Construction of a Xylanase A Variant Capable of Polymerization

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Abstract

The aim of our work is to furnish enzymes with polymerization ability by creating fusion constructs with the polymerizable protein, flagellin, the main component of bacterial flagellar filaments. The D3 domain of flagellin, exposed on the surface of flagellar filaments, is formed by the hypervariable central portion of the polypeptide chain. D3 is not essential for filament formation. The concept in this project is to replace the D3 domain with suitable monomeric enzymes without adversely affecting polymerization ability, and to assemble these chimeric flagellins into tubular nanostructures. To test the feasibility of this approach, xylanase A (XynA) from B. subtilis was chosen as a model enzyme for insertion into the central part of flagellin. With the help of genetic engineering, a fusion construct was created in which the D3 domain was replaced by XynA. The flagellin-XynA chimera exhibited catalytic activity as well as polymerization ability. These results demonstrate that polymerization ability can be introduced into various proteins, and building blocks for rationally designed assembly of filamentous nanostructures can be created.

Materials and Methods

Construction of the flagellin-xylanase A fusion gene

A deletion mutant of flagellin lacking the D3-domain-coding region (∆D3-FliC) was created as described previously [7] using the pKOT1 construct (kind gift from Kenji Oosawa; Nagoya University, Japan). Furthermore, the Pmel cleavage site was replaced by a cassette containing the recognition sites of several restriction enzymes using site directed mutagenesis following the manufacturer’s instruction (QuickChange II Site-Directed Mutagenesis Kit, Stratagene). This modified ∆D3-Flic gene was amplified using forward primer 5’-ACATGATAT-GATGGCACTAGTCATTTAGACAAAGCCG-3’ and reverse primer 5’-ACATGGATCTCTAAACGAGGAGGAC-
GTTTTG-3', and then it was inserted into a pET19b vector (Novagen) using the XhoI and BamHI sites. To minimize the number of extra N-terminal amino acids which may disturb polymerization ability of the expressed protein, the original N-terminal 10 His-tag was shortened to 6-His and the enterokinase cleavage site together with the NdeI site was removed from the construct applying 2-step site directed mutagenesis. This redesigned plasmid served as a cloning vector for the production of the N-terminal His6-tagged version of Flc-xylanase A. The gene of xylanase A was synthesized by Genescript Corporation (Piscataway, NJ, USA) based on the protein sequence of xylanase A from *Bacillus subtilis* (UniProt P18429), and it was inserted into the ΔΔ3-FliC gene between the XhoI and SacI restriction sites using the Rapid DNA Ligation Kit (Fermentas). The obtained plasmid construct (containing the gene of Flc(XynA)) was transformed into *E. coli* TOP10 cells (Invárogen) using the CaCl2 procedure, checked by XhoI and SacI digestion, and sequenced to confirm the presence of the desired DNA sequence and the correct reading frame. The Flc(XynA) construct contained a His6-tag and a few flanking amino acids (the MGHHHHHHGGS segment) attached to its N-terminus.

**Protein expression and purification**

The pET19b-based construct was transformed into BL21-CodonPlus (DE3)-RIL cells to overexpress the His-tagged Flc(-XynA) fusion protein. 100 ml overnight culture was inoculated into 2 L of LB broth, supplemented with ampicillin (100 μg/ml) and incubated at 37°C to reach an OD600 of 0.6. After addition of IPTG to a final concentration of 0.5 mM, incubation was continued at 20°C for another 12 hours. The bacterial pellet was collected by centrifugation at 4000 g for 30 min and stored at −20°C.

Purification of Flc(XynA) was performed as follows: the bacterial pellet from 2 L culture was resuspended in 80 ml 20 mM sodium phosphate buffer containing 500 mM NaCl and 50 mM imidazole, pH = 7.4, and 2 mini Complete tablets (EDTA-free) were added to inhibit unwanted proteolytic degradation. The sample was sonicated on ice to lyse the cells for 1 min 4 times at power of 10W. The solution was centrifuged at 50000 rpm 30 min at 4°C to remove cell debris and aggregates. The supernatant was prepurified on a SP FF cation exchange column (Amersham) to remove contaminating proteases [10]. Then the flow-through fraction was applied to a 5 ml HisTrap Chelating Ni-affinity column (Amersham Pharmacia Biotech). The absorbed proteins were eluted using a linear imidazole gradient (50 mM–200 mM), the His6-tagged Flc(XynA) protein was eluted at about 150 mM imidazole concentration. Collected fractions were dialyzed against PBS buffer and stored at −20°C.

**Limited proteolysis**

Limited proteolysis by trypsin was performed in 10 mM phosphate buffer, pH 7.0, containing 150 mM NaCl at room temperature at a protein concentration of 0.9 mg/ml. In a typical digestion experiment, the protease was added to the protein solution at a 1:300 or 1:30 (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% or 15% (w/v) polyacrylamide slab gels.

**Assay of xylanase activity**

Xylanase activity was determined by measuring the increase in reducing groups during the enzymatic hydrolysis of xylan by the dimitosalicylic acid method of Bernfeld [12]. The reaction mixture consisting of 250 μl of 1% xylan solution in 50 mM phosphate buffer, pH 6.0, and 100 μl of enzyme solution at 4.2 μM concentration was incubated at 37°C for 10 min. After addition of 375 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 within the range of 0–200 μg/ml. Measurements were repeated three times and averaged.

**Polymerization experiments**

The polymerizing ability of the Flc(XynA) fusion protein was investigated by inducing nucleation and polymerization by ammonium sulfate (AS) [13]. Protein solutions of 1 to 1.5 mg/ml were prepared in 20 mM Tris-HCl (pH 7.8) containing 150 mM NaCl and 4 M AS was added to various final concentrations in the range of 0.4 M to 0.8 M. Filament formation was observed after 24 h of incubation at room temperature. For copolymerization of flagellin and Flc(XynA), monomer solutions of Salmonella SJW1660 flagellin capable of forming L-type straight filaments [1] and Flc(XynA) in 20 mM Tris-HCl, 150 mM NaCl (pH 7.8) were mixed at various protein ratios in the range of 1:1 to 1:5 (w/w), and polymerized by the addition of AS to various final concentrations. The mixtures were left at room temperature for 2 days to accomplish polymerization. Filaments were observed by dark-field optical microscopy with an Olympus BX50 microscope. They were also visualized by atomic force microscopy using a DigiScope1000 (Aist-NT) microscope, the scanned area was typically 4 μm ×4 μm.

**Results**

**Design and preparation of the Flc(XynA) fusion construct**

This work aims at the creation of a flagellin-based fusion protein in which the D3 domain of Flc is replaced by the xylanase A enzyme from *B. subtilis* (Fig. 1). In order to obtain a fusion protein which preserves both the catalytic activity of XynA and the polymerization ability of Flc, it is essential to use appropriate linkers for insertion of XynA into the middle portion of flagellin. The linkers should allow proper folding of both partners.
Investigation of polymerization properties

Polymerization ability of the purified FliC(XynA) protein was investigated. Filament formation was induced by adding ammonium sulfate (AS) to a monomer solution of 1 mg/ml at a final concentration in the range of 0.4–0.8 M. We found that FliC(XynA) effectively polymerized into filaments. At 0.4 M AS, aggregates of long filaments were observed under the dark field microscope (Fig. 2A). At higher AS concentration, shorter filaments were observed (not shown). However, the stability of FliC(XynA) filaments required high precipitant concentration (>0.3 M AS), and they dissociated within a few hours after changing the buffer to 20 mM Tris-HCl (pH 7.8) containing 150 mM NaCl.

Highly stable filaments were obtained by mixed copolymerization of FliC(XynA) with flagellin at 1:1 or 1:2 ratios (Fig. 2B) which remained intact even after the removal of AS from the solution by spinning down the sample and dissolving the pellet in 20 mM Tris/150 mM NaCl (pH 7.8). Densitometric analysis of SDS-PAGE gels of copolymer samples revealed that FliC(XynA) was incorporated into the filaments in a proportion similar to the applied mixing ratio (Fig. 2C). Filaments were also observed by AFM (Fig. 2D). Their length was typically in the range of 200 nm–1200 nm, and they showed a straight polymorphic form.

Structural stability of mixed copolymers was probed by proteolytic digestion. Native filaments are known to be highly resistant against tryptic hydrolysis even at room temperature [7]. On the other hand, depolymerized flagellin subunits are quickly degraded under the same conditions since their terminal regions are disordered in the monomeric state [2]. As shown in Figure 3A, FliC(XynA) containing filaments were highly resistant against tryptic degradation even at a 30:1 (w/w) ratio. They remained essentially intact even after an overnight incubation with the protease. These results demonstrate that FliC(XynA) has the ability to form stable filaments when copolymerized together with wild type flagellin, and the XynA portion of subunits has a well-folded, compact conformation. This latter conclusion is also supported by limited proteolysis of monomeric FliC(XynA) (Fig. 3B) which reveals that the natively disordered terminal regions of the fusion protein are highly sensitive to tryptic cleavage but the central portion shows significant stability against proteolytic digestion.

Catalytic properties of FliC(XynA)

Catalytic properties of the FliC(XynA) fusion protein in the monomeric as well as the polymeric state were studied and compared to recombinant wild type xylanase A. XynA can degrade xylan by cleavage of β-1,4-xylosidic linkages. Reducing sugars liberated during the course of hydrolysis were determined by the method of Bernfeld [12] based on absorbance measurements at 575 nm. Color development was compared to a standard xylose curve that was calibrated with D-xylose within the range of 0–200 μg/ml. Activities for the FliC(XynA) fusion protein in the monomeric and polymeric state were determined at 37°C in 50 mM phosphate buffer, pH 6.0 (Table 1). Activity measurements were done at substrate concentration significantly below the saturation level. There are two reasons for this. One is the low solubility of xylan. Another important factor is that the critical flagellin concentration for filament assembly is about 0.2 μM. To study catalytic activity of FliC(XynA) in the filamentous form we had to choose a protein concentration which ensures that the subunits are dominantly in the filament form. Color development by visual inspection clearly indicated that FliC(XynA) was enzymatically active in both the monomeric and polymeric forms. As shown in Table 1, monomeric FliC(XynA) exhibited a xylan
degrading activity similar to that of the recombinant wild-type enzyme. Mixed copolymers were used to investigate the enzymatic activity of FliC(XynA) subunits in the filamentous state. The amount of FliC(XynA) subunits incorporated into the copolymer was estimated by densitometric analysis of SDS-PAGE gels of the corresponding filaments. Our data indicate that the activity of polymeric FliC(XynA) was virtually identical to that of the monomeric form. These experiments demonstrate that XynA maintains its catalytic activity upon insertion into the variable central part of flagellin, and filament formation has no significant influence on the catalytic efficiency of subunits.

**Discussion**

Nanobiocatalysis, in which enzymes are incorporated into nanostructured materials, has emerged as a rapidly growing area [13,14]. Fabrication of nanostructured biocatalysts via self-assembly is an attractive approach which has the potential to allow fabrication of multi-enzymes nanoscale factories. In an early attempt, Baxa et al. [15] fused the prion domain of the amyloidogenic Ure2p protein with various enzymes and demonstrated that the structures of the appended enzymes were modulated very slightly by incorporation into filaments. Our data indicate that the activity of polymeric FliC(XynA) was virtually identical to that of the monomeric form. These experiments demonstrate that XynA maintains its catalytic activity upon insertion into the variable central part of flagellin, and filament formation has no significant influence on the catalytic efficiency of subunits.

The observed polymerization properties of FliC(XynA) were reminiscent to those of flagellin variants containing small truncations in the N-terminal part of the molecule [17] which also formed straight filaments of decreased stability. In the core of the flagellar filament there is a pair of concentric double-tubular structures composed of the inner-tube and the outer-tube [6]. Our previous studies have shown that the inner-tube structure is quite independent from the outer-tube structure [18–19]. The main driving force behind polymerization is the interaction between the outer-tube domains of subunits, and the basic structure of the flagellar filament can be formed with the interactions in the outer-tube alone. The very terminal regions of flagellin are known to construct the inner tube and the wall of the narrow central channel of flagellar filaments [6,18]. They are not essential for polymerization, but they do play an important role in stabilization of the filament structure [17]. Cryo-EM studies revealed that small terminal truncations of subunits resulted in misfolding of the inner tube structure [18], and destabilization of the filament. The similar
Interestingly, this kind of flagellar display has severe limitations. Specifically, ligands or characterize its immunogenic properties. The surface of flagellar filaments, and study its interaction with the insertion of foreign peptides or domains [20–22]. The primary aim of these studies was to display the inserted unit outside the cell on mimic flagellar polymerization properties of FliC(XynA) suggest conformational strains in the filament core. Close inspection of the available 3D structure of the flagellar filament [6] revealed that the wall of the central channel is composed of the helical C-terminal portion of subunits while their N-terminal part is buried under this layer. The FliC(XynA) fusion protein contains a His6-tag and a few additional flanking amino acids attached to its N-terminus. It seems that these extra N-terminal residues cannot be readily accommodated and imposes a conformational strain on the structure of the inner-tube, reflected in the decreased stability. Copolymerization with intact flagellin may significantly relieve these distortions. Work is under way to develop a FliC(XynA) variant in which the fusion tag used for easy purification is attached to the C-terminus or removable by selective proteolytic digestion.

The central part of flagellin has been a favorite target site for insertion of foreign peptides or domains [20–22]. The primary aim of these studies was to display the inserted unit outside the cell on the surface of flagellar filaments, and study its interaction with specific ligands or characterize its immunogenic properties. However, this kind of flagellar display has severe limitations.

**Figure 3. Proteolytic sensitivity of FliC(XynA) in the polymeric and monomeric form.** (A) Copolymers of flagellin and FliC(XynA) obtained by 0.6 M As at 1:1 mixing ratio (w/w) were incubated with trypsin at a 30:1 (w/w) ratio. (B) Proteolysis of monomeric FliC(XynA) at a protein to protease ratio of 300:1 (w/w). At the indicated time points, portions were removed from the reaction mixtures, mixed with electrophoresis sample buffer and boiled for 3 min. Experiments were done in 20 mM Tris–HCl, 150 mM NaCl (pH 7.8) at room temperature at a 1 mg/ml protein concentration.

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Subunits of flagellar filaments are exported by the flagellum-specific export machinery through the narrow central channel of filaments [3]. In the majority of cases flagellin-based fusion constructs are not exported by the flagellar export system because of size limitations or other compatibility problems (related to the amino acid composition, surface properties, the presence of disulfide bonds etc.).

Notwithstanding, these problems do not mean that fusion proteins not applicable to flagellar display are unable to form filaments in vitro. As we realized, these fusion proteins can be overexpressed in bacteria, and after purification from the cells they are usable to build filamentous assemblies. This idea was tested in this study by exploring the filament forming and enzymatic properties of the FliC(XynA) fusion protein. Our experiments show that replacing the D3 domain of flagellin with an enzyme protein using appropriate linkers can result in a functional fusion product that exhibits polymerization ability and catalytic activity as well. Recent studies have demonstrated the versatility of employing bioengineered flagella for the generation of a variety of nanoparticle arrays and nanotubes [23–26]. Our approach opens up the way for creation of building blocks for flagella-based filamentous nanostructures with designed catalytic properties.

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**Author Contributions**

Conceived and designed the experiments: BT MDM FV. Performed the experiments: VS AM BT. Analyzed the data: VS FV. Wrote the paper: FV.

**Table 1.** Catalytic activity of recombinant xylanase A and the FliC(XynA) fusion protein in the monomeric and polymeric forms.

| Sample                        | OD575 | D-xylose deliberated (µg/ml) |
|-------------------------------|-------|-----------------------------|
| native xylanase A             | 0.175 | 26.7                        |
| FliC(XynA) monomer            | 0.156 | 23.8                        |
| FliC/FliC(XynA) copolymer 2:1 (w/w) | 0.153 | 23.3                        |
| FliC/FliC(XynA) copolymer 1:1 (w/w) | 0.163 | 24.9                        |

Measurements were done at 1.2 µM enzyme (catalytic unit) concentration in 50 mM phosphate buffer (pH 6.0) at 37°C. The amount of FliC(XynA) subunits incorporated into the copolymers was estimated by densitometric analysis of SDS-PAGE gels of filament samples.

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1. Vonderviszt F, Namba K (2008) Structure, function and assembly of flagellar axial proteins. In: Scheibel T, ed. Fibrous Proteins. Landes Bioscience, pp 50–76.
2. Vonderviszt F, Kanto S, Aizawa S, Namba K (1989) Terminal regions of flagellin are disordered in solution. J Mol Biol 209: 127–133.
3. Minamino T, Imada K, Namba K (2008) Mechanisms of type III protein export for bacterial flagellar assembly. Mol Biosyst 4: 1105–1113.
4. Yonekura K, Maki S, Morgan DG, DeRosier DJ, Vonderviszt F, et al. (2000) The bacterial flagellar cap as the rotary promotor of flagellin self-assembly. Science 290: 2148–2152.
5. Samatey FA, Imada K, Nagashima S, Vonderviszt F, Kumasaka T, et al. (2001) Structure of the bacterial flagellar protofilament and implications for a switch for supercoiling. Nature 410: 331–337.
6. Yonekura K, Maki-Yonekura S, Namba K (2003) Complete atomic model of the bacterial flagellar filament by electron cryomicroscopy. Nature 424: 643–650.
7. Muskota A, Seregelyes C, Sebestyen A, Vonderviszt F (2010) Structural basis for stabilization of the hypervariable D3 domain of Salmonella flagellin upon filament formation. J Mol Biol 403: 607–615.
8. Henrisat B, Davies G (1997) Structural and sequence-based classification of glycoside hydrolases. Curr Opin Struct Biol 7: 637–644.
9. Murakami MT, Arni RK, Vieira DS, Degreve L, Ruller R, et al. (2005) Correlation of temperature induced conformational change with optimum catalytic activity in the recombinant G/11 xylanase A from Bacillus subtilis strain 168 (1A1). FEBS Lett 579: 6505–6510.
10. Saijo-Hamano Y, Namba K, Oosawa K (2000) A new purification method for overproduced proteins sensitive to endogenous proteases. J Struct Biol 132: 142–146.
11. Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, et al. (2005) Protein identification and analysis tools on the ExPASy server. In: Walker JM, ed. The proteomics protocols handbook Humana Press, NJ. pp 571–607.
12. Bernfeld P (1955) Amylases α and β. Methods Enzymol 1: 149–158.
13. Kim J, Grate JW, Wang P (2008) Nanobiocatalysis and its potential applications. Trends Biotech 26: 639–646.
14. Ge J, Lu D, Liu Z, Liu Z (2009) Recent advances in nanostructured biocatalysts. Biochem Eng J 44: 33–59.
15. Baxa U, Speransky V, Steven AC, Wickner RB (2002) Mechanism of inactivation on prion conversion of the Saccharomyces cerevisiae Ure2 protein. Proc Natl Acad Sci USA 99: 5253–5260.
16. Baldwin AJ, Bader K, Christodoulou J, MacPhee CE, Dobson CM, et al. (2006) Cytoskeleton display on amyloid fibrils. J Am Chem Soc 128: 2162–2163.
17. Vonderviszt F, Aizawa SI, Namba K (1991) Role of the disordered terminal regions of flagellin in filament formation and stability. J Mol Biol 221: 1461–1474.
18. Mimori-Kiyosue Y, Vonderviszt F, Namba K (1997) Locations of terminal segments of flagellin in the filament structure and their roles in polymerization and polymorphism. J Mol Biol 270: 222–237.
19. Mimori-Kiyosue Y, Vonderviszt F, Yamashita I, Fujiyoshi Y, Namba K (1996) Direct interaction of flagellin termini essential for polymorphic ability of flagellar filament. Proc Natl Acad Sci USA 93: 15108–15113.
20. Stocker BA, Newton SM (1994) Immune responses to epitopes inserted in Salmonella flagellin. Int Rev Immunol 11: 167–178.
21. Ezaki S, Tsukao M, Takagi M, Imanaka T (1998) Display of heterologous gene products on the Eschericia coli cell surface as fusion proteins with flagellin. J Ferri Bioeng 5: 500–503.
22. Westerlund-Wikstrom B (2000) Peptide display on bacterial flagella: principles and applications. Int J Med Microbiol 289–293.
23. Kumara MT, Muralidharan S, Tripp BC (2007) Generation and characterization of inorganic and organic nanotubes on bioengineered flagella of mesophilic bacteria. J Nanosci Nanotechnol 7: 2260–2272.
24. Woods RD, Takahashi N, Adams A, Pezzi RJ, Aizawa SI, et al. (2007) Bifunctional nanotube scaffolds for diverse ligands are purified simply from Escherica coli strains coexpressing two functionalized flagellar genes. Nano Lett 7: 1809–1816.
25. Kumara MT, Tripp BC, Muralidharan S (2007) Layer-by-layer assembly of bioengineered flagella protein nanotubes. Biomacromolecules 8: 3718–3722.
26. Kumara MT, Tripp BC, Muralidharan S (2007) Exciton energy transfer in self-assembled quantum dots on bioengineered bacterial flagella nanotubes. J Phys Chem C 111: 5276–5280.