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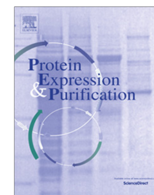

A scalable method for biochemical purification of *Salmonella* flagellin

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ABSTRACT

Flagellins are the main structural proteins of bacterial flagella and potent stimulators of innate and adaptive immunity in mammals. The flagellins of *Salmonella* are virulence factors and protective antigens, and form the basis of promising vaccines. Despite broad interest in flagellins as antigens and adjuvants in vaccine formulations, there have been few advances towards the development of scalable and economical purification methods for these proteins. We report here a simple and robust strategy to purify flagellin monomers from the supernatants of liquid growth culture. Phase 1 flagellins from *Salmonella enterica* serovars Typhimurium (i epitope) and Enteritidis (g,m epitopes) were purified directly from conditioned fermentation growth media using sequential cation- and anion-exchange chromatography coupled with a final tangential flow-filtration step. Conventional porous chromatography resin was markedly less efficient than membrane chromatography for flagellin purification. Recovery after each process step was robust, with endotoxin, nucleic acid and residual host–cell protein effectively removed. The final yield was 200–300 mg/L fermentation culture supernatant, with ~45–50% overall recovery. A final pH 2 treatment step was instituted to ensure uniformity of flagellin in the monomeric form. Flagellins purified by this method were recognized by monoclonal anti-flagellin antibodies and maintained capacity to activate Toll-like Receptor 5. The process described is simple, readily scalable, uses standard bioprocess methods, and requires only a few steps to obtain highly purified material.

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Introduction

The flagella of proteobacteria are large multi-protein structures extending from the cell surface that rotate helically to impart motility. The central filament portion, constituting the bulk of the flagellar structure, is a homogeneous multimer of the flagellin protein present at up to 30,000 subunits per flagellum. The folded *Salmonella* flagellin protein assumes an "L" shaped structure, comprised of 4 unique domains (designated D0–D3). The ~250 amino acids comprising the N- and C-termini form D0 and D1, and have been documented as mostly invariant among Gram-negative and Gram-positive bacteria, including spirochetes that express flagella within the periplasmic compartment [1]. The central polypeptide portion comprises the D2 and D3 regions which are variable in amino acid sequence and length, and bear the epitopes that impart serotype specificity. Flagellins are transported extracellularly from the cytoplasm through the narrow channel of the basal body, whereupon they aggregate into helical flagella filaments under the direction of the FliD flagellar capping protein, with D0 and D1 forming the core and D2 and D3 the outer flagellar surface [2]. The integral residues for flagellar packing are contained within D0 and D1, where interactions between contact residues on adjacent monomers stabilize the flagellar structure [2,3].

Flagella are virulence factors and protective antigens for several bacterial pathogens [4–11]. Antibodies against flagellin have been shown to mediate protection in animal models against infections caused by several important bacterial pathogens (e.g., *Salmonella*, *Pseudomonas*, *Burkholderia*), and have been found to arrest motility and increase opsonophagocytic uptake *in vitro* [12,13]. The flagellin proteins of β and γ proteobacteria contain conserved motifs within D0 and D1 that are recognized by the mammalian innate immune Toll-like Receptor 5 (TLR5)[1] [14]. Signaling through TLR5 causes

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[1] *Abbreviations used:* TLR5, Toll-like Receptor 5; NTS, non-typhoidal *Salmonella*; TMB, 3,3′,5,5′-tetramethylbenzidine; MV, membrane volumes; GMP, Good Manufacturing Practice.

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production of cytokines and chemokines that direct inflammation and promote the induction of adaptive immunity. Accordingly, exposure to flagellin, either by natural infection or immunization, results in high levels of serum anti-flagellin antibodies and robust CD4+ T-cell responses [4,15,16]. The powerful immunostimulatory properties of flagellin have further improved immune responses towards co-administered protein antigens after admixture, covalent linkage, or incorporation into non-covalent complexes [17–19]. We and others have also documented that flagellins are effective carrier proteins that enhance immune responses to chemically linked bacterial polysaccharides [5,20–22].

The unique protein epitopes present within *Salmonella* flagellins are characteristic and conserved for individual serovars, and provide the basis in part for serotyping in the Kauffman–White scheme [23]. In sub-Saharan Africa, invasive infections in children with non-typhoidal *Salmonella* (NTS) serovars Enteritidis and Typhimurium are common, and associated with high fatality rates [24]. Based on the notion that antibodies against *Salmonella* O polysaccharides and flagellin proteins are independently protective, we have developed a promising candidate conjugate vaccine comprised of lipopolysaccharide-derived core and O polysaccharide coupled to the phase 1 flagellin protein from the same serovar [5,25]. Given the cost-constraints for vaccines for the developing world, a method by which flagellin could be obtained economically is a key requirement for transition of flagellin-based conjugates towards broad use in human vaccines. We previously reported the engineering of *Salmonella enterica* serovar Enteritidis and Typhimurium strains that are greatly attenuated and constitutively secrete high levels of flagellin as monomers. These "reagent strains" are safer to manipulate from an occupational health standpoint and can serve as robust expression systems from which to purify large amounts of flagellin [26].

Despite the broad interest in flagellins as components of immunoprophylactic strategies, relatively few advances have been made towards the development of purification methods. The original flagellin purification method, that remains widely employed in the published literature, is based on mechanical shearing of flagella from the bacterial surface coupled with differential low- and high-speed centrifugation to remove cell debris and pellet flagella, respectively [7,14,27]. An improvement to this method exploits the differential pH 2 stability of flagellin monomers and flagella multimers, whereby exposure to low pH causes cell-associated flagella to disaggregate into monomer subunits that are soluble and stable at pH 2. Deflagellated cells are subsequently removed by low-speed centrifugation and the supernatant flagellins are then precipitated with ammonium sulfate [28]. Neither of these methods is optimal, however. Cell-associated flagella frequently shear under the agitation conditions required for aeration in liquid culture, hence conditions that maximize flagella recovery are associated with poor bacterial growth. Furthermore, neither method employs selective nucleic acid or endotoxin removal, or protein fractionation. An ion-exchange method has been reported, whereby flagellin in the boiled supernatants of liquid growth culture are concentrated with 30 kDa centrifugal filters and passed through cation-exchange resins by negative chromatography. The flow-through fraction is then subjected to anion exchange resin chromatography and endotoxin removal using polymyxin B [29,30]. Yields were not reported for this method. Negative chromatography in early bioprocess steps necessitates greater binding capacity however due to higher contaminant levels; furthermore, the use of polymyxin B is generally associated with reduced product yields [31]. Boiling of protein preparations also introduces the possibility for product breakdown.

Herein, we report a simple, efficient and scalable method for purification of flagellins, applied to the phase 1 flagellins of *S.* Enteritidis (g,m epitopes) and *S.* Typhimurium (i epitope). Flagellins were purified with high yield and purity from liquid growth culture supernatants that overcomes the limitations and caveats of the aforementioned conventionally used methods.

Materials and methods

Strains and growth media

Salmonella Enteritidis CVD 1943 is a derivative of Malian invasive strain R11 and is deleted in the following genes: *guaBA*, *clpP* and *fliD*. *S.* Typhimurium CVD1925, a derivative of the wild-type invasive Malian strain I77, has been described, and has deletions in *guaBA*, *clpP*, *fliD* and the gene for phase 2 flagellin, *fljB* [26]. Both strains were grown in chemically defined media [32]. For growth in shake flasks, media was formulated with 13.3 g/L potassium phosphate monobasic, 4 g/L ammonium phosphate dibasic, 6.8 g/L citric acid monohydrate, 1.5% glycerol, 1 ml/L polypropylene glycol, 0.004% guanine, 0.005 M magnesium sulfate, 0.0001 M ferric citrate, and 1 ml/L each of trace vitamin (5 g/L thiamine hydrochloride, 10 g/L nicotinic acid, 10 g/L calcium pantothenate, 10 g/L pyridoxine hydrochloride, 10 g/L Vitamin B12) and trace element (2.5 g/L cobalt chloride, 15 g/L manganese chloride, 1.5 g/L copper chloride, 3 g/L boric acid, 2.5 g/L sodium molybdate, 2.5 g/L zinc acetate dihydrate, 1 ml/L sulfuric acid) solutions. For growth in fermenters, the same media formulation was used but with higher amounts of guanine (0.025%). All media were adjusted to pH 7 prior to inoculation with bacteria.

Analytical tests

Endotoxin levels were assessed by Endosafe PTS® chromogenic Limulus amebocyte lysate assay (Charles River, MA). Nucleic acid levels were measured using the quanti-iT Picogreen DS DNA assay kit (Life Technologies, Carlsbad) per the manufacturer's instructions, with the supplied standards and a fluorometer (Molecular Devices, CA). Proteins were monitored for size by SDS–PAGE with 4–20% Tris–Tricine gels and stained with Coomassie blue (Thermo-Pierce, MA), or transferred to nitrocellulose membranes and probed with a 1:10,000 dilution of monoclonal antibody 15D8 (Bioveris, MD) that broadly recognizes flagellins. Protein size was monitored by HPLC-SEC using a BioSep SEC 4000 (Phenomenex, CA) column and measurement of absorbance at 280 nm and 215 nm on a BioAlliance 2796 with a 2414 dual wavelength UV detector (Waters, MA). Protein concentrations were determined by bicinchoninic acid assay (BCA) (Thermo-Pierce, MA) using the manufacturer's instructions, and flagellin standards obtained as described [5]. In order to account for interference by the culture media in the BCA assay, determination of phase 1 flagellin protein (FliC) levels in growth culture supernatants was accomplished by densitometry analysis of Coomassie stained SDS–PAGE separated samples relative to FliC standards.

ELISA

Analyses were conducted by coating purified FliC from CVD 1943 at 5 μg/ml in 96-well plates (Greiner) in 0.05 M sodium carbonate (pH 9.6), for 3 h at 37 °C and blocking overnight with 10% dried milk in PBS. Following each incubation, the plates were washed with PBS containing 0.05% Tween 20 (PBST) (Sigma, MA). FliC coated wells were incubated with various concentrations of a panel of monoclonal antibodies that were produced from mice immunized with *S.* Enteritidis FliC and selected by positive or negative ELISA reactivity with phase 1 flagellins from *S.* Enteritidis, *S.* Typhimurium, *S.* Paratyphi A, *S.* Paratyphi B and *S.* Typhi (Antibody and Immunoassay Consultants LLC, Rockville, MD).

Monoclonal antibodies CB7IH2 and AE9IB4 are broadly reactive by ELISA with all of the above flagellins; monoclonal antibodies CA6IE2 and JB11IG4 recognize only *S.* Enteritidis FliC. Unique epitope specificity for each antibody was independently confirmed by sandwich ELISA, using various combinations for capture and detection. For all analyses, antibodies were diluted in PBST containing 10% milk. Bound antibody was detected by incubation with goat-anti-mouse IgG conjugated to peroxidase (KPL), and detected with 3,3′,5,5′-Tetramethylbenzidine (TMB) used as the substrate (KPL).

Fermentation growth and harvest

Flasks were inoculated with glycerol stocks directly, and grown for 12 h at 37 °C with shaking at 250 rpm. The 12 h culture was used to directly inoculate the fermentation culture to an initial OD 600 nm of 0.15. Fermentation was accomplished by growth for 6–7 h at 37 °C and maintenance of pH 7, with 30% dissolved oxygen and agitation in cascade mode, 15 LPM air-flow in a 20 L Biostat Fermentor (Braun, Germany). The fermentation culture was clarified by continuous centrifugation followed by 1 μm (Ultra Capsule GF)–0.2 μm (Ultra Capsule HC) depth filtration. Supernatants were held at 4 °C between processing steps.

Ion-exchange membrane chromatography

All membrane chromatography steps were conducted at ~25 °C, with flow rates of 0.5 membrane volumes (MV) per minute, and capacities of ⩾0.05 grams FliC per milliliter MV, using AKTA chromatography systems (GE Life Sciences, NJ) that were monitored and controlled with the Unicorn software package.

Cation exchange chromatography

Flagellin was bound and washed directly from fermentation supernatants using two 5″ 70 ml Sartobind S cation-exchange membranes linked in series (Sartorius, Bohemia, NY). Fermentation supernatants were first diluted ~4-fold and brought to 50 mM acetic acid pH 3.4 with conductivity of 4.7 mS/cm. The adjusted supernatant was loaded onto the membranes in 50 mM acetic acid pH 3. Membranes were then washed with 18 MV of 50 mM acetic acid pH3, then 15 MV of 50 mM acetic acid/1.5 M NaCl/5 mM EDTA/0.1% Tween 20 pH 3, then 16 MV of 50 mM acetic acid pH 3. FliC was eluted by raising the pH with 20 mM Tris pH 8.

Anion-exchange chromatography

The eluate from cation-exchange membrane chromatography was further purified using two 5″ 70 ml Sartobind Q anion-exchange membranes linked in series (Sartorius, Bohemia, NY). Membranes were pre-equilibrated with 20 mM Tris pH 8. Cation-exchange eluates were brought to 1.5 mS/cm pH 8.2 with 20 mM Tris and 5 M NaOH prior to loading on a Q membrane in 20 mM Tris pH 8. Membranes were then washed with 18 MV of 20 mM Tris pH 8, and proteins were eluted with 15 MV of 20 mM Tris/150 mM NaCl pH 8. Protein containing fractions were confirmed by SDS–PAGE Coomassie and pooled for further purification steps.

Tangential Flow Filtration (TFF)

Pooled flagellin containing elution fractions from anion-exchange membrane chromatography were concentrated to ~10 mg/ml and diafiltered against 10 diavolumes of 150 mM NaCl (saline) solution with 30 kDa Pellicon flat sheet TFF membranes (Millipore, MA) using approximately 0.2 m^2 membrane area per liter of starting material, and an equilibrated transmembrane pressure of ~1 PSI.

Monomerization to FliC subunits

Concentrated flagellin protein in saline was brought to 0.1% Tween 20 and the pH was lowered to 2 with 5 M HCl with stirring at ~25 °C. After 30 min, the solution was brought to 10 mM phosphate buffer (PBS) pH 7 with 5 M NaOH, and sterile filtered at 0.2 μm (Millipore, MA).

Flagellin stimulation of epithelial cells

Monolayers of HEK293-κB-Luc cells seeded at 1.7×10^4 cells/well in 96-well plates for NF-κB activation analyses were treated in duplicate for 4 h with media or purified flagellin proteins. Extracts were prepared, and luciferase activity was assessed by the Firefly Luciferase Assay system (Promega, WI) according to the manufacturer's instructions using a Lmax II plate luminometer (Molecular Devices, CA).

Results and discussion

Accumulation of FliC in fermentation culture supernatants

Bacteria were grown in chemically defined minimal growth media to reduce the contaminant background from exogenous biological components, where culture densities of 16–18 OD 600 nm were reliably obtained. Maximal levels of accumulated flagellin in CVD 1943 fermentation growth supernatants, indicated by the ~52 kDa band in SDS–PAGE analysis, accompanied the increase in cell division during logarithmic growth and peaked in late logarithmic phase (Fig. 1). Remarkably, flagellin was the overwhelming major protein in culture supernatants when assessed by SDS–PAGE. Comparable growth characteristics and flagellin secretion patterns were observed for *S.* Typhimurium CVD 1925 (not shown). We found that growth beyond log phase produced conditions that degraded flagellin protein, presumably due to bacterial lysis and release of cytoplasmic proteases (data not shown). Hence, cultures were harvested directly prior to entering stationary phase. Clarification of growth cultures was accomplished by centrifugation and microfiltration to 0.2 μm.

Binding and elution by cation-exchange chromatography

Direct protein binding precludes the need for concentration and buffer exchange prior to ion-exchange chromatography. By establishing conditions for product binding in early stages, the overall process time, number of steps and potential product loss is reduced. We found that flagellins could be bound directly from

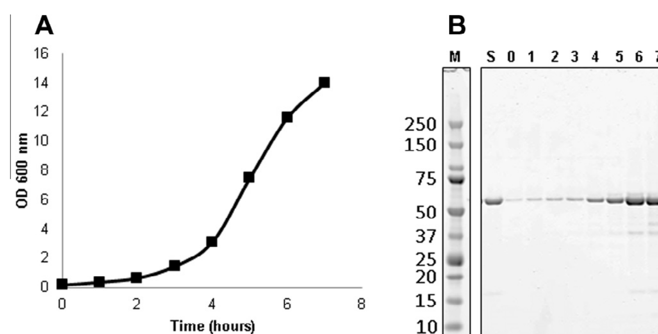

Fig. 1. Fermentation growth and accumulation of flagellin in culture supernatants of *Salmonella* Enteritidis reagent strain CVD 1943. Kinetics of growth and FliC accumulation in CVD 1943 fermentation culture were monitored by optical density at 600 nm (A) and SDS–PAGE with Coomassie staining (B).

growth media supernatants onto cation-exchange membrane sorbents at pH 3, that is ⩾1 pH units below the calculated ∼4.5 flagellin isoelectric point (pI), at reduced conductivity levels (Fig. 2). Comparable binding and elution profiles were found for flagellins from CVD 1943 or CVD 1925 fermentation supernatants (not shown).

A high 254 nm absorbance signal was noted in the flow through during the loading step (Fig. 2A). This is likely representative of unbound bacterial nucleic acid and exogenous guanine in the growth medium, which would be excluded by charge repulsion from the cation exchange sorbent. When analyzed by SDS–PAGE with Coomassie blue staining, no discernable protein bands were detected in the flow through (Fig. 2B). A large peak characterized by higher absorbance at 254 nm relative to 280 nm was also seen after exposure to 1.5 M NaCl, which would suggest further removal of residual nucleic acid. We found, unexpectedly, that flagellin was retained on cation-exchange membranes in the presence of this high salt concentration, as no flagellin bands were evident in the Coomassie stained SDS–PAGE of these fractions. This could be the result of tight binding to a charged pocket on flagellin, alone or in combination with hydrophobic interactions with the base fiber of the membrane matrix. As comparable performance was seen for CVD 1943 and CVD 1925 FliC, the site responsible for this interaction is likely located on a region of the protein that is conserved between the two different flagellin types.

Flagellin elution was accomplished by raising the pH with 20 mM Tris pH 8, that is >1 pH unit above the calculated pI. Proteins eluted as a single peak with an absorbance ratio of 280 > 254 nm, indicating a low ratio of nucleic acid to protein. Recovery of protein from the fermentation supernatant was high (∼70–80%, Table 1), with robust removal of nucleic acid (>75%). We found, however, that endotoxin removal in this step was poor. It is presumed that free LPS that is present in large micelles and liposomes is removed during the clarification step, and residual endotoxin is tightly bound as LPS monomers to the protein. As the phosphate groups of lipid A in LPS are negatively charged, it is not expected that they would be removed by the cation-exchange sorbent.

Interestingly, flagellin was retained more efficiently by strong cation-exchange sulfonate functionalized membranes that are macroporous compared to conventional porous sulfonate functionalized cation-exchange resins (Fig. S1). Membranes effectively captured ∼70% of the FliC input protein, with ∼3% and ∼9% found in the flow-through and 1 M NaCl wash fractions respectively. For resins, ∼52% of FliC was found in the flow-through fraction, ∼23% was removed in the 1 M NaCl wash fraction, and ∼12% was present

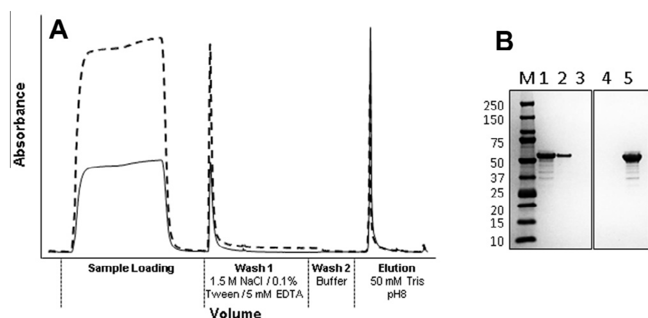

Fig. 2. Binding, wash, and elution of secreted FliC in CVD 1943 fermentation supernatants by cation-exchange membrane chromatography. (A) Chromatogram with absorbance at 280 nm (solid line) and 254 nm (dashed line); (B) SDS–PAGE with Coomassie stain of (M) Molecular weight standards, (1) fermentation culture supernatant, (2) diluted and pH adjusted fermentation supernatant, (3) column loading flow-through, (4) peak absorbance fraction from wash 1, (5) peak absorbance elution fraction. All lanes are from the same gel and exposure.

in the pH 8 elution fraction. Despite the fact that flagellins are secreted as monomers, concentration-dependent aggregation could occur within growth media or the column microenvironment, as multimers phenotypically resembling flagella filaments form at flagellin concentrations ⩾5 mg/ml [33]. The substantial level of nucleic acid in the fermentation supernatant also introduces the possibility for formation of large complexes with flagellin. Treatment with benzonase prior to loading onto a cation exchange resin did not improve retention (data not shown). The wide pore sizes in membranes can likely accommodate flagellin multimers or complexes, whereas inaccessibility of these aggregates to the porous bead interior likely impacts full accessibility to the charged groups of conventional ion-exchange resins. This could result in tethered binding to the bead surface through a single protein anchor, rather than uniform binding across the multimer to charged sorbent groups. In addition to the improved binding capacity at the binding step, ion-exchange membranes are convective and hence not diffusion limited. This permits higher flow-rates and better scalability than conventional ion-exchange resins. Thus, they are better suited for large-scale Good Manufacturing Practice (GMP) production campaigns as they enable reduced process time, performance parameters translate more readily from process development scale, and they are amenable to single-use application.

Anion exchange membrane chromatography

Tris-based buffers are compatible with anion-exchange media, hence no buffer exchange was necessary prior to initiation of anion-exchange chromatography. Flagellin protein eluates in 20 mM Tris pH 8 after cation-exchange membrane chromatography were fully bound by quaternary ammonium (Q) strong anion-exchange membranes (Fig. 3). Various salt levels were assessed for elution (data not shown), and we found that 150 mM NaCl was the minimal concentration required to remove the bulk of the bound flagellin (Fig. 3). Residual endotoxin and nucleic acid were effectively removed by this step (>99.9% and >98% respectively, Table 1), with 60–75% protein recovery at this step.

Concentration and monomerization

Salmonella Enteritidis and Typhimurium FliC proteins demonstrate molecular weights of ∼52 kDa and 50 kDa, respectively, by SDS–PAGE (Fig. 4). They were fully retained by 30 kDa flat sheet tangential flow filtration that was used for concentration and diafiltration after elution from anion-exchange membranes (Fig. 4). The TLR5 moiety of flagellin monomers becomes buried upon incorporation into flagella filaments, hence TLR5 activity for flagella is lower than that of flagellin monomers [14]. In order to standardize biological activity and maintain the purified protein as a homogenous species, it is preferable to obtain flagellin as monomers rather than as multimers, which can be heterogeneous in size and stability. Flagellins are stable and soluble at pH 2, where it has been documented that flagella will disassociate into flagellin monomers [28]. Manipulation of the pH to disaggregate flagellin multimers that may have formed during the purification process is less harsh and more easily controlled than other commonly used methods such as heat treatment [14]. TFF retentates were brought to pH 2 transiently and then returned to neutral pH with phosphate buffer in the presence of 0.1% non-ionic detergent Tween 20. We have found that this helps maintain flagellin in monomeric form at concentrations exceeding those that typically lead to aggregation. Monomerization by this method did not cause overt discernible protein degradation by SDS–PAGE, concentrated and monomerized flagellin produced a single peak at the expected flagellin monomer size by HPLC-SEC (Fig. 4). Several minor bands

Table 1
Total protein yield and reduction in nucleic acid and endotoxin levels after sequential purification steps for FliC from 1 L of *S.* Enteritidis CVD 1943 or *S.* Typhimurium CVD 1925 fermentation culture supernatants.

	Protein mg (% yield)[a]		Nucleic acid mg[d]		Endotoxin units[e]	
	CVD 1943	CVD 1925	CVD 1943	CVD 1925	CVD 1943	CVD 1925
Fermentation supernatant	438[b] (100)	688[b] (100)	0.827	1.5	3.38×10^7	n.d.[f]
Post-S membrane	344[c] (79)	478[c] (69)	0.178	0.370	4.18×10^7	6.52×10^7
Post-Q membrane	209[c] (48)	364[c] (53)	0.002	0.008	4.15×10^3	4.80×10^3
30 kDa TFF concentrated retentate	204[c] (47)	311[c] (45)	0.001	0.003	2.75×10^3	2.90×10^3

[a] Relative to FliC amount in culture supernatant.
[b] Total FliC determined by SDS–PAGE Coomassie/Densitometry with FliC standards.
[c] Total protein levels determined by BCA assay with FliC standards.
[d] Total double stranded DNA levels determined by quanti-iT Sybr Green.
[e] Total endotoxin levels determined by Limulus amebocyte lysate assay.
[f] Not done.

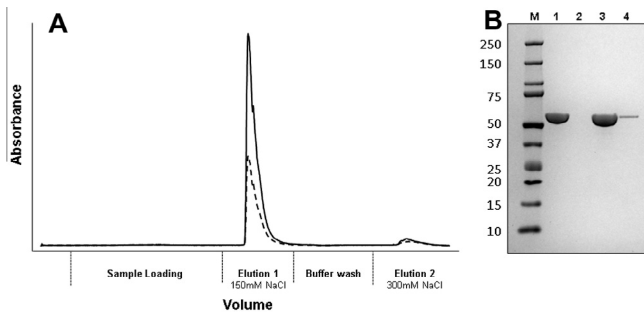

Fig. 3. Anion-exchange membrane chromatography on cation-exchange membrane eluates of CVD 1943 secreted flagellin. (A) Chromatogram of A280 nm (solid black line) and A254 nm (dashed line) with indicated treatment steps denoted; (B) SDS–PAGE with Coomassie stain for: (1) anion exchange starting material, (2) flow through fraction, (3) 150 mM NaCl eluate, (4) 300 mM NaCl eluate. Molecular weight marker is denoted (M).

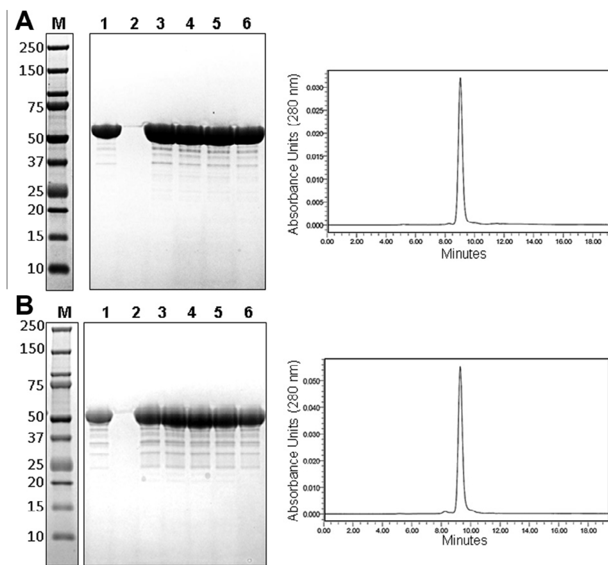

Fig. 4. Concentration and monomerization of flagellins after anion-exchange chromatography. (A) CVD 1943 and (B) CVD 1925: (left panel) in-process material was analyzed by SDS–PAGE with Coomassie staining: (M) Molecular weight standards, (1) 150 mM NaCl anion exchange membrane eluate [10 µg], (2) 30 kDa TFF permeate, (3) 30 kDa TFF concentrated retentate [25 µg], (4) 30 kDa TFF concentrated-diafiltered retentate [25 µg], (5) post-pH 2 incubation [25 µg], (6) 0.2 µm filtrate [20 µg]; (right panel) SEC-HPLC of 0.2 µm filtered purified FliC measuring absorbance at 280 nm.

were seen by SDS–PAGE that migrated below the level of flagellin. We have previously noted a similar banding pattern in flagellin preparations prepared by the shear method, for which it was determined by mass-spectrometry peptide sequencing that they derive from progressive breakdown of the N- and C-flagellin protein termini.

The purified flagellin preparations reacted equivalently by Western blot with a general anti-flagellin monoclonal antibody, producing a single major uniform band at the expected molecular weight (Fig. 5A). By assessing reactivity with antibodies against different conserved and variable epitopes, it is presumed that protein folding within distinct portions of flagellin can be interrogated. The successful binding for multiple different epitopes strongly suggests that the overall protein is folded in the native conformation. Flagellin purified from CVD 1943 demonstrated robust recognition by ELISA with a panel of monoclonal antibodies specific for common or unique *S.* Enteritidis FliC epitopes (Fig. 5B), thus signifying that flagellin purified by this method is properly folded.

Innate immune bioactivity

Salmonella flagellins are ligands for mammalian TLR5 and hence potent activators of the innate immune system. Binding to TLR5 is accomplished through a conserved contact interface between the extracellular portion of TLR5 and the D1 region of flagellin, where functional interaction is dependent on protein structure and orientation [34]. Signaling through TLR5 is facilitated by the central immune transcription factor NF-κB [35,36]. We confirmed the TLR5 bioactivity of the purified flagellin preparations *in vitro*, using HEK293 cells stably transformed with a luciferase report under control of the NF-κB promoter [35]. Activation of the luciferase

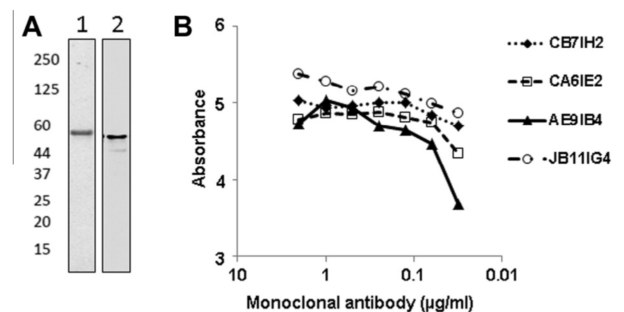

Fig. 5. Reactivity of final purified flagellin proteins with anti-flagellin antibodies assessed by Western blot and ELISA. (A) Post 0.2 µm filtration final purified samples were loaded at 0.25 µg/well, separated by SDS–PAGE and transferred to nitrocellulose membranes for detection with a pan flagellin antibody: (1) CVD 1943 FliC, (2) CVD 1925 FliC; (B) CVD 1943 FliC coated onto ELISA wells, reacted with different monoclonal antibodies against common (CB7IH2, AE9IB4) or serovar specific (JB11IG4, CA6IE2) epitopes of *S.* Enteritidis FliC, tested in multiple concentrations; results represent absorbance values from replicate wells.

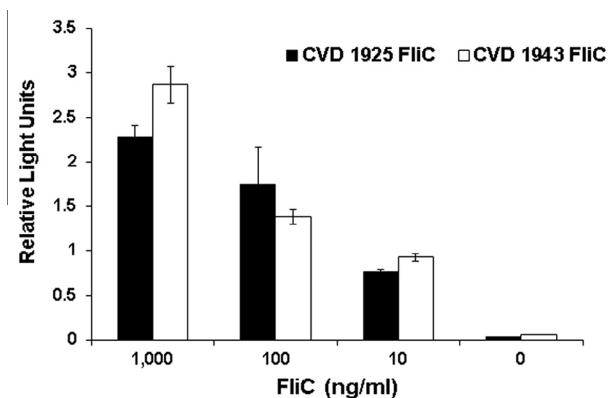

Fig. 6. Functional innate immune bioactivity of *S.* Enteritidis CVD 1943 and *S.* Typhimurium CVD 1925 flagellins purified from fermentation culture supernatants. HEK293 cells stably transformed with a firefly luciferase reporter gene under control of NF-κB were seeded in 96-well plates and treated with media alone or the indicated concentration of CVD 1925 or CVD 1943 FliC. Luciferase levels were determined, and are presented as the average and standard error from duplicate wells.

reporter was found to be concentration-dependent, with a strong signal seen even with very low flagellin amounts (Fig. 6). Comparable specific activity was also observed between flagellins purified from *S.* Enteritidis CVD 1943 or *S.* Typhimurium CVD 1925. This further supports the conclusion that the TLR5 signaling domain is correctly folded in flagellins purified by this method.

Conclusions

We report herein a novel and scalable bioprocess strategy for purification of *Salmonella* flagellins from bacterial culture supernatants, applied to the phase 1 flagellins of *S.* Enteritidis and *S.* Typhimurium. We anticipate that this purification scheme will be economical under large-scale production as the process requires only a few steps, can be completed in only a few days, and is accomplished with standard techniques, apparatus and media that are commonly used in bioprocess manufacturing. We have further found that this method is effective for purification of FliC from *S.* Typhi (d epitope, not shown). Thus, we expect that this purification approach will be efficacious for purification of flagellins from other *Salmonella* serovars that are important causes of disease in humans, such as *S.* Paratyphi A (*Salmonella* serogroup A), or group C *Salmonella* serovars such as *S.* Newport or *S.* Choleraesuis. Given the high degree of homology within D0 and D1 among flagellins from different bacteria, this method could also possibly be used to produce vaccines for other important bacterial pathogens where flagellin is an established vaccine antigen, such as the FlaA or FlaB flagellins of *Pseudomonas aeruginosa* [37].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pep.2014.07.005.

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