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PRODUCTION OF NATIVE FLAGELLIN FROM *Salmonella* Typhimurium IN A BIOREACTOR AND PURIFICATION BY TANGENTIAL ULTRAFILTRATION

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Abstract - Flagellin is the structural protein and most abundant component of bacterial flagella. The flagellum filament contains around 20,000 – 100,000 subunits of 50 kDa flagellin that can have diverse biotechnological applications such as vaccine adjuvant and cellular protector during chemo- and radiotherapy. The main aim of this work was to study a production process of purified native FliC flagellin of *Salmonella* Typhimurium. The culture conditions in shakers were established with medium devoid of animal-derived components. In bioreactors, culture conditions were established in order to obtain flagellin from the culture supernatant by tangential ultrafiltration (TUF). The concentrated 750 kDa cut-off TUF fraction had a purification factor of 1.5 and a recovery yield of 52.2% for flagellin. The volumetric production of flagellin using the described procedure achieved around 307 mg/L of culture, which represented a significant improvement over previously reported methods. These results permit the development of production and purification processes that can be easily scaled up.
Keywords: Flagellin; Purification; Tangential ultrafiltration; *Salmonella* Typhimurium.

INTRODUCTION

The bacterial flagellum is a complex structure that allows movement and survival of bacteria. Flagella are vital structures for pathogens, promoting cell adhesion and invasion (Ramos *et al.*, 2004). The bacterial flagella consist of three different parts: the basal body (driving force); the hook (works as a universal joint) and the filament (the long helical tubular filament). The flagellar filament is composed of a single protein, flagellin. About 20,000 – 100,000 flagellin subunits are polymerised with the aid of a tip chaperone to form the long filament shaft on the cell surface that can extend up to ten times the size of the bacterial cell itself (Yonekura, 2002; Adkins *et al.*, 2006). In *Salmonella* Typhimurium, the FliC flagellin has a molecular mass of approximately 50 kDa and shows four structural domains. The terminal helical D0 and D1 domains are located inside the filament, are required for filament assembly and are highly conserved among different bacterial species. The D2 and D3 domains are exposed on the surface of the filament and show great diversity of amino-acid sequence among different bacterial species. These surface-exposed domains are rich in B cell epitopes and represent the targets of H antigen serotyping (Chng, 2010).

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In addition, flagellin (Figure 1) is also a potent activator of innate immune responses. Its effects are mediated by the specific binding of the D1 domain (blue rectangle) to Toll-like receptor 5 (TLR5) (Smith, 2003). In mammals, this interaction triggers a signaling cascade that results in expression of co-stimulatory molecules and production of cytokines by cells like macrophages and dendritic cells, leading to a more efficient activation of adaptive immune responses (Didierlaurent, 2004). Indeed, *Salmonella* flagellin, both as native or recombinant protein, has been intensively studied as an adjuvant in different vaccine formulations via parenteral or mucosal routes, either admixed or genetically fused to the target protein antigen (Gerwitz *et al.*, 2001a; Gerwitz *et al.*, 2001b; Hayashi *et al.*, 2001; Moors *et al.*, 2001; Liaudet *et al.*, 2002; Huleatt *et al*., 2007; Uematsu *et al.*, 2008; Bargieri *et al*., 2008; Bargieri *et al*., 2010; Braga *et al*., 2010;). *Salmonella* flagellin has been successfully tested as a cell protector, both *in vitro* and *in vivo*, against the toxic effects of chemical and radiological procedures commonly used in the treatment of cancer (Ramos *et al.*, 2004; Vijay-Kumar *et al.*, 2008; Sanders *et al.*, 2008; Wang *et al.*, 2008).

The need for new and safe vaccine adjuvants requires the generation of flagellin with a high degree of purification. Only a few previous studies reported the purification of *Salmonella* flagellin under well-defined experimental conditions and none of them focused on the establishment of procedures allowing downstream upgrade of the purification process (Montie *et al.*, 1983; Ibrahim *et al.*, 1985; Logan *et al.*, 1987). Basically, the reported small-scale procedures involve cultivation of the microorganism in shake flasks or bioreactors and purification of flagellin based on mechanical shearing of flagella, differential centrifugation, depolymerization and repolimerization of flagella by pH changes, followed by precipitation with ammonium sulphate and dialysis or acetone precipitation (Ibrahim *et al.*, 1985; Braga *et al.*, 2008). These processes are laborious, time-consuming, have low recovery yields and result in material highly contaminated with endotoxin.

Our group has successfully applied tangential ultrafiltration, with different cut-off sizes, to the purification of various biomolecules derived from microorganisms (Gonçalves *et al*., 2003; Takagi, *et al*., 2008; Oliveira *et al*., 2010). In the present report we describe conditions that allow production of *Salmonella* FliC*i* flagellin in bioreactors with subsequent purification in high yields by tangential ultrafiltrafiltration. The reported procedure proved to be simple and fast, and permit easy scale-up optimization that attends the demand of highly purified *Salmonella* flagellin.

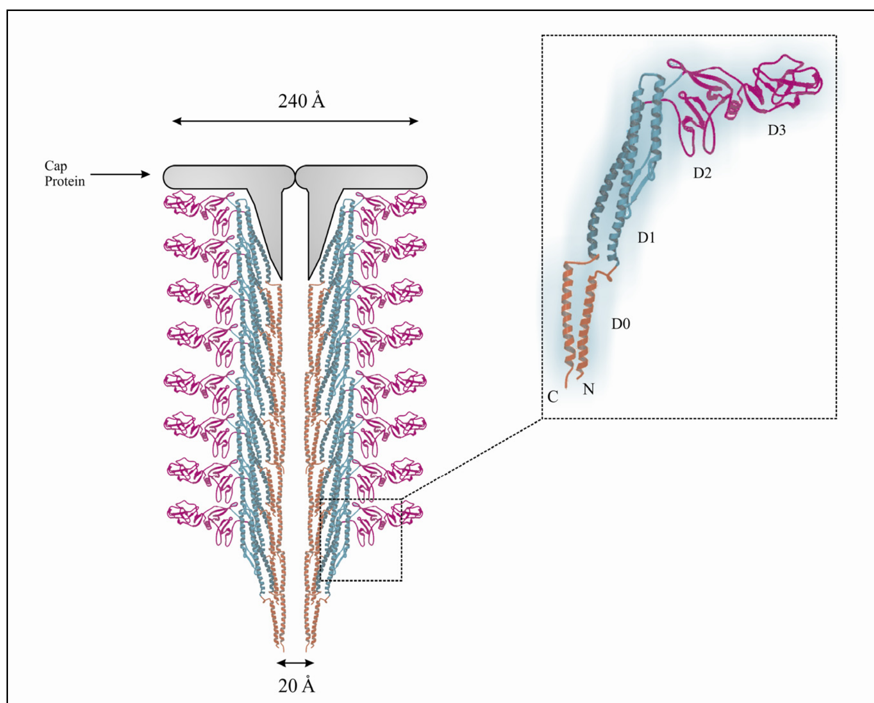

Figure 1: Schematic longitudinal view of the *S.* Typhimurium flagellum and ribbon diagram of the flagellin monomer. The flagellin structural domains are coloured as follows: the terminal α-helix chains (D0, brown), the central α-helix chains (D1, blue), and the β-sheet hypervariable regions (D2 and D3, pink). The α-helix regions (brown and blue), necessary for filament architecture and motility functions, are embedded in the flagellum inner core.

MATERIAL AND METHODS

Strain

Attenuated *Salmonella enterica* sv Typhimurium LDV 322 strain, derived from *aro A* SL3261 strain, expresses only FliC as phase 1 H antigen (Massis *et al.*, 2008).

Culture Medium

Two complex media were used: 1) LB: Tryptone (BD) – 10 g/L; Yeast Extract (BD) – 5 g/L and NaCl – 5 g/L and 2) Soy peptone - Soytone (BD) – 20 g/L; Yeast Extract (BD) – 20 g/L and NaCl – 5 g/L.

Growth of Cultures in Shakers

The experiments were carried out in a New Brunswick shaker (Shaker series 25) with different media. Cultivation was performed in 2 L flasks containing 1 L of LB medium or Soytone-yeast extract medium kept at 240 rpm for 16 h at 35ºC.

Growth of Cultures in Bioreactors

The experiments were carried out in a Bioflo 3000 bioreactor (New Brunswick Scientific) in 2 L of Soytone-yeast extract medium, pH 7.0 (pH probe – L3580 – Mettler Toledo), adjusted with addition of 5 N NaOH, at 35°C and pO_2 (probe: SN0473419 - Mettler Toledo) controlled at 30% of air saturation. Glucose was added when the pH started to increase, indicating that the carbohydrates present in the medium had been consumed.

Flagellin Purification

Two procedures were tested according to the cultivation of the cells. For cultures prepared in shake flasks, cells were centrifuged (Avanti™ centrifuge J-251 - Beckmann) at 11,325 g during 30 min, resuspended in 25 mM Tris-HCl buffer, pH 7.0, containing 150 mM of NaCl and incubated for 30 min at 37 ºC under 150 rpm (New Brunswick Shaker series 25). The cells were then submitted to homogenisation using a bench mixer (homogeniser model X-520 - Schaft T10F -Ingenieurbüro CAT, M.Zipperer GmbH), in order to release the flagellar filaments and then centrifuged at 5,000 g for 30 min. The flagellin-containing supernatant was submitted to tangential ultrafiltration with a 100 kDa cut-off membrane (PLCHK 100 –50 m^2 Cat. No PXC100C50 – Millipore ®) to eliminate molecules with less than 100 kDa from the culture medium or metabolites produced by the bacterial strain (Figure 2). Flagellin production was also determined by precipitation with 4 volumes of acetone, incubation at -20°C for 1 h and centrifugation. The recovered pellet was suspended in 500 µL of 150 mM NaCl.

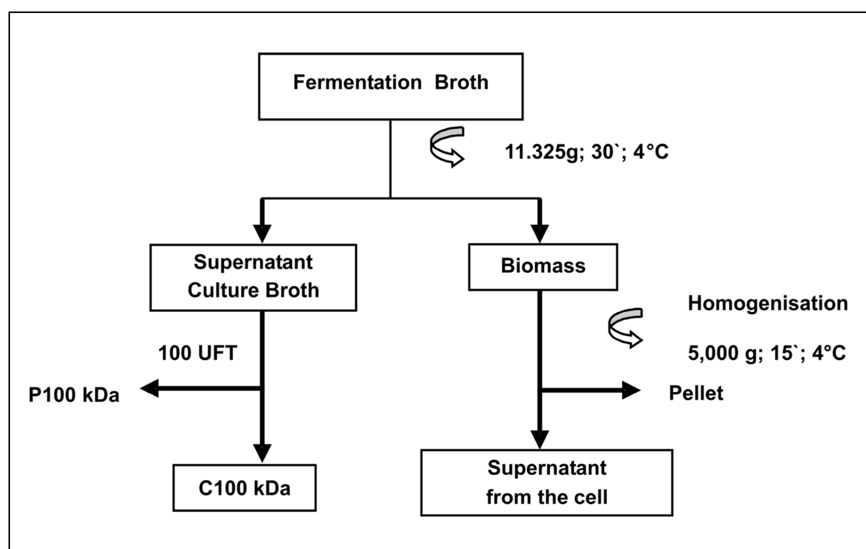

Figure 2: Flagellin purification flow diagram based on aerated culture flasks.

Cultures prepared in the bioreactor were submitted to centrifugation (Avanti™ centrifuge J-251 - Beckmann) at 11,325 g for 30 min at 4°C. The resulting supernatant was saved and named supernatant medium broth. The cell pellet was suspended in 25 mM Tris HCl, pH 7.5, and submitted to flagellin extraction with a Sorvall homogeniser Omni Mixer, Model 17220, centrifuged at 5,000 g for 30 min at 4°C. The collected supernatant from the cell was frozen for subsequent measurement of the flagellin content. The supernatant medium broth was concentrated with a tangential ultrafiltration membrane of 750 kDa cut-off (UFP-750-E-8A GE Healthcare®) and washed with 6 volumes of 25 mM Tris-HCl buffer, pH 7.0. This step resulted in two fractions: 750 kDa concentrate (750 kDa) and 750 kDa ultrafiltrate (UF750 kDa). The fraction UF750 kDa was concentrated with a tangential ultrafiltration membrane with a cut-off of 300 kDa (PELLICON 2 – 300 K - PTMK-C – 0.5M^2 CAT SK1P041W8 - Millipore®), resulting in a 300 kDa concentrate and 300kDa ultrafiltrate, identified as 300kDa and UF300kDa, respectively (Figure 3). All samples were analyzed by SDS-PAGE and Western Blotting.

SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a mini-slab gel apparatus (Bio Rad) according to the Laemmli method. Proteins were stacked in 4.5% acrylamide gel and sorted in 12% acrylamide gel submitted to a constant voltage of 150 V. Proteins were stained with Coomassie blue (Laemmli, 1970). Protein contents were determined by Lowry's method (Lowry *et al.,* 1951), and densitometry by using GS-800 Densitometer and Quantity one software. The flagellin concentrations (mg/L) were determined after multiplying the percentage of relative protein (calculated from densitometry) and the protein concentration determined by Lowry`s method.

Western Blotting

The proteins were transferred to nitrocellulose membrane (Hybond-C Extra; Amersham) at 1 A for 2 h using the buffer conditions described by Towbin *et al*. (1979). The membrane was blocked with 5% low-fat milk in PBS for 1 h at room temperature and incubated for 1 h with rabbit antiserum against *Salmonella* FliC antigen. After washing with 0.05% Tween-20 in PBS (PBS-T), bound antibodies were reacted with goat anti-rabbit IgG conjugated with peroxidase (Sigma). After incubation for 1 h with the conjugate and washing with PBS-T, bound antibodies were detected with o-phenylenediamine (Sigma).

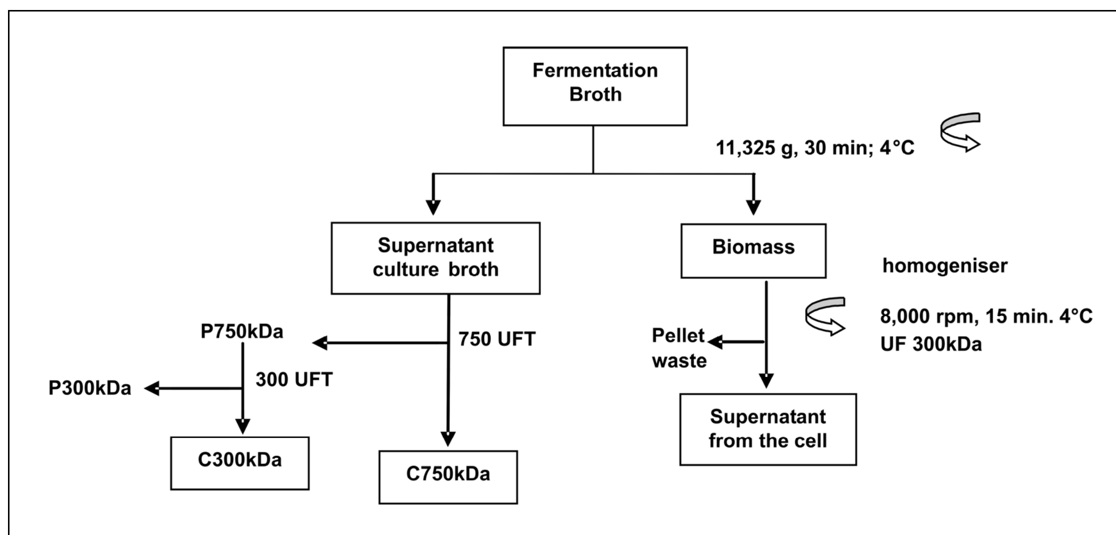

Figure 3: Flagellin purification flow diagram based on bioreactors.

RESULTS AND DISCUSSION

The first critical step in *Salmonella* flagellin isolation was the extraction of the flagellar filaments from the bacterial cell surface. Previous attempts to obtain flagellin from *S*. Typhimurium cultures employed low speed agitation (80 rpm) followed by cell shearing in a bench mixer (Vortex) and precipitation of released flagellar fragments with cold acetone (Braga *et al.*, 2008). However, our previous observations indicated that the cultivation of bacteria at higher agitation speed (240 rpm) increased both cell growth and flagellin expression (Oliveira *et al*., 2010). In order to increase the shearing forces required to detach flagellin from the bacterial cell without promoting significant cell lysis, we tested two blender type homogenisers: the model X-520 homogeniser (Schaft T10F -Ingenieurbüro CAT, M.Zipperer GmbH) and the Omni Mixer (Sorvall) with the helical axis fitted into a hermetic reservoir (Figure 4). For flagellin removal, the S. Typhimurium LDV322 strain was cultivated in LB medium at 35°C for 18 h using an agitation speed of 240 rpm. The flagellin isolation procedure was conducted using both homogenisers according to the flowchart presented in Figure 2. As shown in Figure 5, the flagellin recovery yields obtained after extraction with both homogenisers were similar. The following experiments were carried out with the Omni Mixer Sorvall due to the safer handling procedures regarding manipulation of bacterial cells. In addition, cultivation at a higher agitation speed (240 rpm) resulted in increased amounts of flagellin released by the bacterial cells into the growth medium. Indeed, flagellin was detected in Comassie blue-stained polyacrylamide gels even without concentration of flagellin by acetone precipitation (Figure 5 lane 5).

Figure 4: The two types of homogenisers used in the present study. (1) Sorvall Omni Mixer, which has a helical axis inside a reservoir; (2) CAT, which has two overlapping axes, a fixed and a mobile one.

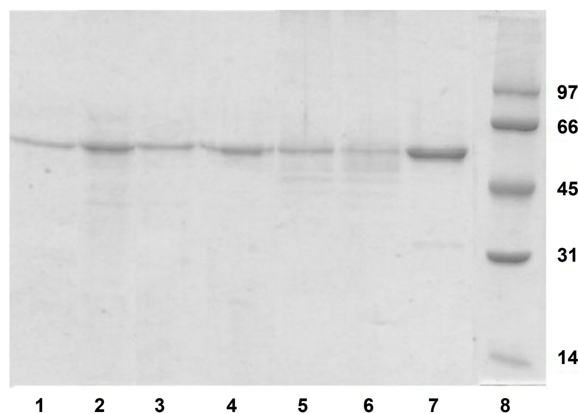

Figure 5: S. Typhimurium FliC flagellin obtained by different procedures. Samples: 1) Flagellin extracted using the CAT blender; 2) Flagellin extracted using the CAT blender and precipitated with acetone; 3) Flagellin extracted using the Sorvall Omni Mixer; 4) Flagellin extracted using Sorvall Omni Mixer and precipitated with acetone; 5) Cell-free culture supernatant concentrated using an Ultrafiltration membrane with 100 kDa cut-off; 6) Cell-free culture supernatant concentrated using a Ultrafiltration membrane with 100 kDa cut-off and precipitated with acetone; 7) Purified FliC flagellin; 8) Molecular weight markers. Each lane contains approximately 7.5 µg of protein.

In a second step, we evaluated whether FliC flagellin production by the *S.* Typhimirium LDV322 strain would be affected after cultivation of the strain using an alternative culture medium without animal-derived components (tryptone). Such an alternative culture would fit into the WHO recommendation for production of microbial-derived vaccine-components [WHO, 2006]. For this purpose, experiments were carried out in Soytone medium (2%), containing no animal-derived components, supplemented with yeast extract (2%) and sodium chloride. Cell growth was higher in Soytone medium (DO_{600nm} of 6.0) when compared with cell cultures prepared with LB medium (DO_{600nm} of 2.5). Similarly, expression of flagellin was also higher in cells cultivated in Soytone medium when compared to cells cultivated under the same experimental conditions in LB medium (Oliveira *et al.*, 2010).

The next step in the improvement of the flagellin purification procedure was the replacement of the aerated shaker flasks by cultures prepared in a bioreactor, in which significantly higher cell densities can be achieved. The experiment in the bioreactor was carried out with Soytone medium using a discontinuous cultivation mode with intermittent glucose pulses. The culture pH was kept at 7.0 with addition of sodium hydroxide and the pO_2 controlled at 30% of air saturation at a temperature at 35ºC. Figure 6 shows the cell growth profile with a maximum specific growth rate of 0.7 h^{-1} and a biomass of 8.5 g/L reached at the end of cultivation in the bioreactor. During cultivation, the agitation speed increased up to a maximum value of 900 rpm at the 8th hour, which remained constant until the end of the cultivation period (10 h). From the 8th hour onward, cell growth was limited by oxygen and pO_2 decreased to 5%. Consequently, cell growth was limited by oxygen restriction after 8 h of cultivation. Production of flagellin was affected by the oxygen limitation and the maximal value achieved was 300 mg/g protein (Table 1). As shown by the SDS-PAGE analysis, flagellin was released into the culture supernatant during cultivation in the bioreactor (Figure 7). The high concentration of flagellin in the culture supernatant permitted the purification of the protein directly from the spent medium without the need to remove flagella from the cells.

Table 1: Kinetics parameters in batch cultivation of S. Typhimurium SDV 322

Parameter	Value
Maximum specific growth rate, $\mu_{max}(h^{-1})$	0.7 ±0.02
Maximum flagellin concentration - $P_{máx}$ (mg/L)	1490 ±0.50
Maximum Biomass concentration - $X_{máx}$ (gDCW/L)	12.5 ±0.15
Specific production of flagellin (mg flagellin/g DCW)	117.30± 0.2
Relative purity (mg flagellin/ g total protein)	330.00±0.25
Cell yield coefficient ($Y_{x/s}$) - g/g	0.33 ±0.01

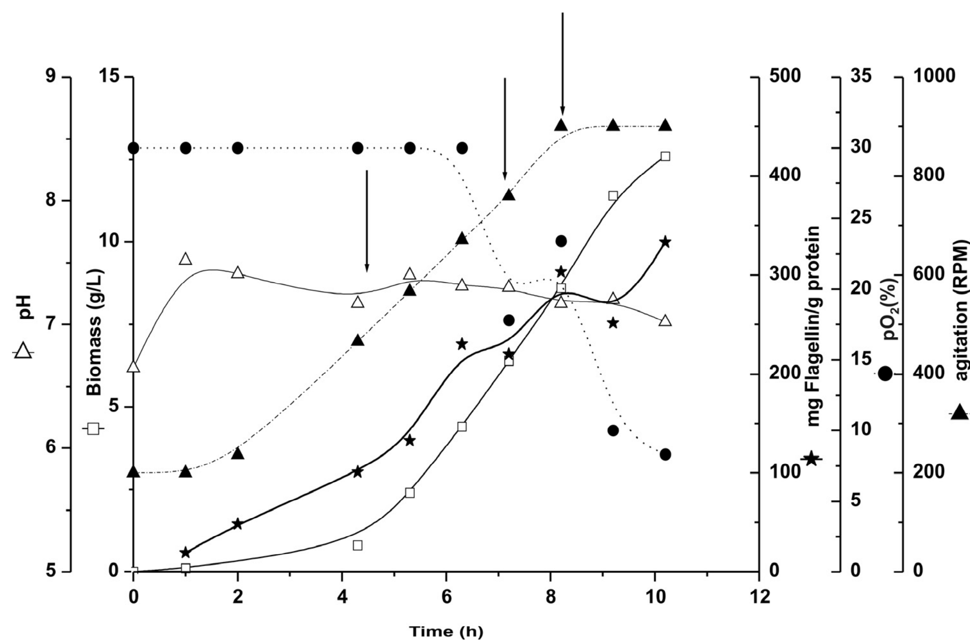

Figure 6: Biomass ( ), relative purity of flagellin (✯), pH (Δ), agitation (▲), pO_2 (%) (●) in the batch cultivation of S. Typhimurium SDV 322 strain. The arrows show the glucose addition.

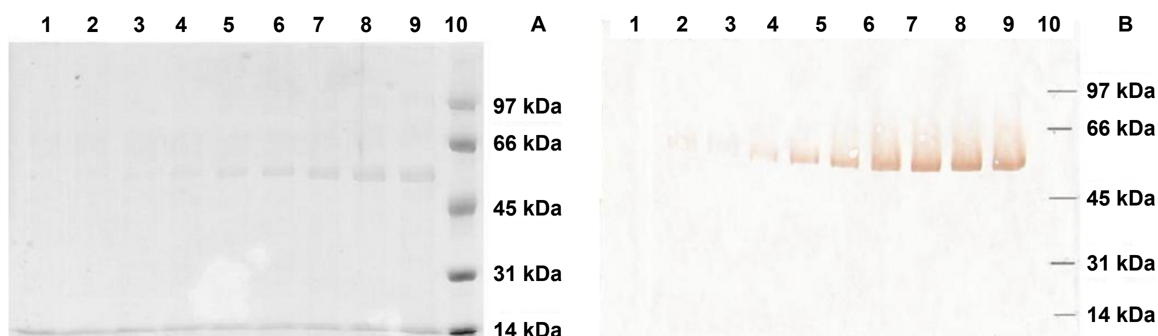

Figure 7: Flagellin released in the culture supernatant of S. Typhimurium cultivated in bioreactor using Soytone broth. (A) SDS-PAGE. (B) Western Blotting developed with anti-flagellin antibody. Samples sorted according to the incubation time in the bioreactor: 1- 2 h; 2- 4.3 h; 3- 5.3 h; 4- 6.3 h; 5- 7.2 h; 6- 8.2 h; 7- 9.2 h; 8- 10.2 h; 9- 11.2 h; 10- Molecular weight markers.

Flagellin purification was initiated after centrifugation of the bacterial culture at 11,325 g for 30 min at 4°C. The cell-free supernatant was saved and submitted to the tangential ultrafiltration steps with different molecular weight cut-off filters, as indicated in the flowchart depicted in Figure 3.

The flagellin purification steps based on S. Typhimurium cultures prepared in bioreactors are listed in Table 2. The relative purity of flagellin with regard to contaminating proteins (PRprot) and the corresponding purification factor (PF) are indicated. The tangential ultrafiltration step with a 750 kDa cut-off membrane eliminated most of the contaminating proteins in a single step. The purification factor (PFprot) of the concentrated fraction with the 750 kDa cut-off membrane was 1.5 relative to the initial material, with a recovery yield of 52.2%. A significant loss of flagellin was detected in the ultrafiltered sample (UF[750kDa]), corresponding to 16.3% of total flagellin, but this protein could easily be recovered by another ultrafiltration step with a 300 kDa cut-off membrane (Figure 8). However, the purification factor of this step is quite low (FP=0.1) (Table 2). The flagellin volumetric production with tangential ultrafiltration step was around 307 mg of protein per litre of culture.

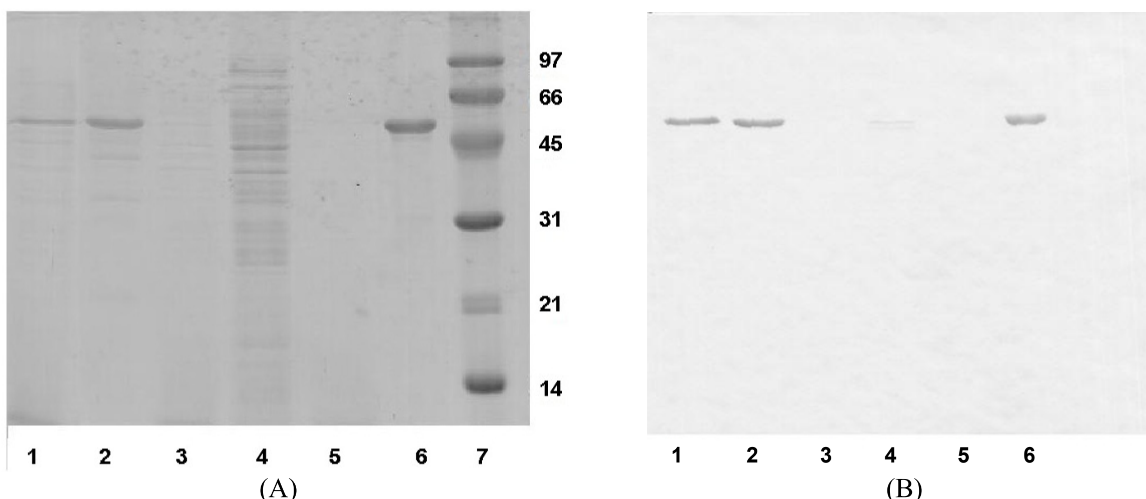

Figure 8: Purification of FliC flagellin using tangential ultrafiltration. (A) SDS-PAGE and B) Western Blotting Samples: 1) Supernatant; 2) C750kDa; 3) P750kDa; 4) C300kDa; 5) P300kDa; 6) Purified FliC; 7) Molecular weight markers.

Table 2: Purification of flagellin FliC.

Purification Steps	**Total Protein (mg)**	***FliC (mg)**	**□ RPprot mgFliCi/ mg Protein**	**•PF global**	**♦PF Step**	**Rec (%)**
Supernatant broth	2,097±0.20	1,030±0.11	0.49	1.0	1	100
C750kDa	744±0.10	537±0.10	0.72	1.5	1.5	52.2
P750kDa	1,154±0.25	167±0.05	0.15	0.30	0.3	16.3
C300kDa	506±0.12	20±0.10	0.04	0.10	0.3	1.9
P300kDa	802±0.15	0	0.00	0.00	0.0	0

Average of total protein and FliC protein in mg. All values are the average of two experiments

□ Relative purity: RP = mg Flagellin/mg Total protein

• Global purification factor: $PF_{global} = RP_{step}/RP_{initial}$

♦ Step purification factor: $PF_{step} = RP_{step}/RP_{previous\ step}$

Smith *et al.* (2003) have purified flagellin through shearing of flagellar filaments and ultracentrifugation. The pellets were suspended in PBS and the flagellar filaments then submitted to exhaustive dialysis by tangential ultrafiltration with a 300 kDa cut-off membrane, which achieved high purity. However, no information was given about the final flagellin yield or the volumetric production. The results obtained in this study show that replacement of an animal-derived medium by a soybean-based culture medium resulted in an improved flagellin production. In addition, the use of the bioreactor for growth of the *S.* Typhimurium strain further enhanced the flagellin recovery yield based on a single-step procedure. The high speed agitation condition increased cell growth and release of flagellin into the growth medium, making the purification step simpler, faster and safer regarding handling of the bacterial cells. The procedure described here represents, therefore, a significant improvement over previously described flagellin purification methods, allowing application of additional purification steps, such as ion exchange chromatography, to achieve WHO standards for bacterial-derived products for vaccine use and further scale-up.

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