on heavy chain, in the vicinity of ganglioside-binding site, and its structure is stabilized during treatment with formaldehyde [19]. Generally, reactivity of specific MoAbs with indicated samples were proportionate, leading us to the conclusion that all TTd preparations mostly contain "complete" TTd molecules, i.e. molecules containing all tested epitopes. The samples obtained after TTd-B purification by ammonium-sulphate precipitation or HIC were the exceptions. The reactivity of MoAb71 with these samples was not proportional to the reactivity of other MoAbs; expression of epitope recognized by MoAb 71 (located on heavy chain) was lower compared to other tested epitopes. This implies that ammonium-sulphate precipitation or HIC allows isolation of "damaged" TTd molecules, which is of great importance since more or less pronounced fragmentation of tetanus antigen prior to formaldehydization may occur [4,8,13,14]. Finally, it could be observed that the reactivity of used MoAbs with obtained TTd preparations (Fig. 6) was not in strict accordance with their previously determined purity (Table 2.). This could be explained by the nature of standard antiserum used for Lf content determination. Namely, it represents a mixture of numerous antibodies (polyclonal antibodies) that specifically recognize various epitopes on a TTd molecule.

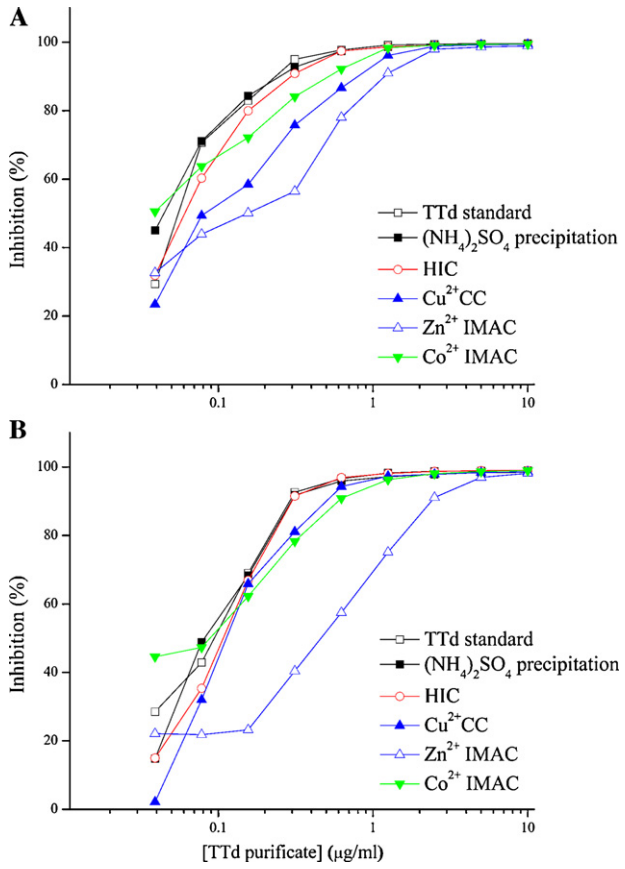

Fig. 7. Inhibition of binding of IgG from standard human anti-tetanus serum (NIBSC code: TE-3) to TTn (2 μg/ml) adsorbed onto ELISA microplate by TTd purificates obtained out of native TTd-A (A) and TTd-B (B) using assigned chromatographic techniques. Mixtures of standard anti-tetanus antiserum (0.1 IU/ml) and specific TTd purificates in rising concentrations incubated for 1 h at room temperature were used as samples. TTd preparation supplied by NIBSC, Potters Bar, UK, was used as a standard. All samples were assayed in triplicate and mean percentage of inhibition are presented.

The determination of antigenicity is important for the confirmation that the molecule in question is really TTd. However, the determination of isolated TTd immunogenicity is of a higher importance. According to the European Pharmacopeia, the immunogenicity test has to be routinely performed on guinea pigs as one of the release tests. In order to reduce the use of animals, we decided to indirectly evaluate the immunogenicity of the obtained TTd preparations trough their ability to inhibit the binding of IgG molecules from standard human anti-tetanus sera to TTn. The protective capacity of standard sera, expressed in international units per milliliter of sera, represents its ability to neutralize TTn and, therefore, prevent the appearance of TTn-induced pathology. We hypothesized that the inhibitory potential of TTd preparations would reflect the abundance of epitopes crucial for the induction of protective TTn-specific immune response, providing the basis for comparison of their immunogenicity according to the results obtained by competitive ELISA. In both cases (TTd-A and TTd-B) concentrations needed for complete inhibition were the lowest for samples obtained by ammonium sulphate precipitation and HIC, and these concentrations were identical to the one for TTd standard, providing complete inhibition under the same conditions (Fig. 7). This could be a consequence of their higher purity, but not exclusively, as Lf content of samples obtained from TTd-A and TTd-B by methods based on interaction with immobilized metal ions differ significantly, while their concentrations providing complete inhibition are very similar.

4. Conclusion

The purpose of the research presented in this paper is to provide the basis for improvement of TTd purification step in a large-scale production of TTd. In methods based on interaction with immobilized metal ions (CC and IMAC), the yields were low and purity (Lf content) was highly dependent on characteristics of the starting material, being lower for samples where TTn fragmentation occurred. On the contrary, the purification of TTd by HIC represents a good alternative to widely used ammonium-sulphate precipitation. HIC-obtained TTd preparations were similar to those obtained by ammonium-sulphate precipitation in respect to yield, purity and immunogenicity. In addition, the characteristics of starting material had the lowest impact on TTd preparations when HIC was the applied method of purification with their immunogenicity similar to standard TTd preparation. Initially, purification of TTd by salt precipitation seems to be a cheaper process, primarily due to low cost of ammonium-sulphate, but it is less specific and accompanied by tedious in process validation and standardization, handling of high sample volumes and waste managing problems. On the other hand, purification procedure based on tangential ultrafiltration along with HIC would provide a possibility to obtain TTd of higher purity in the same or better yield by a process that could be fully controlled and easier to validate. Proper handling of cartridges for tangential ultrafiltration and HIC column would allow their use over a long period that would justify their high initial cost. Hence, taking all the facts into account, HIC as a method for TTd purification would have a beneficial cost effect.

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