Optimization of tetanus toxoid ammonium sulfate precipitation process using response surface methodology

Marija Brgles\textsuperscript{a}, Pero Prebeg\textsuperscript{b}, Tihana Kurtović\textsuperscript{c}, Jelena Ranić\textsuperscript{c}, Martina Marchetti-Deschmann\textsuperscript{d}, Günter Allmaier\textsuperscript{d}, and Beata Halassy\textsuperscript{a}

\textsuperscript{a}Centre for Research and Knowledge Transfer, University of Zagreb, Zagreb, Croatia; \textsuperscript{b}Faculty of Mechanical Engineering and Naval Architecture, University of Zagreb, Zagreb, Croatia; \textsuperscript{c}Bacterial Vaccine Department, Institute of Immunology, Zagreb, Croatia; \textsuperscript{d}Institute of Chemical Technologies and Analytics, Technische Universität Wien, Vienna, Austria

\textbf{ABSTRACT}
Tetanus toxoid (TTd) is a highly immunogenic, detoxified form of tetanus toxin, a causative agent of tetanus disease, produced by \textit{Clostridium tetani}. Since tetanus disease cannot be eradicated but is easily prevented by vaccination, the need for the tetanus vaccine is permanent. The aim of this work was to investigate the possibility of optimizing TTd purification, i.e., ammonium sulfate precipitation process. The influence of the percentage of ammonium sulfate, starting amount of TTd, buffer type, pH, temperature, and starting purity of TTd on the purification process were investigated using optimal design for response surface models. Responses measured for evaluation of the ammonium sulfate precipitation process were TTd amount (Lf/mL) and total protein content. These two parameters were used to calculate purity (Lf/mgPN) and the yield of the process. Results indicate that citrate buffer, lower temperature, and lower starting amount of TTd result in higher purities of precipitates. Gel electrophoresis combined with matrix-assisted laser desorption ionization–mass spectrometric analysis of precipitates revealed that there are no inter-protein cross-links and that all contaminating proteins have pIs similar to TTd, so this is most probably the reason for the limited success of purification by precipitation.

\textbf{Introduction}
Tetanus toxoid (TTd) is a deactivated form of tetanus toxin (TTx), a protein produced by \textit{Clostridium tetani} (\textit{C. tetani}) causing tetanus disease. \textit{C. tetani} is an anaerobic bacterium, forming spores present in the environment, particularly in the soil of warm and moist areas.\textsuperscript{[1]} Tetanus disease is still causing a large number of deaths (213,000 deaths in 2002) mostly as a neonatal and maternal tetanus in the countries with insufficient health care and a limited availability of the vaccine.\textsuperscript{[1]} Tetanus is readily preventable through immunization with tetanus toxoid vaccine, which consists of a nontoxic but highly immunogenic protein.

Tetanus toxin is a 150 kDa protein consisting of an N-terminal light chain (50 kDa) and a C-terminal heavy chain (100 kDa) bridged by a disulfide bond. Intracellular form of TTx is a one-chain form, whereas extracellular form is cleaved by endogenous proteases to a two-chain form, required for neurotoxicity,\textsuperscript{[2–5]} linked through a disulfide bridge and noncovalent interactions. N-terminal part of the heavy chain (HN) is responsible for membrane translocation, whereas C-terminal part (HC) participates in receptor binding.\textsuperscript{[6,7]} Light chain (L) exhibits metalloproteinase activity and cleaves synaptobrevin, resulting in prevention of synaptic vesicle fusion with the plasma membrane,\textsuperscript{[8,9]} i.e., blocking of the neurotransmission thereby eliciting spastic paralysis—typical symptom of tetanus.\textsuperscript{[1]}

Conventional production of tetanus toxoid vaccine includes growth of toxigenic strain of \textit{C. tetani} in a liquid medium that enhances toxin production followed by toxin harvest by filtration, detoxification by formaldehyde, and several steps of purification and sterilization. The toxoid is adsorbed to aluminum or calcium salts to increase immunogenicity.\textsuperscript{[1]} Formaldehyde reacts with proteins mostly through amino group of the N-terminal amino acid residue and the side chains of Arg, Cys, His, and Lys.\textsuperscript{[10,11]} TTx consists of three domains: HC, HN, and L, each of which have their function: receptor recognition, membrane translocation, and synaptobrevin cleavage (respectively), and diminishing any of these functions by chemical modification of amino acids in these domains would result in detoxification. Which modification precisely is responsible for detoxification remains to be determined.\textsuperscript{[12]} It was shown that formaldehyde treatment of TTx does not alter secondary structure of the protein and that TTd remains highly immunogenic and actually increases immunogenicity most probably due to the increased stability after cross-linking.\textsuperscript{[13]}
Purification of TTd is traditionally performed by ammonium sulfate precipitation but other purification procedures are being investigated as well. Gel filtration chromatography, metal affinity chromatography, and hydrophobic interaction chromatography were also investigated with the aim of improvement of production process. However, ammonium sulfate precipitation is still the principally used method in tetanus toxoid vaccine production. Ammonium sulfate precipitation is one of the oldest techniques for the purification of proteins and is still used, e.g., in reducing of sample complexity prior to proteomic analysis.

Tetanus toxoid of high purity is a prerequisite for a tetanus toxoid vaccine. The aim of our research was to explore whether purification of TTd using ammonium sulfate precipitation can be improved by changing solution conditions such as pH, buffer composition, temperature, and starting purity of TTd. These factors were tested using design of experiments (DoE) methodology, optimal design for response surface models. In comparison to one-factor-at-a-time experimentation, DoE reduces the number of experiments and enables identification of not only the factors significant for a process but also the possible interactions of these factors. Response surface methodology in biotechnology has been applied to many areas of research in biology and the bioprocessing industries.

Materials and methods

Chemicals
Iodoacetamide (IAA), 1,4-dithio-DL-threitol (DTT), α-cyano-4-hydroxycinnamic acid (CHCA), Coomassie Brilliant Blue R250 and all peptide standards were from Sigma (St. Louis, MO, USA). Trypsin (proteomics grade) was obtained from Roche Diagnostics (Mannheim, Germany). All organic solvents were of analytical or LiChrosolv grade from Merck (Darmstadt, Germany) and ultrapure water was obtained fresh on each day by Simplicity purification system (Millipore, Billerica, MA, USA). All other chemicals were from Sigma (St. Louis, MO, USA) or Kemika (Zagreb, Croatia).

Production of tetanus toxoid
Tetanus toxin was obtained from Clostridium tetani (Harvard strain). Toxin was detoxified by formaldehyde treatment and then ultrafiltrated. Buffer exchange was also performed using ultrafiltration on 100-kDa membrane (Millipore, Billerica, MA, USA).

Quantification of tetanus toxoid
Quantification of TTd was performed by radial immunodiffusion (RID). Agarose gel was prepared as 1% (w/w) agarose solution in Dulbecco’s phosphate buffered saline (DPBS) and brought to boil, and after cooling to around 60°C mixed with standard antitetanus toxoid antibody (22 mL of agarose with 0.45 mL of antibody) it is poured into 10 × 10-cm plastic plate. Prior to use, 20 holes were punched per one gel and samples as well as standards (6 µL) were applied in duplicate (or triplicate). Assay was repeated at least three times (in three different days), and for DoE four times. Diameters of immunoprecipitates were read after 48 h using a magnifier, and the quantification was performed according to the calibration curve. Before the actual quantification assay, orientation assay was performed to enable sample dilution estimation. One lyophilized TTd batch calibrated against international NIBSC standard was used as a standard. Calibration curve ranged from 15 to 75 Lf/mL (limes of flocculation [Lf] is the unit for TTd quantity).

Ammonium sulfate precipitation
Ammonium sulfate precipitation was performed by the addition of an appropriate amount of solid ammonium sulfate to 2 mL of TTd solution. Precipitation was performed overnight (16 h) at a defined temperature. Samples were centrifuged for 30 min at 3,000 g and supernatants were decanted from the precipitates.

Protein quantification
Protein quantification in TTd samples was performed according to the following equation:

\[
(A_{228.5\text{nm}} - A_{234.5\text{nm}}) \times 0.3175 \times \text{dilution factor [mg/mL]}
\]

Gel electrophoresis and detection
Electrophoresis under denaturing conditions (SDS-PAGE) was performed using 4–12% Bis-Tris precast gels and MES running buffer, on an XCell Sure Lock system from Invitrogen (Carlsbad, CA, USA), according to the manufacturer’s instructions. Detection of protein bands was performed using acidic Coomassie Brilliant Blue R250 solution. When two-dimensional gel electrophoresis was performed, isoelectric focusing was done on a 7-cm IPG strip (pH 3–10, linear) according to the manufacturer’s instructions, in total applying 6000 Vh.

Sample preparation for matrix-assisted laser desorption ionization–mass spectrometry
In-gel digestion, peptide extraction, and purification were performed as previously described. Purified peptide solutions were dried and stored at 4°C until analysis. Prior to analysis, peptides were dissolved in 5 µL of CHCA solution (3 mg/mL, in 0.1% TFA (trifluoroacetic acid)/ACN (acetonitrile, 50:50, V/V)), and two spots, each 2 µL, were spotted onto the MALDI–MS microplate-format stainless steel target followed by drying at room temperature.

MALDI-TOF–MS analysis
Measurements were performed on an AXIMA TOF2 and AXIMA CFR+ instrument (Shimadzu—Kratos Analytical,
Manchester, UK). These instruments are equipped with 20 Hz nitrogen lasers (337 nm) and were operated in the positive ion mode with the accelerating voltage of 20 keV applied. Delayed extraction was used for all experiments to optimize resolution (optimal delay time was set according to the m/z range of interest). Typically, peptide mass fingerprint (PMF) mass spectra were acquired by averaging around 300 unselected single laser shots. For postsource decay (PSD) fragment ion experiments, the laser fluent was increased slightly above the threshold to promote fragmentation and 3000–5000 unselected laser shots were used to accumulate a final PSD spectrum.

Raw data generated on AXIMA instruments were converted into mzXML files, processed using mMass,[25] and searched against NCBI nr database. Searches were performed with the following fixed parameters: precursor ion mass tolerance of ±0.6 Da, product ion mass tolerance of ±1.2 Da, two missed trypsin cleavages, carbamidomethylation of Cys and with variable modification: deamidation.

Response surface for processes with high variation

Statistical calculations were performed using a trial version of Design-Expert Software version.

Response surfaces (RS) approximate criteria functions using low-order polynomials, mostly simple linear and quadratic or certain specific polynomials like the orthogonal Legendres polynomials.

General matrix formulation of this model can be written as:

\[ y_{RS} = B^T \beta \]

where \( B \) is a k-tuple of the used polynomial functions, while \( \beta \) is a k-tuple of the unknown corresponding coefficients. If a mostly linear polynomial is used, \( B \) and \( \beta \) are

\[ B^T = \{ 1 \ x_1 \ldots \ x_i \ldots \ x_k \} \]
\[ \beta^T = \{ \beta_0 \ \beta_1 \ldots \ \beta_i \ldots \ \beta_k \} \]

The unknown coefficients \( \beta \) are usually determined using the least square regression analysis by fitting the response surface approximation into existing data:

\[ \beta = (B_{1-n}B_{1-n}^T)^{-1}B_{1-n}y_{1-n} \]

where \( y_{1-n} \) is n-tuple of n known response values, while \( B_{1-n} \) is \( k \times n \) matrix with the calculated values of selected basis functions at locations 1–n.

Results and discussion

Aim of this study was to explore possibilities of ammonium sulfate precipitation for purification of TTd. The precipitation process was investigated as a one-step process which is somewhat different from the one performed for industrial-scale purification where precipitation is performed in two steps. First step is a low ammonium sulfate concentration (CAS) precipitation resulting in the precipitation of mostly contaminating proteins and leaving TTd in solution, whereas the second step precipitates majority of TTd with a higher concentration of ammonium sulfate. Nevertheless, the goal of the experiments described here was to study the possibilities of the precipitation process itself and not necessary its direct implementation into production. Factors considered here as possibly having influence on the process of the ammonium sulfate precipitation of TTd and therefore having impact on the purity of TTd are CAS, starting concentration of TTd (Lf/mL), starting purity of TTd (Lf/mgPN, PN denotes protein nitrogen), buffer, and temperature. We have performed four sets of experiments investigating these factors as summarized in Table 1 using DoE methodology, IV optimal design. Raw experimental data for each set of experiments, i.e., factors tested and responses measured are presented in Supplementary material (Tables S1–S4). The aim of purification was of course to obtain the purest TTd possible with the highest yield; therefore, two responses of the ammonium precipitation process were measured; total protein concentration and the amount of TTd (Lf/mL) and from these two and the starting concentration of TTd (Lf/mL), we calculated purity in terms of Lf/mgPN as well as the yield (the amount of TTd in the precipitate/starting TTd). Purities higher than 1000 Lf/mgPN were rarely achieved in performed experiments. It should be noticed that the determined TTd purity value depends on several factors and is influenced by the limitations of the methods used for quantification of TTd amount and the total protein content, e.g., the quality of anti-TTd antibodies, the precision of the determined value of TTd standard (in the case of the RID method), and other. Flocculation method although officially recommended by the authorities is hampered by the subjectivity of the person reading the flocculation.[26–29] Precision obtained by the RID is burdened by the precision of the precipitate readings which are rather small but the method was reported to be just as suitable as the flocculation method.[28] Quantification by radial immunodiffusion was shown to be more precise and of much higher throughput than the flocculation assay in our hands. However, variation was still quite high (Tables S1–S4), and it has been shown that one of the most influential parameters starting TTd concentration was also burdened by the imprecision of quantification. This was best demonstrated with the starting TTd concentrations. They were prepared from one starting TTd solution in each set of experiments, but when appropriate dilutions were prepared and again quantified, achieved values differed up to 25% from the targeted (Table 1). We find this a matter of variation of the TTd quantification method and not an error in dilution preparation.

To gain better understanding of the samples itself and determine possible impact on the precipitation process, we performed biochemical characterization of TTd samples first.

Identification of proteins contaminating TTd

TTd samples of different purity (284 and 736 Lf/mgPN) were subjected to SDS-PAGE and a subsequent identification by MALDI–mass spectrometry (Figure 1A). Identification of the proteins was possible since the whole genome of C. tetani is sequenced.[30] Identification data are summarized in Table 2 and are showing that besides TTx protein at expected
molecular mass truncated heavy chain was also detected. Truncated forms of TTx were previously reported. Interestingly, although proteins react with formaldehyde and modifications of N-termini, Lys, Arg, His, Cys, Tyr, Trp, and Phe occur, all protein bands (in the SDS-PAGE) could be identified through at least one peptide which indicates that some peptide stretches are inaccessible to formaldehyde, what is to be expected for a native protein. In addition, Figure 1A shows that there are no inter-protein cross-links, which was also expected as the used formaldehyde concentration is rather small (50 mM) as well as protein concentration and only intra-molecular reactions occur. As is seen from Table 2, all proteins in the TTd sample have similar theoretical pIs around 5.5. This was experimentally confirmed when unpurified TTd sample (after ultrafiltration step) was subjected to 2D gel electrophoresis (Figure 1B). Similarity of pIs could be the possible reason for partial success of purification by ammonium sulfate precipitation since protein precipitation depends on protein’s pI. It also explains precipitation of TTd sample below pH 5.5 that was observed in preliminary experiments. In the case of TTd used in this study, the largest contamination was S-layer protein/N-acetylmuramoyl-L-alanine amidase, and its role and presence in such large amount together with TTx could only be speculated and will be the topic of future research.

**Preliminary ammonium sulfate precipitation of TTd (DoE1)**

In this first set of experiments, we aimed to test the influence of four factors (CAS, starting amount of TTd, pH, and temperature) on the purity of TTd obtained by ammonium sulfate precipitation (Table 1). Percentage of ammonium sulfate ranged from 14 to 22% and citrate buffer was used as it is suitable for acidic to neutral range. First set of experiments (DoE1) was planned for a full quadratic model using IV-optimal design. In addition to 15 experiments necessary for determination of quadratic model coefficients, 7 experiments were replicated for determination of random error, while additional 15 were used to calculate model error. Analysis of purity response surface model for obtained experiment responses has resulted in a model without quadratic members. Final ANOVA for purity is presented in the upper part of Table S5, while the model equation and some basic statistic measures are given in lower part. Although adjusted $R^2$ is relatively low, it is in a good correlation with predicted $R^2$. It is also worth to mention that obtained response surface models of protein concentration and TTd concentration had much higher $R^2$ measures, ranging from 0.9 to 0.96 (data not shown). Results indicated that lower temperature favors purity of the precipitates as well as higher pH of those tested.

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**Table 1.** Parameters used in four experimental setups for determination of critical factors in tetanus toxoid purification by ammonium sulfate precipitation.

| DoE1 | DoE2 | DoE3 | DoE4 |
|------|------|------|------|
| Number of runs | 37 | 32 | 52 | 26 |
| $\omega$ ([NH₄]₂SO₄)/% | 14, 16, 18, 20, 22 | 14, 16, 18, 20, 22 | 16, 18, 20, 22 | 16, 18, 20, 22 |
| Aimed Lf/mL | 800, 1100, 1400 | 500, 700, 900 | 100, 300, 500 | 100, 500, 900 |
| Measured Lf/mL | pH 5.5: 675, 931, 1159 | pH 6.3: 686, 1103, 1434 | pH 7.0: 866, 1240, 1415 | 284 Lf/mgPN: 126, 314, 522 |
| Buffer | 25 mM citrate, pH 5.5, 6.3, 7.0 | 25 mM phosphate, pH 7.0 | 25 mM phosphate, pH 7.0 | 25 mM phosphate, pH 7.0 |
| $\Theta/°C$ | 6, 24, 37 | 6 | 6, 37 | 6 |
| Lf/mg PN | 213 | 334 | 284, 736 | Phosphate 244 |
| Block | — | 2 blocks (two operators) — | — | — |

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Figure 1. (A) SDS-PAGE of tetanus toxoid samples, left is the sample of 284 Lf/mgPN and right is the sample of 736 Lf/mgPN. Protein bands were identified using mass spectrometry (identification data are presented in Table 2). (B) 2D electrophoresis of tetanus toxoid sample.
Protein concentration model had an additional significant interaction between pH and CAS in the model while the TTd concentration model also contained quadratic members of pH and CAS (data not shown). Figure S1 shows the purity model in the design space, with pH and temperature fixed to the values that have resulted in the highest purity (pH 7.0, 6°C), while Figure S2 shows the purity model contour plot in the design space as well as prediction statistics in the area with the highest predicted purity values. Figure S3 shows the purity model normal plot of residuals. Very high variation was obtained in DoE1 set of experiments which possibly influences quality of the model and is certainly reflected in the weight of the conclusion resulting from DoE1. Therefore, DoE1 was regarded only as an orientation for further experiments quality of the model and is certainly reflected in the weight of the conclusion resulting from DoE1. Therefore, DoE1 was regarded only as an orientation for further experiment especially since some experimental limitations were observed as well. Obtained purity model clearly indicated that the highest purity values were on the boundary of design space, so the next set of experiments was planned to investigate the purity behavior in the area of lower starting TTd concentrations and at fixed pH and temperature (pH 7.0, 6°C). To confirm this, an additional numerical optimization with an objective to minimize the protein concentration and maximize the TTd concentration has been conducted using their response surface models. The results have confirmed that maximum purity can be expected in the area directly shown by the purity model (Figure S1).

**Influence of starting amount of TTd on TTd ammonium sulfate precipitation process (DoE2)**

Second set of experiments (DoE2) tested only two parameters; CAS and the starting concentration of TTd, at five and three levels, respectively, and was performed using phosphate buffer, pH 7, at 6°C, and performed by two operators (Table 1). Sixteen experiments were planned for a full quadratic model using IV-optimal design, an adequate number to determine quadratic model coefficients, random error, and assess model error. Additionally, each experiment was performed separately by different operators and at different time points, which has been treated as experiments in two blocks. Variations of the results obtained by both operators were somewhat high, but these reflect the limitations of the available methods used, especially quantification of TTd. Contribution to the variation of the test by the performer was tested since two persons performed DoE2 separately (only using the same starting TTd solutions). First block had lower variation and the differences in variations from the two blocks reflected differences in the skill of the two performers. Surprisingly, purity response surface model obtained for the experiment responses has resulted in a model in which the starting TTd amount was almost an insignificant factor (Figure 2). Only A1B interaction related to the starting TTd amount was significant, while the other starting TTd concentration-related members were kept to keep hierarchy of the model. Final ANOVA for purity is given in the upper part of the Table S6, while the model equation and some basic statistic measures are given in the lower part. Adjusted $R^2$ is relatively low but it is in reasonable correlation with the predicted $R^2$. As for DoE1, response surface models of protein concentration and TTd concentration once again had much higher $R^2$ measures, ranging from 0.91 to 0.98 (data not shown). The obtained protein concentration and TTd concentration response surface models, however, clearly showed that starting TTd concentration is significant in both of them, as is seen in Figure 2. Since purity is actually a quotient of TTd concentration and protein concentration, it seems that starting TTd concentration had similar influence to protein concentration and TTd concentration which resulted with almost vanishing of its influence to the purity model. Normal plots of residuals for purity, protein concentration, and TTd concentration are shown in Figures S4–6. Obtained DoE2 purity model shows a small indication that the highest purity values are on the lower boundary of starting TTd concentrations, while the maximum one, with respect to concentration of ammonium sulfate, is at ≈ 21%.

![Table 2. Proteins identified in tetanus toxoid sample from Figure 1A.](data:image/epubx-table)

| Protein band | Peptide identified by MS | Protein identified | Theoretical $M_o$/pl |
|-------------|--------------------------|--------------------|---------------------|
| A           | DFWGNPLR, LYTYSLETTLFR, VNGQAGILFOQWVR | Tetanus toxin | 150.7 kDa/5.79 |
| B           | IYINFDR, DNTRYTLDTEGR | S-layer protein/N-acetyl muramoyl-L-alanine amidase | 118.7 kDa/5.34 |
| C           | IYINFDR | Tetanus toxin$^a$ | – |
| D           | DFWGNPLR, LLAYVEWLR, VVSYDNEWGYSTR | Methylaspartate ammonia-lyase | 45.6 kDa/5.32 |
| E           | GYEIPEDATWNR, NOSLESREPTEYER | Glyceraldehyde 3-phosphate dehydrogenase | 36.5 kDa/5.87 |
| F           | DDFWNLDR, EIFAELDR | Putative surface/cell-adhesion protein | 36.7 kDa/5.06 |
|             |             | 3-Hydroxybutyryl-coA dehydrogenase | 30.6 kDa/5.56 |

$^a$H2 fragment.

**Table 2. Proteins identified in tetanus toxoid sample from Figure 1A.**

The obtained DoE2 purity model shows a small indication that the highest purity values are on the lower boundary of starting TTd concentrations, while the maximum one, with respect to concentration of ammonium sulfate, is at ≈ 21%. To confirm that an additional numerical optimization was conducted with an objective to minimize the protein concentration and maximize the TTd concentration using their response surface models. The results have confirmed that maximum purity can be expected at the lower starting TTd concentration boundary. However, numerical optimization also suggested that the optimum value for CAS is close to 19%, which slightly disagrees with the direct purity model. Another interesting point is that the obtained experiments did not agree with the previously performed experiments in DoE1 in the overlapped design space between those two sets of experiments. Theses set of experiments have resulted in a significantly lower purity values than in DoE1 in the overlapped design space. These discrepancies are most likely a result of the already discussed variation inherent to the TTd quantification.
Influence of the temperature, starting amount, and starting purity of TTd on TTd ammonium sulfate precipitation process (DoE3)

For the third set of experiments (DoE3), number of CAS was reduced, but a new parameter was introduced—starting purity (Table 1). In addition, since the first two experimental sets indicated higher purities for lower starting TTd concentrations, starting amounts of TTd were investigated at lower values. Temperature was also returned to the model and was tested at two levels (6, 37°C). Initially, a total of 26 experiments was planned for a full quadratic model using IV-optimal design. This is an adequate number of experiments to determine the quadratic model coefficients, random error, and to assess the model error for the model that included the first three parameters. However, before the conduction of these experiments, it was decided to check the influence of the starting purity parameter, and additional 26 experiments were performed. Final ANOVA for DoE3 purity model is given in upper part of Table S7, while the model equation and corresponding statistic measures are given in the lower part. Adjusted $R^2$ and predicted $R^2$ are much better than for the first two sets of experiments, probably because a smaller part of design space was investigated with respect to CAS and starting TTd concentration. Similarly as before, response surface models of protein concentration and TTd concentration had much higher $R^2$ measures, ranging from 0.97 to 0.99. Figure 3A shows purity model in design space, where temperature is fixed to the lowest value since it produces the highest purity, while starting purity is fixed to the lower value (284 Lf/mgPN). Figure 3B shows purity model contour plot in the design space together with prediction statistics in the area with the highest predicted purity values. Figure 3C shows purity model normal plot of residuals. The third set of experiments confirmed findings of the second set of experiments, which means that the highest value of purity was found on the lowest starting TTd concentrations. The maximum purity value is still on the lower starting TTd concentration bound, but further experiments with starting TTd concentrations lower than 100 are not practically convenient due to the test’s sensitivity for the quantification of TTd.

Maximum value of purity with respect to CAS is reached within the investigated interval and goes from 19 to 21% for starting TTd concentrations 500 and 100 Lf/mL, respectively. This confirmed DoE2 result obtained by numerical optimization with minimization of protein concentration and maximization of TTd concentration that resulted with the optimum of 19% ammonium sulfate and the starting TTd concentration of 500 Lf/mL. This set of experiments has confirmed also that lower temperatures have positive impact on purity. Surprisingly, results showed that the starting purity of TTd has no significant effect on the final purity of the precipitates, i.e., the samples whose starting purity was around 2.6 higher resulted in only 12% (on average) higher final purity and 20% (on average) higher yield. This has an important implication on the large-scale purification, meaning that the low purity material usually obtained from cell cultures and used for purification is suitable for ammonium sulfate precipitation, i.e., would not gain from prepurification. Also, the low amounts of TTd usually used for ammonium sulfate purification are actually beneficial for the purification and concentration during ultrafiltration process usually performed before the ammonium sulfate precipitation is not needed.

In DoE3 set of experiments, in addition to measuring protein and TTd amount in precipitates, TTd concentrations were also measured in all the supernatants to calculate recoveries and gain insight on possible discrepancies resulting from imprecision of the methods used. Recovery (amount of TTd in precipitate + supernatant) for TTd amount was found to be 92.3 ± 5.65 and 86.9 ± 5.99% and for the total protein amount 97.5 ± 8.66 and 93.4 ± 6.00% (data correspond to lower and higher starting purity sample sets respectively). Some experimental constraints contribute to total error such as small sample losses during decantation of supernatants and the deviations in volume which were not taken into calculation since the precipitate is not completely dry and 2 mL of water was used to resuspend the precipitates (of different sizes), thereby causing variations in volume which were not taken into calculation. It can therefore be concluded that recoveries are satisfying and methods used for quantification of TTd and total protein amount are of reasonable precision.
Influence of the buffer and starting TTd amount on TTd ammonium sulfate precipitation process (DoE4)

To corroborate data obtained from previous sets of experiments and expand them, the fourth set of experiments was designed (DoE4) to cover almost an entire range of tested factors from DoE2 and DoE3. Since some of the DoE1 findings were not confirmed with later DoE2 results in the overlapped design space, we aimed to investigate the possibility that some other parameters, which were not controlled, have influenced responses. It was speculated that the citrate buffer which was used in DoE1 could be an important factor since phosphate buffer was used in the other two sets of experiments. Therefore, starting TTd amount was tested in a wide range (100, 500, 900 Lf/mL) and the two types of buffer were tested (citrate and phosphate) as a third, categorical, parameter (Table 1). In this set of experiments, 26 experiments are planned for a full quadratic model using IV-optimal design. This is an adequate number of experiments to determine quadratic model coefficients, random error, and assess model error. Final statistics of DoE4 purity model are given in Table S8, in the same

![Figure 3. Analysis of the third set of experiments (DoE3). (A) 3D surface plot of purity model in design space at 6°C, purity 284 Lf/mgPN. Each experimental result is denoted by a circle (red above and pink below the response surface plot) connected to the surface plot by vertical line to denote the distance from it. (B) Contour plot of purity model in design space with prediction statistics at 6°C (purity 284 Lf/mgPN). (C) Purity model normal plot of residuals. TT0, starting TTd concentration; PUR, purity.](a)

![Figure 4. Analysis of the fourth set of experiments (DoE4). (A) 3D surface plot of purity model (phosphate buffer). (B) 3D surface plot of purity model (citrate buffer). (C) Contour plot of purity model in design space with prediction statistics (citrate buffer). Each experimental result is denoted by a circle (red above and pink below the response surface plot) connected to the surface plot by vertical line to denote the distance from it. (D) Purity model normal plot of residuals. (E) Contour plot of yield model in design space with prediction statistics (citrate buffer). TT0, starting TTd concentration; PUR, purity; PUR0, starting purity.](b)
manner as for the previous experiment sets. Figure 4A shows purity model in design space for the phosphate buffer, while Figure 4B shows the purity model for citrate buffer. Table S8, Figure 4A and B indicate that the buffer is a significant parameter, and that citrate buffer results with higher purity values. Since phosphate and citrate buffer have significantly different level of initial purity (Table 1), influence of the buffer (which was found as a significant parameter in DoE3), i.e., its bias to the buffer type itself can be excluded. Figure 4C shows purity model with citrate buffer contour plot in design space together with prediction statistics in the area with the highest predicted purity values. The values of purity model with phosphate buffer are 50 Li/mgPN lower in that area. Figure 4C shows purity model normal plot of residuals.

The fourth set of experiments has confirmed results of the DoE2 and DoE3 experiments and identified the buffer type as a significant parameter. Finally, since this test has confirmed position of the area with maximal purity, it is interesting to show the yield model, as it indicates the efficiency of the process. Figure 4D shows yield model with citrate buffer contour plot in design space together with the prediction statistics in the area with the highest predicted purity values.

Conclusion

The four conducted sets of experiments have clearly shown that the process of TTd purification by ammonium sulfate precipitation is a process with a high variation and that protein concentration and TTd quantification methods exhibit high variation also. Therefore careful planning and an appropriate number of experiments and repetitions were performed to obtain reasonable models and draw conclusions from the data obtained. Our results indicate that lower starting amounts of TTd result in higher purities of precipitates. Also, precipitation at 6°C (in comparison to 37°C) as well as citrate buffer (in comparison to phosphate buffer) were shown as beneficial regarding purity. Purity of the starting sample significantly influenced the purity of the precipitates. Also, these results demonstrate a moderate efficiency of ammonium sulfate precipitation to yield TTd of high purity, corroborating the need for other purification methods.

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References

[1] WHO. Tetanus vaccine, WHO position paper. Weekly Epidemiol. Rec. 2006, 81, 198–208.
[2] Helting, T.B.; Parschat, S.; Engelhardt, H. Structure of Tetanus Toxin. J. Biol. Chem. 1979, 254, 10728–10733.
[3] Abnert-Hilger, G.; Weller, U.; Dauzenroth, M.-E.; Habermann, E.; Gratzl, M. The tetanus toxin light chain inhibits exocytosis. FEBS Lett. 1989, 242, 245–248.
[4] Sciavi, G.; Papini, E.; Henna, G.; Montecucco, C. An intact interchain disulfide bond is required for the neurotoxicity of tetanus toxin. Infect. Immun. 1990, 58, 4136–4141.
[5] Kriegelstein, K.G.; Henschen, A.H.; Weller, U.; Habermann, E. Limited proteolysis of tetanus toxin. Eur. J. Biochem. 1991, 202, 41–51.
[6] Emsley, P.; Fotinou, C.; Black, I.; Fairweathers, N.F.; Charles, I.G.; Watts, C.; Hewitt, E.; Isaacs, N.W. The structures of the HC fragment of tetanus toxin with carbohydrate subunit complexes provide insight into ganglioside binding. J. Biol. Chem. 2000, 275, 8889–8894.
[7] Calvo, A.C.; Oliván, S.; Manzano, R.; Zaragoza, P.; Aguilera, J.; Osta, R. Fragment C of tetanus toxin: New insights into its neuronal signaling pathway. Int. J. Mol. Sci. 2012, 13, 6883–6901.
[8] Rossetto, O.; Scorzo, M.; Megighian, A.; Montecucco, C. Tetanus neurotoxin. Toxicon 2013, 66, 59–63.
[9] Galeo, M.; Schiavo, G. Central effects of tetanus and botulinum neurotoxins. Toxicon 2009, 54, 593–599.
[10] Metz, B.; Kersten, G.F.A.; Hoogerhout, P.; Brugghe, H.F.; Timmermans, H.A.M.; Jong, A.; Meiring, H.; Hove, J.; Hennink, W.; Crommelin, D.J.A.; Jiskoot, W. Identification of formaldehyde-induced modifications in proteins. J. Biol. Chem. 2004, 279, 6235–6243.
[11] Toews, J.; Rogalaki, J.C.; Clark, T.J.; Kast, J. Mass spectrometric identification of formaldehyde-induced peptide modifications under in vivo protein cross-linking conditions. Anal. Chim. Acta 2008, 618, 168–183.
[12] Thaysen-Andersen, M.; Jørgensen, S.B.; Wilhelmsen, E.S.; Petersen, J.W.; Hojrup, P. Investigation of the detoxification mechanism of formaldehyde-treated tetanus toxoid. Vaccine 2007, 25, 2213–2227.
[13] Metz, B.; Tilstra, W.; van der Put, R.; Spruit, N.; van den IJssel, J.; Robert, J.; Hendriksen, C.; Kersten, G. Physicochemical and immunochemical assays for monitoring consistent production of tetanus toxoid. Biologicals 2013, 4, 231–237.
[14] Latham, W.C.; Jenness, C.P.; Timperi, R.J.K.; Michelsen, C.B.H.; Zipilivjan, E.M.; Edsall, G.; Ley, H.L. Purification and characterization of tetanus toxoid and toxin. I. Fractionation of tetanus toxoid by gel filtration. J. Immunol. 1965, 95, 487–493.
[15] Latham, W.C.; Michelsen, C.B.; Edsall, G. Preparative procedure for purification of toxoids by gel filtration. Appl. Microbiol. 1967, 15, 616–621.
[16] Fleš, M.; Pende, B. Fractionation of tetanus toxoid by Sephadex gel filtration and immunochemical characterization of the fractions. Croat. Chem. Acta 1967, 39, 81–87.
[17] Stojićević, I.; Dimitrijević, L.; Dovezenski, N.; Živković, I.; Petrušić, V.; Marinčikov, E.; Inić-Kanada, A.; Stojanović, M. Tetanus toxoid purification: Chromatographic procedures as an alternative to ammonium-sulphate precipitation. J. Chromatogr. B 2011, 879, 2213.
[18] Foster, P.R.; Dunmill, P.; Lilly, M.D. Salting-out enzymes with ammonium sulphate. Biotechnol. Bioeng. 1971, 3, 713–718.
[19] Mahn, A.; Ismail, M. Depletion of highly abundant proteins in blood plasma by ammonium sulfate precipitation for 2D-PAGE analysis. J. Chromatogr. B 2011, 879, 3645–3648.
[20] Gilmour, S.G. Response surface designs for experiments in bioprocessing. Biometrica 2006, 62, 323–331.
[21] Mountzouris, K.C.; Gilmour, S.G.; Jay, A.J.; Rastall, R.A. A study of dextran production from maltodextrin by cell suspensions of Gluconobacter oxydans NCIB 4943. J. Appl. Microbiol. 1999, 87, 546–556.
[22] Mountzouris, K.C.; Gilmour, S.G.; Rastall, R.A. Continuous production of oligodextrins via controlled hydrolysis of dextrin in an enzyme membrane reactor. J. Food Sci. 2002, 67, 1767–1771.
[23] Ehresmann, B.; Imbault, P.; Well, J.H. Spectrophotometric determination of protein concentration in cell extracts containing tRNA's and rRNA's. Anal. Biochem. 1973, 54, 454–463.
[24] Grgles, M.; Kurtović, T.; Kovačić, L.; Križaj, I.; Barut, M.; Lang Balija, M.; Allmaier, G.; Marchetti-Deschmann, M.; Halassy, B. Identification of proteins interacting with ammodytoxins in tRNA's and rRNA's. Int. J. Mol. Sci. 2004, 5, 2213–2227.
analysis of mass spectrometric data. *Anal. Chem.* **2010**, *82*, 4648–4651.

[26] Iwaki, M.; Horiuichi, Y.; Komiya, T.; Fukuda, T.; Arakawa, Y.; Takahashi, M. Toxoid flocculation assay by laser light-scattering. *J. Immunol. Methods* **2007**, *318*, 138–146.

[27] Lyng, J.; Bentzon, M.W. The quantitative estimation of diphtheria and tetanus toxoids. 1. The flocculation test and the LF-unit. *J. Biol. Stand.* **1987**, *15*, 27–37.

[28] Ljungqvist, L.; Lyng, J. Quantitative estimation of diphtheria and tetanus toxoids. 2. Single radial immuno-diffusion tests (Mancini) and rocket immuno-electrophoresis test in comparison with the flocculation test. *J. Biol. Stand.* **1987**, *15*, 79–86.

[29] Preneta-Blanc, R.; Rigsby, P.; Sloth Wilhelmsen, E.; Tierney, R.; Brierley, M.; Sesardic, D. Calibration of replacement international standards of diphtheria and tetanus toxoids for use in flocculation test. *Biologicals* **2008**, *36*, 315–326.

[30] Brüggemann, H.; Bäumer, S.; Fricke, W.F.; Wiezer, A.; Liesegang, H.; Decker, I.; Herzberg, C.; Martinez-Arias, R.; Merkl, R.; Henne, A.; Gottschalk, G. The genome sequence of *Clostridium tetani*, the causative agent of tetanus disease. *Proc. Natl. Acad. Sci.* **2003**, *100*, 1316–1321.