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Xylan-Degrading Catalytic Flagellar Nanorods

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Abstract

Flagellin, the main component of flagellar filaments, is a protein possessing polymerization ability. In this work a novel fusion construct of xylanase A from *B. subtilis* and *Salmonella* flagellin was created which is applicable to build xylan-degrading catalytic nanorods of high stability. The FliC-XynA chimera when overexpressed in a flagellin deficient *Salmonella* host strain was secreted into the culture medium by the flagellum-specific export machinery allowing easy purification. Filamentous assemblies displaying high surface density of catalytic sites were produced by ammonium-sulfate-induced polymerization. FliC-XynA nanorods were resistant to proteolytic degradation and preserved their enzymatic activity for a long period of time. Furnishing enzymes with self-assembling ability to build catalytic nanorods offers a promising alternative approach to enzyme immobilization onto nanostructured synthetic scaffolds.

Keywords: flagellin, xylanase A, polymerization, self-assembly, flagellar export, nanorod

Abbreviations: FliC, flagellin; XynA, xylanase A from *Bacillus subtilis*; FliC(XynA)-His_6, fusion construct of FliC and XynA with a C-terminal His_6 tag; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; AS, ammonium sulfate; flagzyme, flagellin-enzyme fusion protein.

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Introduction

In nanobiotechnology diverse nanoscale scaffolds (e.g. nanoparticles, nanofibers, nanoporous materials) are developed and used to immobilize enzymes [1-4]. Nanostructured materials offer a large surface area and high effective enzyme loading. They can be synthesized in a variety of sizes controlling diffusional limitation. Enzyme immobilization to nanoscaffolds may enhance enzyme activity and stability, can facilitate easy separation of products and efficient recovery of the catalysts resulting in low cost of industrial applications. Various immobilization techniques are used, including adsorption, covalent attachment or entrapment inside the particles [5,6]. However, immobilization onto a carrier surface, especially by covalent crosslinking, may induce structural changes leading to partial inactivation and loss of functionality. Other typical drawbacks are the heterogeneous conformations of the immobilized enzymes and their different local environments which may affect catalytic efficiency.

To avoid chemical immobilization, a promising approach is to furnish enzymes with polymerization ability and create self-assembling building blocks which are capable of forming filamentous nanostructures exhibiting catalytic activity. The very first steps in this direction have been made by fusing enzymes with amyloid-forming protein domains [7,8] or with the polymerizable protein, flagellin [9]. Bacterial flagellar filaments are rigid tubular nanostructures with a diameter of 23 nm which are composed of thousands of flagellin subunits [10]. Flagellin monomers can be reconstituted into filaments by adding short filaments as seeds or a precipitant such as ammonium sulfate (AS) [11]. The length of filaments can be controlled by the polymerization conditions in the range of 200 nm – 10 μm.

It was demonstrated that the variable central portion of flagellin, forming the surface-exposed D3 domain in the polymeric state, is not involved in filament formation and can be replaced by other proteins without destroying self-assembling ability [13,14]. The prototype of

polymerizable flagellin-based enzymes (flagzymes) had been created by introducing the xylanase A (XynA) enzyme from *Bacillus subtilis* into the middle part of flagellin replacing D3 (Fig. 1) [9]. Appropriate linker segments facilitating functional insertion were selected by computer modeling. The FliC-XynA fusion protein preserved the functional properties of both partners: it exhibited xylan-degrading catalytic activity and was capable of filament formation. However, the FliC-XynA filaments had a marginal stability, presumably because of the purification His_6-tag attached to the N-terminus of the fusion construct, and easily decomposed into subunits upon removal of the AS precipitant. Another problem was related to the bacterial overexpression of the fusion protein because the disordered terminal regions of flagellin [15], controlling the polymerization ability [16], were readily degraded during the purification steps.

In this work we aimed at constructing a flagzyme which can be easily produced and forms stable and catalytically active filamentous nanostructures. To improve polymerization properties of the FliC-XynA flagzyme the His_6 affinity tag was moved from the N-terminus to the C-terminus, while cost effective production of the fusion protein was achieved in a flagellin deficient Salmonella host strain by secreted overexpression through the flagellum-specific export pathway [17].

Materials and Methods

Plasmid Construction, Protein Expression and Purification

The C-terminally His_6-tagged version of FliC-XynA was created using the pKOT1-based plasmid construct containing the coding sequence of D3-domain-deleted FliC driven by the original *Salmonella fliC* promoter. The deleted D3-coding sequence was replaced by a cassette containing multiple cloning sites as it was described previously [9]. The *xynA* gen was inserted between the *XhoI* and *SacI* restriction sites resulting in the *fliC-xynA* fusion construct. The 5'-CACCACCACCACCACCAC-3' oligonucleotide sequence, which encodes for a His_6-tag, was inserted before the STOP codon of the construct using the Quikchange II Site-Directed Mutagenesis Kit (Agilent) following the manufacturer's instructions. This construct was transformed by electroporation into the flagellin deficient non-motile *Salmonella* strain SJW2536 and used for protein production. This mutant strain can not produce flagellin, therefore only the basal body and the short hook part of the filament is formed.

The monomeric FliC-XynA-His_6 was secreted into the supernatant by the flagellar type III export apparatus. To check the level of secretion 1 ml of cell culture supernatant was precipitated by adding 63 µl trichloroacetic acid (TCA). The precipitate was centrifuged and washed twice with acetone, then dissolved in 20 µl SDS sample buffer and analyzed by SDS-PAGE using Coomassie brilliant blue (CBB) staining (Fig.2).

Purification of FliC-XynA was performed as follows: 1L of LB broth supplemented with ampicillin (100µg/ml) was inoculated from a single colony from an LB-Agar plate and it was incubated at 37°C overnight. The supernatant containing the FliC-XynA fusion protein was collected by centrifugation (14.000g for 45 minutes) and it was concentrated by Vivaflow 200 (Sartorius) using 30.000 MWCO PES membrane to a final volume of 25 ml. For the subsequent purification by Ni affinity chromatography, sodium dihydrogen phosphate,

sodium chloride and imidazole was added to the concentrated solution to set their final concentration to 20mM, 500 mM and 50 mM, respectively, and to adjust the pH to 7.4. Then the sample was applied to a 5 ml HiTrap Chelating Ni-affinity column (GE Healthcare) and the absorbed proteins were eluted using a stepwise imidazole gradient from 50mM to 300mM. The His_6-tagged Flic-XynA protein was eluted and collected at 200mM imidazole. After overnight dialysis against 2L 20 mM Tris-HCl (pH 7.0) at 4°C, samples were further purified by anion-exchange chromatography on a MonoQ 5/5 column. The fusion protein eluted at 40 mM NaCl in 20 mM Tris-HCl (pH 7.0). Pure fractions were collected and dialyzed overnight at 4°C against 1 L 50 mM phosphate buffer (pH 6.0), containing 150 mM NaCl, and finally stored at -20°C for further use.

N-terminally His-tagged xylanase A was overexpressed on *E. coli* BL21-CodonPlus (DE3)-RIL cells and prepared as described previously [9].

Protein concentrations were determined from absorption measurements at 280 nm using molar extinction coefficients (ε_{280}=17880 $M^{-1}cm^{-1}$ for flagellin, ε_{280}=82850 $M^{-1}cm^{-1}$ for XynA, and ε_{280}=93280 $M^{-1}cm^{-1}$ for FliC-XynA-His_6) calculated from the known aromatic amino acid contents of the molecules [18]. Purity of protein samples was checked using SDS-PAGE.

Xylan degrading activity of the FliC-XynA nanorods was measured and compared to the xylanase A enzyme as described in [9].

Polymerization Experiments

The polymerization ability of the FliC-XynA-His_6 fusion protein was investigated by inducing nucleation and polymerization by ammonium sulfate (AS) [16]. Protein solutions of 2 mg/ml were prepared in 50 mM phosphate buffer, 150mM NaCl (pH 6.0) and 4M ammonium sulfate was added to various final concentrations in the range of 0.4 M to 1.2 M. Filament formation was observed after 24 h of incubation at room temperature by dark-field optical microscopy

using an Olympus BX50 microscope. Samples were also visualized by atomic force microscopy using a DigiScope1000 (Aist-NT) microscope.

Limited Proteolysis

Limited proteolysis by trypsin was performed in 50 mM phosphate buffer, pH 6.0, containing 150 mM NaCl at room temperature at a protein concentration of 2.0 mg/ml. In a typical digestion experiment, the protease was added to the protein solution at a 1:300 (w/w) ratio. At various times 10 µl samples were taken and mixed with 10 µl electrophoretic sample buffer, followed immediately by heating in a boiling water bath for three minutes. Electrophoresis was performed on 12.5% polyacrylamide slab gels.

Assay of xylanase activity

Xylanase activity was determined by the method of Bernfeld [19]. The reaction mixture containing 85 µl of 1 % xylan solution in 50 mM phosphate buffer, pH 6.0, and 35 µl of 28 µM enzyme solution was incubated at 37°C for 10 min. After addition of 125 µl DNSA solution, the reaction mixture was boiled at 100 °C for 20 minutes. The amount of reducing sugars was determined by absorbance measurement at 575 nm. The standard xylose curve was calibrated with D-xylose (Sigma) within the range of 0 – 250 µg/ml. Measurements were repeated three times.

Results and Discussion

Preparation of FliC-XynA-His6

Our first attempt to furnish the xylanase A enzyme with self-assembling ability produced a moderate success. The flagellin-xylanase fusion protein which contained an N-terminal His_6 purification tag, displayed catalytic activity comparable to the native enzyme, however, it formed filaments of marginal stability [9]. Analysis of the flagellar filament structure [20] revealed that the N-terminal extra residues were responsible for the paralyzed filament formation disturbing the very inner core structure of filaments. Nevertheless, inspection of the 3D structural model suggested that a purification tag attached to the C-terminus could be readily accommodated without imposing sterical constraints because the very C-terminal segment of flagellin forms the inner wall of the central channel of filaments and the His_6 segment can find free space in the channel without interfering with filament formation. Therefore, a novel gene construct was prepared which encoded the FliC-XynA variant with a His_6 purification tag attached to the C-terminus.

Both terminal regions of flagellin are disordered in the monomeric state and are very sensitive to proteolytic degradation [15,16]. When the flagellin-based fusion constructs were produced in *E. coli* expression host cells, vulnerability of the terminal regions made the purification process complicated and inefficient. To avoid this problem we tried secreted overexpression of FliC-XynA-His_6 in a flagellin deficient Salmonella host strain.

In vivo self-assembly of flagellar filaments involves incorporation of flagellin subunits at the distal end below the pentameric HAP2 cap [21]. Flagellin subunits are recognized and translocated by the flagellum-specific export machinery, located at the base of flagellum on the cytoplasmic side, and they are transported through the central channel of the filaments to the tip [17]. The signal which is recognized by the flagellar export system is contained in the

N-terminal disordered region of flagellin [22]. It was demonstrated that attaching the 22-residue long signal to foreign proteins resulted in the export of the fusion constructs by the flagellar export system [23]. The FliC-XynA-His_6 fusion protein contains the intact N-terminal part of flagellin therefore its export through the flagellar export pathway was expected.

To exploit flagellar export for the efficient and easy production of the flagellin-xylanase fusion protein, the plasmid encoding for FliC-XynA-His_6 was introduced into the flagellin deficient SJW2536 Salmonella host strain [24]. This mutant strain is nonmotile because it lacks the helical filaments of flagellum, only the base and the short hook part of the filament is formed covered by the molecular cap made from a pentameric assembly of HAP2 subunits (Fig. 2A). This cap is leaky and allows excessively exported subunits to diffuse out into the culture medium [25]. Upon transformation of SJW2536 with the plasmid encoding the FliC-XynA-His_6 construct we could not observe filament formation and regain of cell motility, however, the fusion protein was produced and secreted into the culture medium in significant amounts as expected (Fig. 2B, lane 3). It seems that FliC-XynA-His_6 subunits are not capable of filament formation *in vivo*. They are translocated through the narrow central channel of the flagellum in an unfolded state. We think that FliC-XynA-His_6 fusion protein can not refold efficiently in the tiny cavity below the pentameric HAP2 cap at the distal end of filaments and released into the outer culture medium.

FliC-XynA-His_6 subunits were purified from the cell culture supernatant applying ultrafiltration followed by Ni-affinity chromatography. Further purification by FPLC anion-exchange chromatography was applied to obtain pure samples free of terminally truncated fragments (Fig. 2B, lane 4) which would impair efficient polymerization.

Polymerization Experiments with FliC-XynA-His_6

To check the polymerization ability of the purified fusion protein filament formation was induced by ammonium sulfate (AS) precipitation. In earlier experiments with N-terminal His-tag the fusion protein was capable of forming filaments upon high precipitant concentration, however, the filaments quickly disassembled under physiological conditions [9].

The FliC-XynA-His_6 fusion protein readily polymerized upon addition of 4M AS in various final concentrations in the range of 0.4 – 1.2 M (Fig. 3A). Depending on the precipitant concentration, filaments of various average lengths were produced. Filaments were stable in 50 mM phosphate buffer, 150mM NaCl (pH 6.0) after removal of the AS precipitant. Even after one week they retained structural integrity.

Stability of FliC-XynA filaments was also investigated applying limited proteolysis by trypsin (Fig. 3C). Flagellin monomers are known to be highly sensitive to proteolytic degradation because of their structurally disordered terminal regions [15]. However, polymerization is accompanied by the stabilization of the disordered segments into α-helical bundles in the filament core, and flagellar filaments can withstand proteolytic attack [26]. To probe the stability and structural integrity of FliC-XynA-His_6 filaments they were digested by trypsin at a protease to protein ratio of 1:300 (w/w). At various time points aliquots were withdrawn and analyzed by SDS-PAGE. As demonstrated by Fig. 3C, filaments were resistant to tryptic degradation for a long period of time, at least for days indicating that they possess a compact stable structure. Under similar conditions FliC-XynA-His_6 monomers, having disordered terminal regions, were digested into smaller fragments within a few minutes. The high proteolytic resistance of FliC-XynA filaments indicated that the xylanase A protein could acquire its well-folded conformation even upon insertion into the variable central part of flagellin.

Catalytic FliC-XynA Nanotubes

Our earlier experiments demonstrated that the N-terminally His_6-tagged FliC-XynA fusion protein had a catalytic activity similar to the native XynA enzyme [9]. Translocation of the His_6-tag from the N-terminus to the C-terminus of the chimera did not influence the activity of the xylanase A moiety introduced into the central portion of flagellin (Table 1). Filaments were built from FliC-XynA-His_6 subunits by 0.6M AS precipitation, and their catalytic properties were compared to the monomeric FliC-XynA-His_6 protein and the recombinantly expressed wild-type xylanese A enzyme.

As shown in Table 1, monomeric FliC-XynA-His_6 exhibited a xylan degrading activity very similar to that of the wild-type enzyme. We found that the FliC-XynA subunits retained their catalytic activity in the polymeric state, however it was about 20% smaller than that of the monomeric flagzyme. This observation demonstrates that incorporation of the subunits into the filamentous structure does not significantly affect their catalytic properties. Nevertheless, the high density of catalytic moieties on the filament surface may have an adverse effect on their accessibility. The spacious xylan substrate bound to the surface-exposed XynA portion of a subunit may restrict accessibility of the neighboring catalytic sites explaining the somewhat decreased catalytic efficiency of FliC-XynA in the polymeric form. The nanorods remained stable and enzymatically active for a long period of time. Incubation at room temperature or 37°C for one week did not cause any significant change in the xylan degrading activity of the nanorods.

Conclusion

The xylanase A enzyme can be inserted into the variable central part of the polymerizable protein, flagellin, retaining its catalytic activity. The C-terminally His_6-tagged version of the fusion protein is secreted by the flagellum-specific export machinery into the cell culture medium when overexpressed in a flagellin deficient *Salmonella* host strain, allowing easy purification and cost effective preparation. Our studies demonstrate that the FliC-XynA-His_6 chimera can self-assemble into stable nanorods displaying thousands of catalytic units on their surface which are in identical local environments. These flagzymes can form stable nanostructures without any additional matrix or support offering a new way of enzyme immobilization which does not require any expensive and complicated chemical crosslinking procedure. The size of nanorods can be controlled by the polymerization conditions. When enzymes are immobilized onto various surfaces or nanostructured supports their catalytic activity is often seriously paralyzed. The XynA enzymes retained their catalytic activity when the fusion protein assembled into flagellar nanorods. It is important to emphasize, that the XynA portion of the fusion protein is displayed on the filament surface as a result of polymerization and readily accessible for the substrate molecules. These studies pave the way for providing various enzymes with self-assembling ability. Flagellin-based catalytically active building blocks can be used to form nanorods by mixed copolymerization which are capable of catalyzing multiple coupled reactions.

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Figure legends

Figure 1

Arrangement of subunits in a catalytic nanorod polymerized from FliC-XynA fusion proteins. The xylanase A enzyme from *B. subtilis* was inserted into the middle part of *Salmonella* flagellin to replace the D3 domain situated on the filament surface. Solid surface representation of the flagellar filament is shown according to [26]. (bar: 5 nm) The C_α backbone trace of XynA (PDB code: 1XXN [27]) is rainbow-colored from blue to red representing the sequence from the NH_2- to COOH terminus. The distance between the solvent-exposed catalytic units on the filament surface is about 5.5 nm.

Figure 2

Secreted overexpression of FliC-XynA-His_6 subunits. (A) FliC-XynA-His_6 was overexpressed in a flagellin deficient Salmonella host strain SJW2536. In this mutant only the base part and the short hook part of the filament is formed covered by the leaky HAP2 cap. FliC-XynA is exported by the flagellar export apparatus through the narrow central channel of the filament and secreted into the culture medium. (OM: outer membrane; PG: peptidoglycan layer; IM: inner membrane) (B) SDS-PAGE analysis of whole cell culture (lane 1), cell culture pellet (lane 2), TCA-precipitated cell culture supernatant (lane 3) and purified FliC-XynA-His_6 (lane 4) fractions.

Figure 3

Filaments reconstructed from FliC-XynA-His_6 subunits as visualized by (A) dark field and (B) atomic force microscopy (white bar: 500 nm). Polymerization experiments were done in 50 mM phosphate buffer, 150 mM NaCl (pH 6.0) at 2 mg/ml protein concentration, and 4 M AS was added to 0.6 M final concentration to initiate filament formation. (C) Resistance of FliC-XynA-His_6 filaments against tryptic degradation. Proteolytic experiments were done at a

protein to protease ratio of 300:1 (w/w) at room temperature. The filament sample contained a small amount of monomers which were quickly degraded but after ~30 minutes the intensity of the major band at 64 kDa did not change significantly indicating the stability and resistance of filaments.

TABLES

Table 1. Comparison of the catalytic activity of recombinant xylanase A and the FliC(XynA)-His$_6$ fusion protein in the monomeric and polymeric forms.

Sample	D-xylose released (µg/ml)
native xylanase A	129
FliC(XynA)-His$_6$ monomer	128
FliC(XynA)-His$_6$ polymer	105

Activity measurements were done at 8.2 µM enzyme (catalytic unit) concentration in 50 mM phosphate buffer (pH 6.0) at 37 ºC. Experiments were repeated three times and averaged.

Figure 1

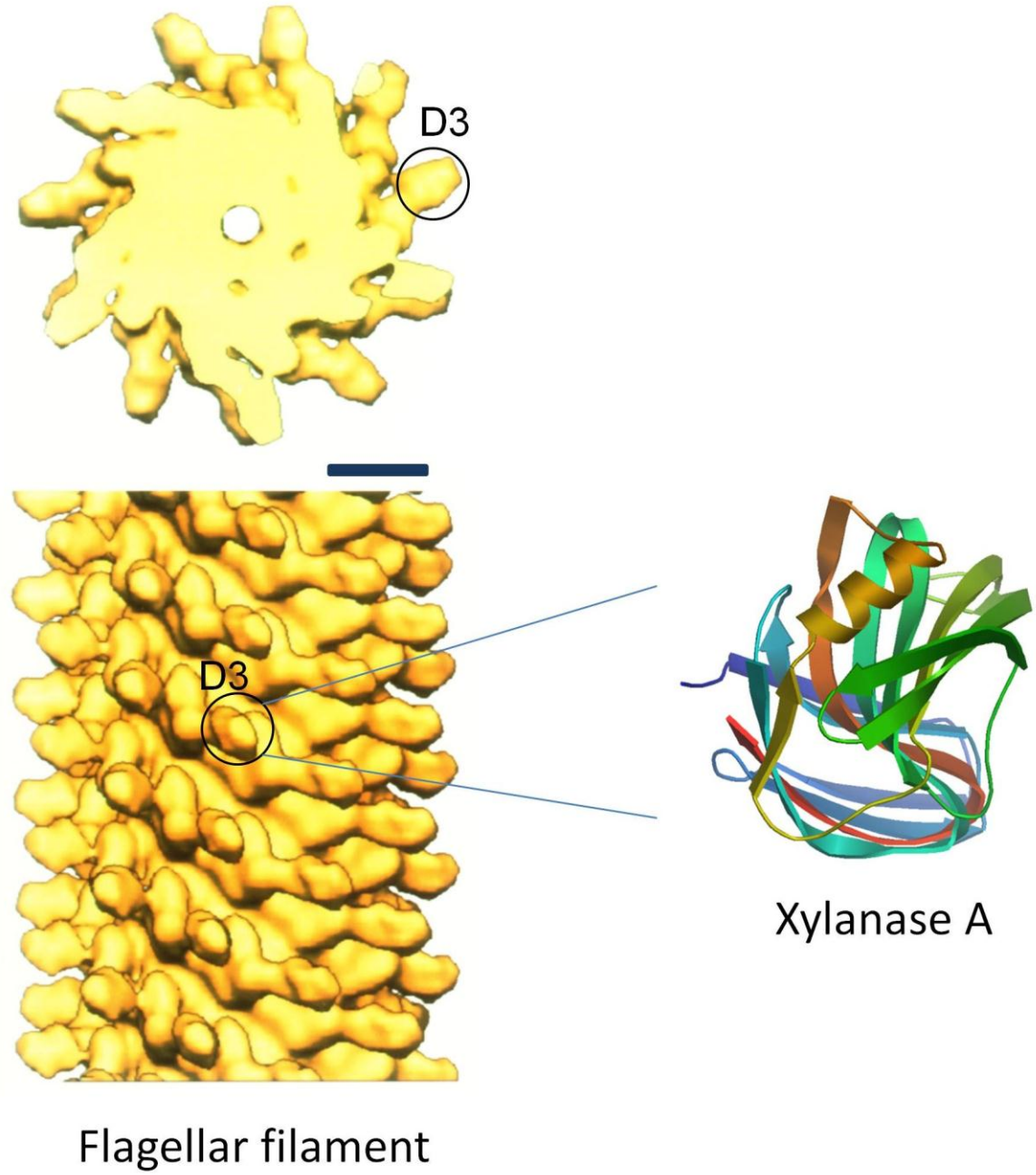
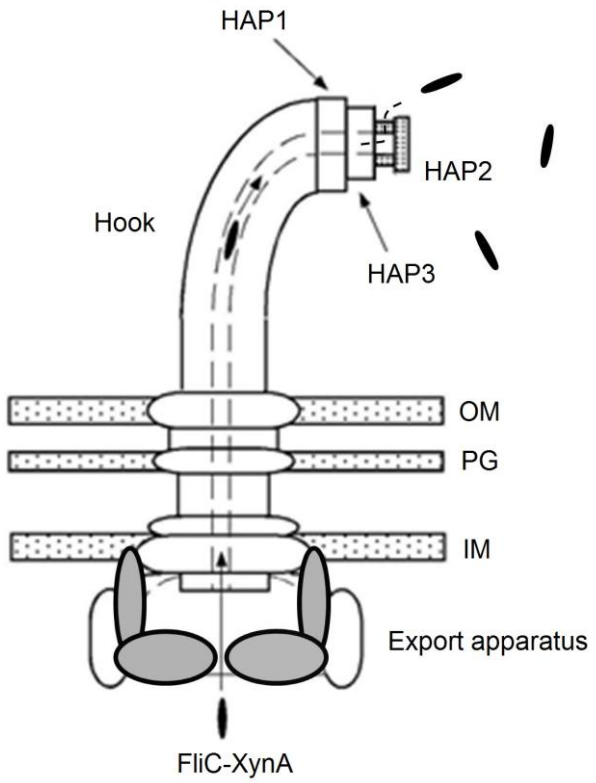


Figure 2

A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HAP3
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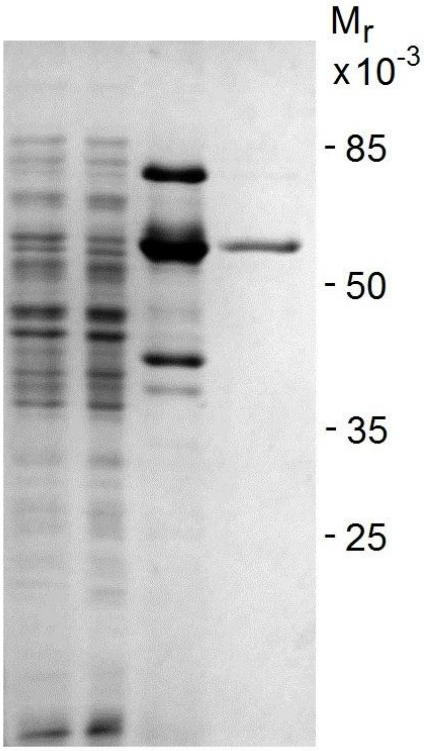
FliC-XynA

B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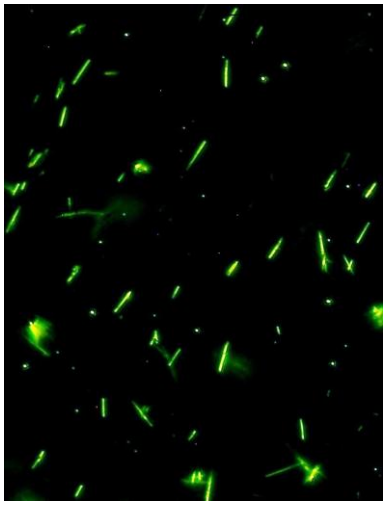
- 85
- 50
- 35
- 25

Figure 3

A



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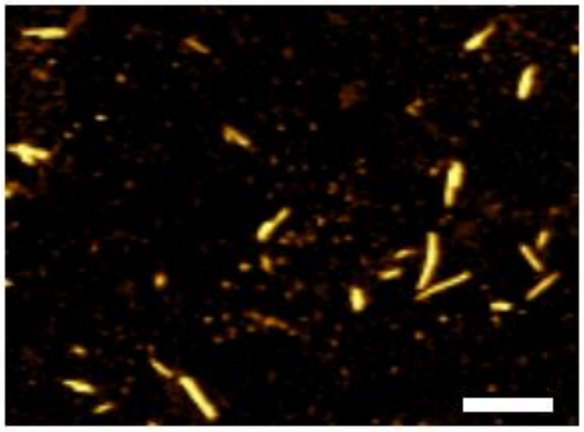
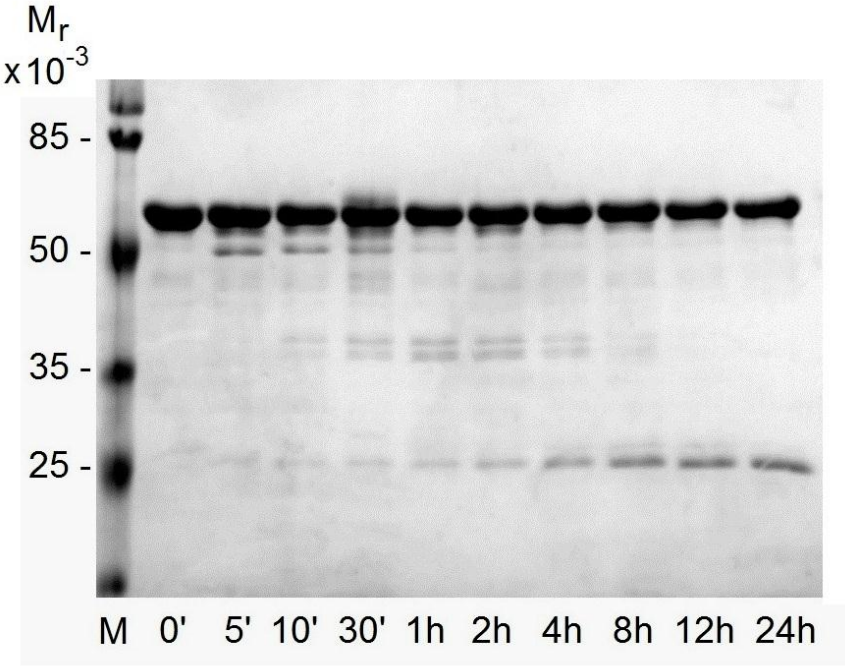
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