An effective, simple and low-cost pretreatment for culture clarification in tetanus toxoid production

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ABSTRACT

Chemically inactivated tetanus toxin (tetanus toxoid, TT), purified from cultures of a virulent *Clostridium tetani* strain, is the active pharmaceutical ingredient of anti-tetanus vaccines. Culture clarification for TT production and is usually performed by filtration-based techniques. Final clarification of the culture supernatant is achieved by passage through 0.2 µm pore size filtering membranes. Large particles removal (primary clarification) before final filtration (secondary clarification) reduces costs of the overall clarification process. With this aim, chitosan-induced particle aggregation was assessed as an alternative for primary clarification. Three chitosan variants were tested with similar results. Optimal clarification of culture supernatant was achieved by the addition of 8 mg chitosan per l of culture. Extrapolation analysis of filter sizing results indicate that 100 l of chitosan-treated supernatant can be finally filtered with a 0.6 m2 normal filtration cartridge of 0.45 + 0.2 µm pore size. The clarified material is compatible with current standard downstream processing techniques for TT purification. Thus, chitosan-induced particle aggregation is a suitable operation for primary clarification.

KEYWORDS Depth filtration; filtration precipitation; microfiltration; purificatonAQ2

Introduction

Clarification during vaccine production can often be divided in two operations: separation of large particles (primary clarification) and removal of sub-micron particles (secondary clarification).[1] Typical primary clarification is done based on low-speed centrifugation, tangential flow filtration (TFF), or depth-filtration. Its aim is to protect and thus maximize the usage of the costly normal filtration membranes employed during the following clarification steps. Secondary clarification is almost exclusively performed by normal or dead-end filtration with membranes of pore sizes ranging from 0.1 to 0.65 µm. Clarification operations are scarcely addressed during vaccine process development although they have a deep impact in following downstream processing steps.

Tetanus toxin, produced by *Clostridium tetani*, is the responsible of a severe disease known as tetanus.[2] This toxin, one of the most potent known, is an heterodimeric protein composed of one heavy chain (100 kDa) linked by a single disulfide bond to a light chain (50 kDa).[3] Tetanus still remains a serious threat to public health, with over 200,000 fatal cases per year around the world.[4] Chemically inactivated tetanus toxin (tetanus toxoid, TT), produced during the culture of a virulent *C. tetani* strain, is an active pharmaceutical ingredient (API) of anti-tetanus vaccines.[5,6] Tetanus toxin expression during bacterial growth is the consequence of the activation of the lytic cycle of a lysogenized-phage. Biomass removal from the fermented broth is most commonly performed by filtration.[7–11] Lowering clarification costs could thus help making a more affordable production of this important API. In this direction, flocculants, a class of materials that favor solid-liquid separation, increase the efficacy of filtration trains without adversely impacting on the recovery of biopharmaceuticals.[12–14] Chitosan stands out from other flocculants due to its characteristic low-cost, high accessibility, non-toxicity, and biodegradability.[15] Its safety is exemplified by its increasing use as an adjuvant during the

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development of new vaccine formulations.[16,17] In this work, chitosan is assessed for the first time as part of a primary clarification operation, with an aim on reducing membrane filtration needs and therefore the costs for secondary clarification operations, during TT production.

Experimental

Toxin production and inactivation

Tetanus toxin was produced in static cultures of a virulent *C. tetani* strain (Harvard Caracas 80), essentially as recommended by WHO.[6] The fermentation medium was the Latham modification of the original Müller and Miller formulation.[18] Cultures were inactivated with formalin for 21 d at 37 °C, and conserved at 4–8 °C until use. The solids content in inactivated fermentation broth harvests were calculated from their OD_{600nm}, employing a linear estimation constructed from previous experiments. Thus, the wet biomass concentration in these samples was estimated to be in the range 1.4–2.0 g/l.

Flocculants

Its biologic origin and different degrees of deacetylation from the original biopolymer chitin, are the main characteristics that differ on commercially available chitosan from different producers. Three chitosan variants were assayed in parallel, designated C1 ("Chitosan-medium molecular weight", product code 448877, Sigma Aldrich, St. Louis, MO, USA), C2 ("Chitosan-low viscosity", product code 448869, Sigma Aldrich, St. Louis, MO, USA), and C3 ("Chitosan powder", product code CHT1K, Federal Laboratories Corporation, Alden, NJ, USA). Chitosan stock solutions (10 g/l) were prepared as described by Ägkervist.[19] Briefly, chitosan was first mixed with an equal amount (w/w) of glacial acetic acid, then 0.1 M NaCl was added up to the final volume and completely dissolved after 12 h mixing. Only freshly prepared solutions were used.

Flocculation tests

Flocculation tests were performed essentially following the process described by Ägkervist.[19] Working chitosan dilutions were constructed in the range 1–1000 mg/l by serial dilution of the stock chitosan solutions in 0.1 M NaCl. Then, 9 ml aliquots of inactivated cultures were transferred to 50 ml conic-base graduated plastic tubes and 1 ml of the corresponding working chitosan dilution was added during 30 s under strong stirring (1 cm diameter three-blade inclined impeller mounted on a DecalabFbr® mechanic homogenizer, at 3000 rpm). Mixing velocity was lowered to 1000 rpm and maintained for 5 min, and the plastic tube content was transferred to 15 ml conic-base graduated plastic tubes for sedimentation. After a 12–16 h settling period at room temperature, 3 ml were carefully extracted from the top of each tube and their OD_{600nm} was determined. A blank tube was constructed for OD_{600nm} measures by adding 1:9 parts of 0.1 M NaCl to an aliquot of the inactivated culture and completely clarifying it by a combination of centrifugation (10,000 g × 10 min) and 0.22 µm pore filtration. Relative turbidity was then calculated as the quotient between the OD_{600nm} of each chitosan dose and the OD_{600nm} of an aliquot of the original inactivated culture, where 1:9 parts of the 0.1 M NaCl solution were added but that was not allowed to settle (full turbidity). Each dose of chitosan was assayed in duplicate.

Protein and TT analysis

Protein extracts were analyzed by 7.5% SDS-PAGE, under reducing conditions, according to Laemmli.[20] Electrophoretic runs were performed in a Mini-protean Tetra cell® (Biorad, USA). Gels were either silver[21] or colloidal Coomassie Brilliant Blue stained.[22]

TT content (Lf/ml) is traditionally determined by the Ramon flocculation technique.[23] However, this cumbersome technique is inappropriate for processing a large number of samples, as in these experiments. A valid alternative to quantify purified TT intermediates, recommended by WHO, is the single radial immunodiffusion method (SRD).[24,25] For quantitation, a standard curve was constructed with serial dilutions of an in-house prepared secondary standard of

purified TT in the 25–100 Lf/ml range. Images of the Coomassie Blue stained precipitate rings were obtained with the aid of an office scanner and their diameters were determined from digital images with Image J software.[26]

Filter sizing

Filter sizing for final clarification of the supernatant after chitosan-induced aggregation and gravity sedimentation was performed on 450 ml of inactivated culture samples. These samples were first transferred to a 1000 ml beaker and 50 ml of an 80 mg/l dilution of the C3 chitosan variant, in 0.1 M NaCl, was added under strong stirring (2 cm diameter three blade inclined impeller mounted on a DecalabFbr® mechanic homogenizer, at 3000 rpm). Mixing velocity was lowered to 1000 rpm and maintained for 5 min, the homogenized mixture was then transferred to a 1000 ml graduated cylinder and left standing 12–16 h at room temperature for sedimentation. The supernatant was collected with the aid of a peristaltic pump (approximately 450 ml) kept for filter sizing. Filter evaluation was conducted under constant pressure (0.3 bar) on 0.45 + 0.2 µm pore size combined, 47 mm diameter (13.5 cm^2 effective area) regenerated cellulose filter membranes (Sartobran P®, Sartorius GmbH, Göttingen, Germany). In-line pressure, filtrate volume data were collected and analyzed with Zero-T software (Sartorius GmbH, Göttingen, Germany). Membrane fouling was modeled from experimental data and sized to the final scale according to the linearizing mathematical equations described by Irritani.[27]

TT purification

TT from the filtrate was purified by a laboratory scale adaptation of the production-scale technique recommended by WHO.[6] The clarified inactivated culture was first submitted to a TT partial-purification step, based on tangential flow concentration followed by diafiltration against 10 volumes of saline (50 kDa MWCO membrane, Vivaflow® 50, Sartorius GmbH). Then, this semi-purified material was purified by the traditional two-step ammonium sulfate precipitation methodology. Initially, solid ammonium sulfate was added under stirring until achieving a 15% w/v concentration and left overnight at room temperature. The supernatant was collected and ammonium sulfate addition was repeated until reaching a final 25% w/v concentration. After this second precipitation step, the supernatant was removed, the precipitate resuspended and diafiltered against 15 volumes of saline (50 kDa MWCO membrane, Vivaflow® 50, Sartorius GmbH, Göttingen, Germany). This purified TT was then filter-sterilized and conserved at 4–8 °C for further analysis.

Electrospray ionization mass spectrometric (ESI MS/MS) peptide mass fingerprinting (PMF)

SDS-PAGE bands were excised and their proteins identified by the PMF technique, based on an ESI MS/MS analysis. The proteomics work was done at the Proteomics Platform of Barcelona Science Park, University of Barcelona, a member of ProteoRed-ISCIII network.

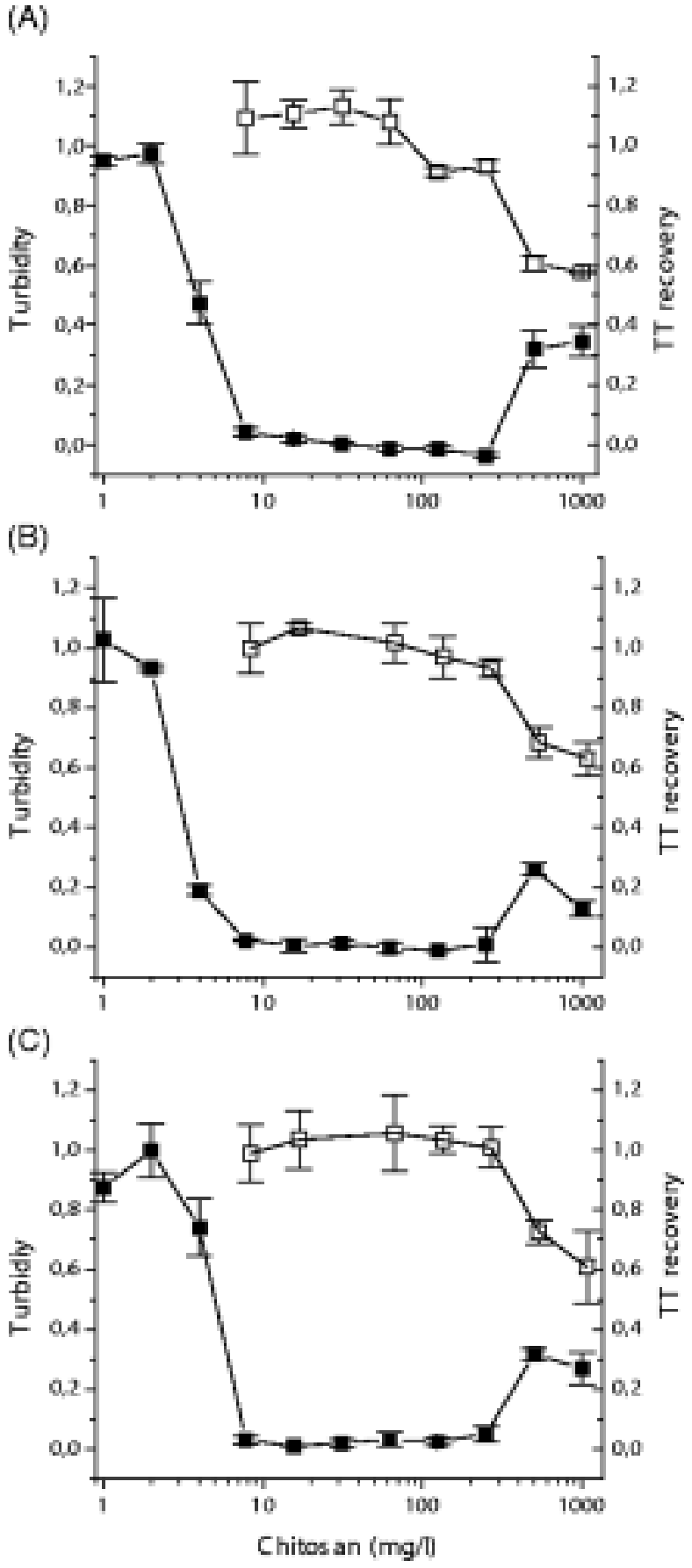
Results and discussion

Chitosan-induced flocculation of inactivated *C. Tetani* cultures

Chitosan-induced particle aggregation and sedimentation were first explored on 9 ml aliquots of inactivated *C. tetani* culture harvests. The three variants of chitosan displayed similar flocculating activity (Fig. 1). An initial steep decrease in turbidity with increasing chitosan doses was observed, followed by a plateau in the 8–250 mg/l region (Fig. 1). Above this dose, the chitosan-induced clarification was less effective. Critical flocculation dosage (cfd) was defined as the chitosan dose obtained by extrapolation to zero turbidity of the steepest part of the turbidity vs. chitosan dosage curve. Mean cfd values were 8.0 mg/l, 7.8 mg/l, and 9.6 mg/l for C1, C2, and C3, respectively, with no statistical differences between them (One-way ANOVA with Tukey *post-hoc* test, $\alpha < 0.05$ inference level). Since this is the first time that flocculation is tested for primary clarification during TT production, comparison with previous reports from literature is limited. Chitosan-induced flocculation of different bacterial culture harvests has shown a strong dependence on pH, ionic strength, chitosan variant (acetylation degree and molecular weight), and bacterial species under the study.[19,28,29] Nevertheless, maximal flocculation was observed in most cases with chitosan doses comparable with those reported here. The study of flocculation for primary clarification in therapeutic proteins production has been almost exclusively related to monoclonal antibodies production. Optimal clarification has been achieved with 200 mg/l chitosan

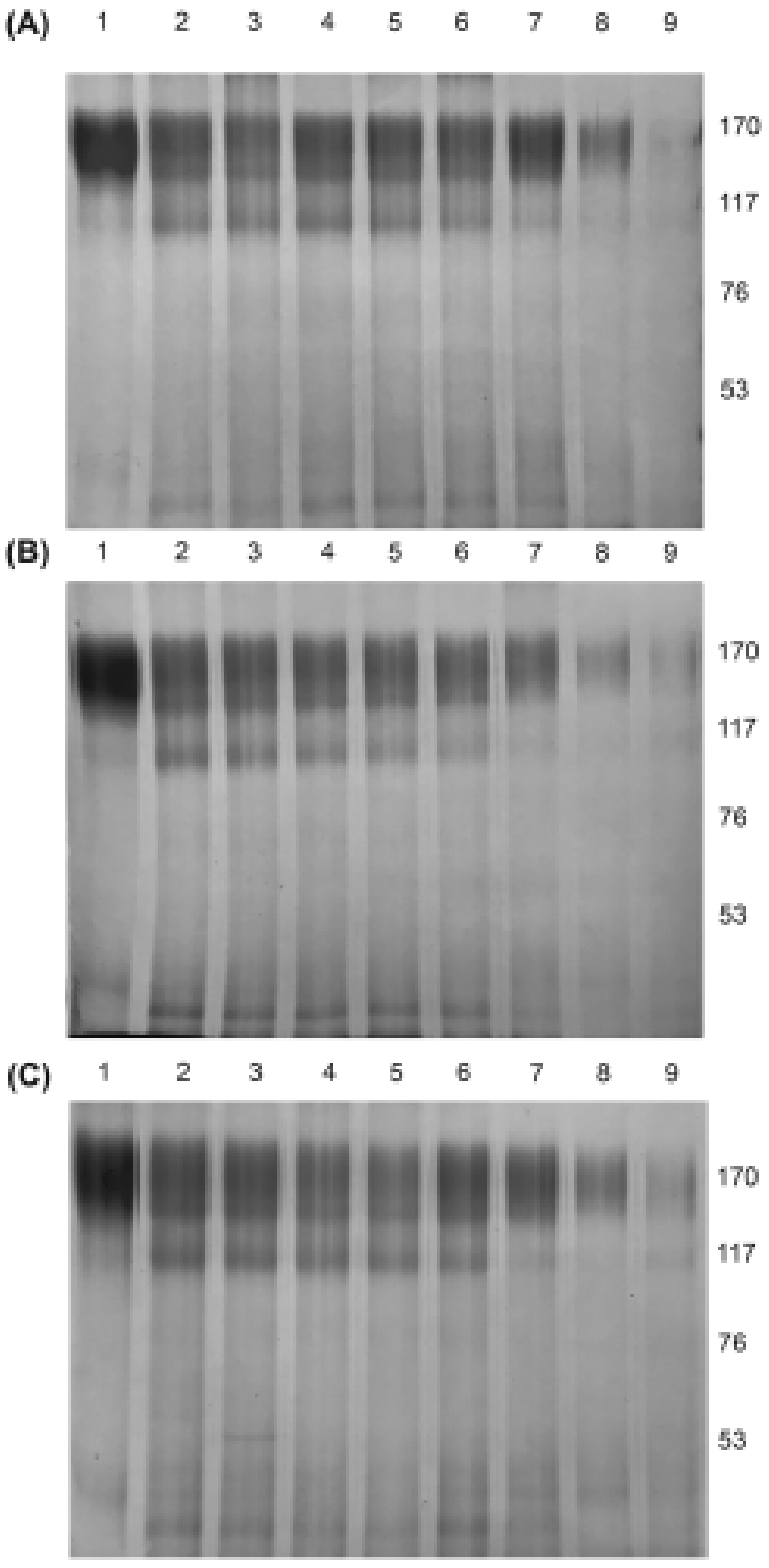
for NS0 cell culture harvests, 500 mg/l polydiallyldimethylammonium chloride (pDADMAC) for Chinese hamster ovary (CHO) cell culture harvests, 850 mg/l Polymin P on tobacco plant cell homogenates.[12,13,30]

Figure 1. Effect of chitosan concentration on turbidity and TT recovery: 1 ml of 10 g/l stock solutions of three variants of chitosan, diluted to the desired concentration, were added to 9 ml of inactivated culture harvests and left settling for 12–16 h at room temperature. The upper 3 ml of each tube were collected and analyzed in parallel for turbidity and TT recovery. Results for chitosan variants C1, C2, and C3 are displayed in panels A, B, and C, respectively. Relative turbidity was calculated as the quotient between the OD600 nm of each sample, blanked against a completely clarified sample, and that of a diluted aliquot of the original inactivated culture that was not allowed to settle (black squares). TT recovery was calculated for each sample as the quotient between the TT concentration determined in it by the SRD methodology and that of a blank tube (white squares). Standard deviation bars are depicted for each mean value.


(A)
(B)
(C)
Turbidity
TT recovery
0,0
0,2
0,4
0,6
0,8
1,0
1,2
1
10
100
1000
Chitosan (mg/l)

Flocculants interact not only with micelles and particulate material (such as intact bacteria and cell debris) but also with biomolecules present in solution, such as DNA and polypeptides.[31] Therefore, the effect on TT recovery was studied at different increasing chitosan doses. TT concentration remained almost invariable for chitosan doses below 250 mg/l, whereas higher doses had a negative impact (25–40% loss) on TT recovery (Fig. 1). As inferred from SDS-PAGE analysis of the supernatant, the TT loss was not selective but reflected a detrimental effect of chitosan on total protein recovery at higher doses (Fig. 2). Another significant aspect was the dose-effect observed on sediment characteristics among the 8–250 mg/l region: below 16 mg/l chitosan, the sediment aspect was compact and homogeneous, while more than 31 mg/l, it appeared coarser and sparser (Suppl. Fig. 1). It could thus be concluded that chitosan doses near or just above the cfd are best suited for primary clarification of inactivated cultures.

Figure 2. SDS-PAGE analysis of chitosan-induced flocculation supernatant: Samples (10 µl) from the supernatant obtained after sedimentation of chitosan-induced flocculation were resolved by 7.5% acrylamide SDS-PAGE and silver stained. Each lane shows the electrophoretic profile of samples obtained with different doses of chitosan (0, 8, 31, 62, 125, 250,500, and 1000 mg/ml for lanes 2–9, respectively). Chitosan variants C1, C2, and C3 were employed for flocculation on panel A, B, and C, respectively. Purified TT was loaded on lane 1 of each gel, for comparison purposes. Molecular weight marker: HMW-SDS marker, code 17061501, GE Health care. Molecular weights, in kDa, are indicated at the right of the figures.



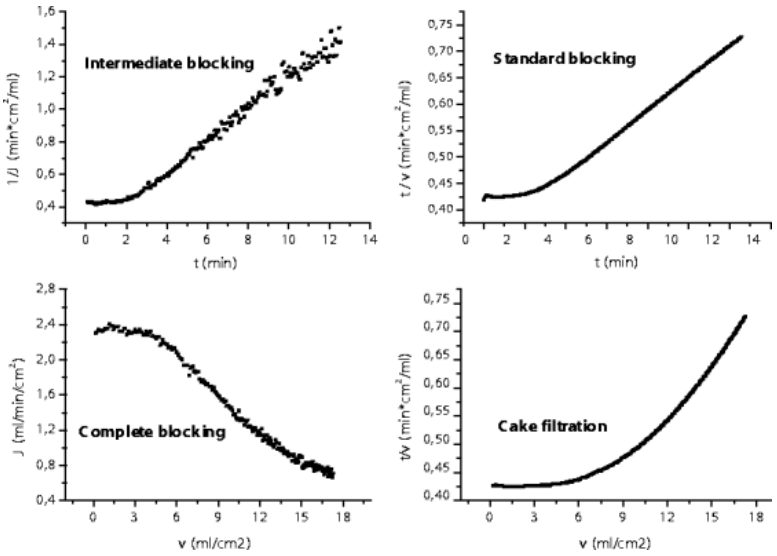
Evaluation and sizing of a simple final filtering step

Biomass and sub-micron particles are usually removed from *C. tetani* cultures by primary clarification based on TFF or depth-filtration and often followed by final dead-end filtration through 0.2 µm pore size membranes.[7–11] Chitosan-induced particle sedimentation was herein assessed as a cheap and simple alternative for primary clarification. Thus, chitosan was added to an inactivated *C. tetani* culture and left standing 12–16 h for particle sedimentation. Due to similar flocculating properties observed for the three chitosan variants evaluated, these experiments were performed only with one of this variants (C3). After sedimentation, the supernatant was collected with the aid of a peristaltic pump and submitted to filter sizing analysis using a 0.45 + 0.2 µm pore size combined cellulose-acetate filter membrane, as described in Section 2.

Fouling dynamics in membrane filtration under constant pressure can generally be interpreted in four models: Complete blocking; Standard blocking, Intermediate blocking, and Cake filtration.[27] Complete blocking model is based on the assumption that particles in suspension are of larger size than membrane pores, thus blocking the pores completely without interacting or superimposing between them. Standard blocking considers pore blocking to be the consequence of particle deposition inside the membrane pore-channels. In the Intermediate blocking model, particles are considered to be larger in size than membrane pores, as in Complete blocking model, but particle to particle interaction is considered on membrane plugging dynamics analysis. Finally, Cake filtration model considers that a growing layer of particles on the membrane surface (cake) is formed during filtration, adding resistance to flow. Model fitting through the corresponding linearizing equations as part of filter sizing analysis allows to obtain additional information on membrane fouling dynamics that aid the filtering-train design.

The linearizing plots calculated from data obtained during a representative filter sizing test are shown in Fig. 3. In this experiment, mean initial filtrate flux under constant pressure (0.3 Bar) through the 13.5 cm^2 filtering device was 2.19 ml/min/cm^2. Filtrate flux dropped during the 12.5 min of the test, achieving a final value of 0.66 ml/min/cm^2. No significant changes were observed in TT content after clarification (Student *t* test, $\alpha < 0.05$). As Fig. 3 shows membrane fouling dynamics in this experimental asset are better described in Standard blocking model. According to this model, pore volume constriction occurs after particle deposition on the walls, it can be concluded that particles in the supernatant are smaller in size than membrane pores. After linear-fitting of the experimental data to this model, scaling parameters for filtration were calculated. The filtrate volume at the moment when flow reaches 20% of its initial value (V_{80}) was 42.92 ml/cm^2. Then, for clarification at typical low-scale industrial TT production level (100 l culture harvest) 800 mg chitosan and 0.49 m^2 area of filtering membrane would be required. Current commercially available 10-inch cartridges of the evaluated material have a surface of 0.6 m^2. According to different authors who assayed 0.2 µm pore size microfiltration TFF membranes, processing 100 l fermentation broth would demand 0.45–0.7 m^2 area of filtering cassettes.[7,9–11] Clarification by depth-filtration would require two 0.11 m^2 area depth-filter capsules of diatomite-cellulose combined media, together with a 0.6 m^2 area of a 0.22 µm pore regenerated cellulose filtering cartridge.[8]

Figure 3. Evaluation of different filter fouling models: Filter sizing experiments were conducted under constant pressure, as mentioned in Section 2. Experimental data were collected and plotted for graphical discrimination among different blocking models. The image shows results of one representative experiment.



Unfortunately, the implementation of flocculants for primary clarification during vaccine production has received little interest. Most research has been conducted toward clarification during monoclonal antibodies production. In those studies, pretreatment with polycationic flocculants (including chitosan) has shown to increase 3- to 7-fold the capacity of the following depth-filter trains.[12,13,30] Although the starting material for monoclonal antibody production is significantly different from *C. tetani* culture harvests, it is expected that chitosan pretreatment should lead to comparable increases on filter capacity in current clarification technologies.

Chitosan effects on subsequent TT purification

The effect of flocculant addition for primary clarification on subsequent downstream processing of biomolecules has received scarce attention in the literature. The presence of traces from poly-cationic flocculants in the clarified material has a negative impact on certain purification strategies, such as ion-exchange chromatography, although it does not significantly affect other strategies, such as protein-A affinity chromatography.[12,32] In case of TT, most producers still follow the traditional ammonium sulfate-induced precipitation strategy.[33] Although some authors have proposed chromatographic techniques as an alternative to this traditional technique, they have not found broad implementation at the industrial level.[34–36] Ammonium sulfate precipitation is based on differential hydrophobic interaction between macromolecules in solution at different salt concentrations that renders them insoluble. Chitosan interaction with macromolecules in solution and particles in suspension involves electrostatic forces, hydrogen bonding and also other interactions such as Van der Waals forces.[31] It could be then argued that chitosan traces, due to their potential effect on particle aggregation, might affect purification by ammonium sulfate purification. To test this possibility, the clarified material obtained by the methodology proposed herein was processed by an adaptation at laboratory level of the methodology used at industrial level. First, recovery and partial purification of TT was performed through diafiltration against saline, then TT was further purified by a two-step ammonium sulfate precipitation followed by resuspension and final conditioning by diafiltration against saline. The optimal concentration required for initial precipitation (removal of high molecular weight impurities) and secondary precipitation (isolation of TT by precipitation from impurities that remain soluble) were in the range recommended by WHO, 15 and 25% w/v, respectively.[6] The content of the different fractions obtained after ammonium sulfate purification was analyzed by a combination of SDS-PAGE resolution (Fig. 4) and mass spectrometric identification of the polypeptides (Table 1). As expected, during the second step of precipitation most impurities remained in the supernatant (lane 3), while TT became insoluble and was later solubilized and diafiltered against saline (lane 1). Results from the mass-spectrometric-based identification were as expected, while TT was identified in the most abundant band of purified fractions (band A), bands B, C, D, E, and F were composed by different proteins, most of them already identified in similar fractions by other authors.[37] It could thus be concluded that chitosan-induced flocculation is compatible with traditional TT purification methods.

Figure 4. SDS-PAGE analysis of TT purification: TT from inactivated cultures clarified by the methodology analyzed in this work was purified by the two-step ammonium sulfate precipitation technique. The performance of TT purification was analyzed by 7.5% acrylamide SDS-PAGE and stained by the colloidal Coomassie Brillant Blue technique. Lane 1: diafiltered sediment from the second precipitation step, Lane 2: crude extract before purification, and Lane 3: diafiltered supernatant from the second precipitation step. Selected bands were exercised and analyzed by mass-spectrometry for polypeptide identification. Molecular weight marker: HMW-SDS marker, code 17061501, and GE Health care. Molecular weights in kDa are indicated at the left of the figure.

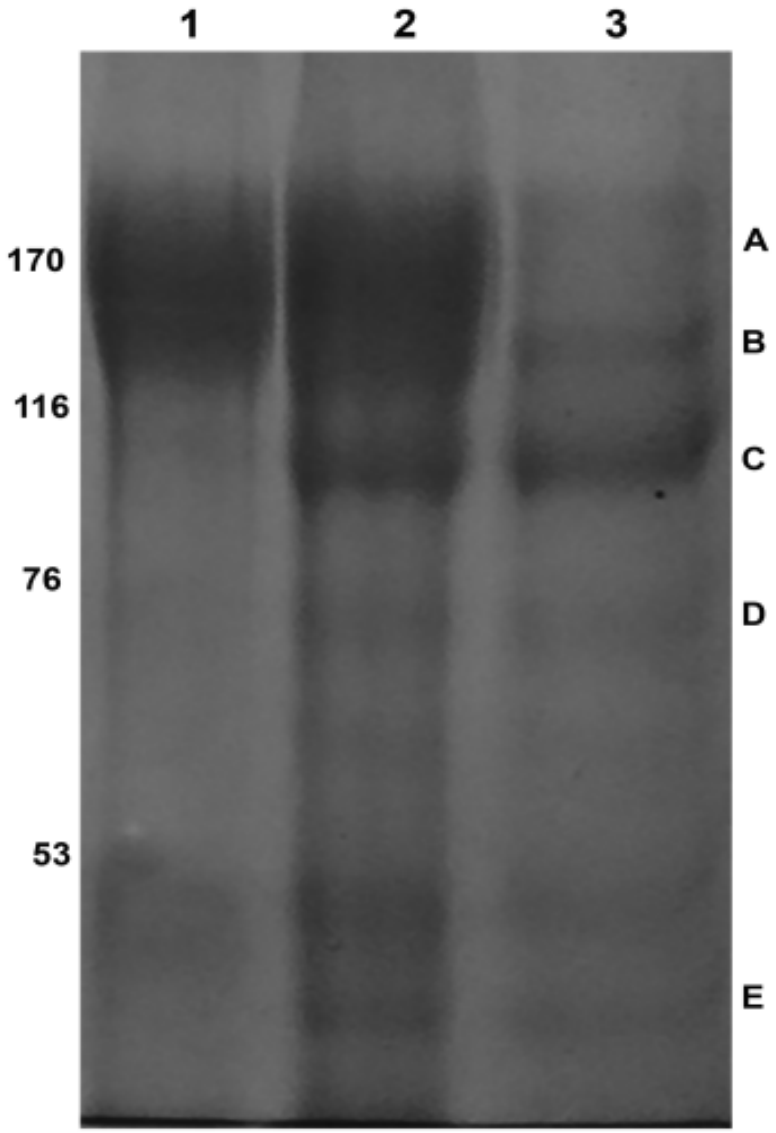


Table 1. Mass spectrometry (ESI-MS/MS) based protein identification.Table Layout

Gel band	Description	Score	# Unique peptides	Coverage (%)	MW (kDa)
A	Tetanus toxin	3271.80	31	94.45	150.6
B	Putative S-layer protein/N-acetylmuramoyl-L-alanine amidase	3180.08	73	91.02	118.6
C	Putative S-layer protein/N-acetylmuramoyl-L-alanine amidase	4906.51	69	87.87	118.6
Note: [a]Gel band E and E* results correlate to samples excised from lanes 3 and 1, respectively.					

Gel band	Description	Score	# Unique peptides	Coverage (%)	MW (kDa)
D	Putative S-layer protein/N-acetylmuramoyl-L-alanine amidase	3263.60	54	84.54	118.6
E[a]	Methylaspartate ammonia-lyase	900.32	69	98.80	45.6
E*[a]	Tetanus toxin	400.32	79	49.89	150.6
	Methylaspartate ammonia-lyase	130.59	26	60.48	45.6
F	Glyceraldehyde-3-phosphate dehydrogenase	918.32	51	94.33	36.5
Note: [a]Gel band E and E* results correlate to samples excised from lanes 3 and 1, respectively.					

Conclusion

Chitosan-induced particle sedimentation is evaluated for the first time as a non-expensive alternative for primary clarification of *C. tetani* cultures during TT production. Filter sizing predicted that complete clarification of the inactivated culture would be achievable at 100 l scale in two simple steps with 0.8 g of chitosan and a 0.6 m^2 area of a 0.45 + 0.22 µm pore size regenerated cellulose filtering cartridge. Thus, simple and non-expensive chitosan-induced particle aggregation can complement or even replace current primary clarification operations employed for culture clarification during TT production.

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