Purification of Flagellin from *Acidovorax avenae* and Analysis of Plant Immune Responses Induced by the Purified Flagellin

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[Abstract] Plants sense potential pathogens by recognizing conserved pathogen-associated molecular patterns (PAMPs) that cause PAMP-triggered immunity (PTI) including the generation of reactive oxygen species, callose deposition, and expression of several PTI-related genes. *Acidovorax avenae* is a Gram-negative bacterium that causes a seedling disease characterized by the deposition of brown stripes on the sheaths of infected plants. We previously reported that flagellin isolated from the rice avirulent *A. avenae* N1141 strain induces PTI, while flagellin isolated from the rice virulent *A. avenae* K1 strain does not induce PTI. To examine the molecular mechanism of specific PTI induction by N1141 flagellin, highly purified flagellin from N1141 or K1 strains is required. Here, we describe a high quality purification method for the *A. avenae* flagellins and for using it in PTI induction study.

Materials and Reagents

1. 0.22 µm sterilization filter (Merck Millipore, catalog number: SLGS033SB)
2. Nitrocellulose membrane (GE Healthcare, code number: 10401196)
3. Collodion-coated grid (Ted Pella, Inc., catalog number: 12575-CU)
4. Cultured rice cells of line OC (*Oryza sativa* C5928; obtained from RIKEN BioResource Center)
5. *Acidovorax avenae* N1141 (MAFF 301141) and K1 (MAFF301755) (National Institute of Agrobiological Sciences genebank, catalog number: MAFF 301141; MAFF301755)
6. Skim milk powder (Wako, catalog number: 190-12865)
7. Sodium hydrogen L(+)-glutamate monohydrate (Wako, catalog number: 198-02035)
8. Luria-Bertani (LB) liquid medium (MO BIO laboratories, catalog number: 12107-05)
9. 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) (Wako, catalog number: 204-07885)
10. NaCl (Nacalai Tesque, catalog number: 31320-05)
11. KCl (Nacalai Tesque, catalog number: 28514-75)
12. 1% (w/v) phosphotungstic acid (pH 6.9) (Sigma-Aldrich, catalog number: 79690)
13. Sodium dodecyl sulfate (SDS) (Wako, catalog number: 191-07145)
14. Molecular-weight marker low (APRO, catalog number: SP-0110)
15. Anti-flagellin rabbit antibody (Eurofins Genomics)

Note: Flagellin purified from A. avenae N1141 strain as antigen was injected in rabbit. Anti-flagellin rabbit antibody was purified from rabbit antiserum (anti-flagellin) using flagellin purified from A. avenae N1141 strain.

16. Goat HRP conjugated anti-rabbit IgG antibody (H + L chain) [Medical & Biological Laboratories (MBL), catalog number: 458]
17. ECL plus Western blotting detection reagents (GE Healthcare, code number: RPN2132)
18. Hybri-Bag (Cosmo Bio, catalog number: S-1001)
19. Potassium ferricyanide (Wako, catalog number: 169-03721)
20. KNO_3 (Nacalai Tesque, catalog number: 28704-85)
21. $(NH_4)_2SO_4$ (Wako, catalog number: 019-03435)
22. $MgSO_4 \cdot 7H_2O$ (Nacalai Tesque, catalog number: 21003-75)
23. $CaCl_2 \cdot 2H_2O$ (Nacalai Tesque, catalog number: 06731-05)
24. $NaH_2PO_4 \cdot 2H_2O$ (Wako, catalog number: 192-02815)
25. $MnSO_4 \cdot 5H_2O$ (Nacalai Tesque, catalog number: 21229-35)
26. $ZnSO_4 \cdot 7H_2O$ (Nacalai Tesque, catalog number: 37011-75)
27. $CuSO_4 \cdot 5H_2O$ (Nacalai Tesque, catalog number: 09605-04)
28. H_3BO_3 (Wako, catalog number: 021-02195)
29. $Na_2MoO_4 \cdot 2H_2O$ (Nacalai Tesque, catalog number: 31621-52)
30. EDTA·2Na (DOJINDO, catalog number: 345-01865)
31. $FeSO_4 \cdot 7H_2O$ (Wako, catalog number: 098-01085)
32. MS vitamin powder 1,000x (Sigma-Aldrich, catalog number: M7150-100ML)
33. 2, 4-dichlorophenoxyacetic acid (Wako, catalog number: 040-18532)
34. Sucrose (Wako, catalog number: 196-00015)
35. 3-mercapto-1,2-propanediol (Wako, catalog number: 131-16451)
36. Glycerol (Nacalai Tesque, catalog number: 17018-25)
37. Bromophenol blue (BPB) (Wako, catalog number: 021-02911)
38. Glycine (Wako, catalog number: 077-00735)
39. Coomassie brilliant blue R-250 (CBB) (Wako, catalog number: 031-17922)
40. Methanol (Wako, catalog number: 137-01823)
41. Acetic acid (Wako, catalog number: 017-00251)
42. Polyoxyethylene sorbitan monolaurate (Tween 20) (Wako, catalog number: 167-11515)
43. KH_2PO_4 (Wako, catalog number: 169-04245)
44. Skimmed milk solution (see Recipes)
45. *Acidovorax avenae* N1141 and K1 strain skimmed milk stock solution (see Recipes)
46. 25 mM TBS buffer (pH 7.4) (see Recipes)
47. 20x R2 Major solution (see Recipes)
48. 1,000x R2 Minor solution (see Recipes)
49. 500x Fe liquid solution (see Recipes)
50. 1,000x MS vitamin solution (see Recipes)

51. 2, 4-dichlorophenoxyacetic acid solution (see Recipes)
52. R2S medium (pH 5.6) (see Recipes)
53. 2x sample buffer (pH 6.8) (see Recipes)
54. Electrophoresis buffer (see Recipes)
55. CBB staining solution (see Recipes)
56. Destaining solution (see Recipes)
57. Transfer buffer (see Recipes)
58. Tris buffered saline with Tween 20 (TBST) buffer (see Recipes)
59. Blocking buffer (see Recipes)
60. 50 mM potassium phosphate buffer (pH 7.9) (see Recipes)
61. Luminol solution (see Recipes)
62. Potassium ferricyanide solution (see Recipes)

Equipment

1. Rotary shaker (TAITEC, model: NR-20)
2. High-speed refrigerated micro centrifuge (TOMY, model: MX-300)
3. High-speed refrigerated centrifuge (Hitachi Koki, model: CR20G)
4. Ultracentrifuge (Hitachi Koki, model: CP70MX)
5. Fiber blender (Panasonic, model: MX-X58-SW)
6. Incubator (TAITEC, model: BR-42FL· MR)
7. Transmission electron microscope (Hitachi, model: H-7100)
8. Plant growth chamber (NK system, model: LH-411SP)
9. Electrophoresis tank (ATTO, model: AE-6530M)
10. Semi-dry blotter (Bio-Rad, catalog number: 1703940JA)
11. Luminescent image analyzer (GE Healthcare, model: ImageQuant LAS 4000)
12. Lumi-counter (ATTO, model: AB-2350)

Procedure

A. Purification of flagellar filaments from *Acidovorax avenae*

1. For pre-culture of *Acidovorax avenae*, add 2 µl of *A. avenae* skimmed milk stock solution to 2 ml of LB liquid medium, and shake (200 rpm) for overnight at 30 °C.
2. Add 100 µl of pre-cultured suspension to one liter fresh LB medium, and shake (200 rpm) for 24 h at 30 °C.
3. Harvest *A. avenae* cells by centrifugation at 6,000 *x g* for 20 min at room temperature.
4. Add 300 ml of 25 mM TBS buffer to the pellet and re-suspend. After centrifugation at 6,000 *x g* for 20 min at room temperature, discard the supernatant.
5. Re-suspend the pellet with 90 ml of TBS buffer, and transfer to fiber blender.

6. To take off flagellum from bacterial cells, shear with fiber blender for 1 min at 4 °C, and incubate for 5 min on ice. Repeat this step 7 times.
7. To remove intact bacterial cells, centrifuge the bacterial suspension at 6,000 *x g* for 30 min at 4 °C and save the supernatant.
8. To remove bacterial cellular debris, centrifuge the supernatant at 16,000 *x g* for 60 min at 4 °C and save the supernatant.
9. Ultracentrifuge the supernatant at 200,000 *x g* for 60 min at 4 °C and discard the supernatant.
10. Add 1.5 ml of ice-cold distilled water to the pellet and re-suspend.
11. Centrifuge the re-suspended pellet at 20,000 *x g* for 20 min at 4 °C, and discard the supernatant. The pellet contains the monomer and polymer flagellins.
12. Re-suspend the pellet with 0.5 ml of ice-cold distilled water and store at -80 °C.

B. Observation of purified flagellar filaments using transmission electron microscopy
1. 50 µl droplet of the purified flagellar filaments (pellet of 20,000 *x g*) are used.
2. Absorb the purified flagellum onto collodion-coated grids, which are supported with carbon and rendered the carbon surface hydrophilic, for 1 min.
3. Stain the grids with 1% (w/v) phosphotungstic acid (pH 6.9) for 1 min and wash with two drops of distilled water.
4. Images are taken as digitized pictures with transmission electron microscope operated at 80 kV.

C. Purity check of flagellin in the purified flagellar filaments by Western blotting
1. Flagellar filaments of *A. avenae* consists of only flagellin protein. Purity of flagellin in the flagellar filaments is analyzed by SDS-PAGE and Western blotting.
2. Add half volume of 2x sample buffer to 100 ng of purified flagellum suspension.
3. Heat the sample at 95 °C for 5 min, and cool on ice.
4. Load 20 ng of sample and 2 µl of Prestained XL-Ladder Low marker of low range onto SDS-PAGE gel.
5. Electrophoresis at constant 100 Volts until the dye reaches the bottom of the gel.
6. Electrotransfer to nitrocellulose membrane using transfer buffer (constant 10 Volts for 3 h).
7. Incubate membrane in 25 ml of blocking buffer for 1 h at room temperature.
8. Wash three times for 5 min each with 25 ml of TBST.
9. Incubate membrane with primary Anti-flagellin rabbit antibody (1:2,500) in 10 ml of TBST for 1 h at room temperature.
10. Wash five times for 5 min each with 25 ml of TBST.
11. Incubate membrane with secondary goat HRP conjugated anti-rabbit IgG antibody (1:2,000) in 10 ml of TBST for 30 min at room temperature.
12. Wash three times for 5 min each with 25 ml of TBST.
13. Incubate membrane with 1 ml of ECL plus Western blotting detection reagent for 3 min at room temperature, and then wrap in Hybri-Bag.

14. Measure the intensity of each band with LAS-4000.

D. H_2O_2 detection and quantification
   1. Suspension cultures of rice cells (line OC) were grown at 30 °C under white fluorescent light irradiation in a plant growth chamber. The cells were diluted in fresh medium every week, and experiment for H_2O_2 detection was performed 4 days after transfer.
   2. Place the cultured rice cells onto filter paper to remove the culture medium. Add 10 mg culture cells into 0.5 ml of fresh medium, and culture the cells in a plant growth chamber for 2 h at 30 °C.
   3. Add the flagellin (100 nM) to the cultured cells, and incubate in a plant growth chamber at 30 °C. Less than 100 µl of the flagellin suspension is desirable for addition to a culture medium.
   4. After incubation, add 10 µl of culture medium to chemiluminescence reaction buffer containing 160 µl of 50 mM potassium phosphate buffer (pH 7.9), 10 µl of 1.1 mM luminol and 20 µl of 14 mM potassium ferricyanide. Quantify H_2O_2 using a lumi-counter.

Representative data

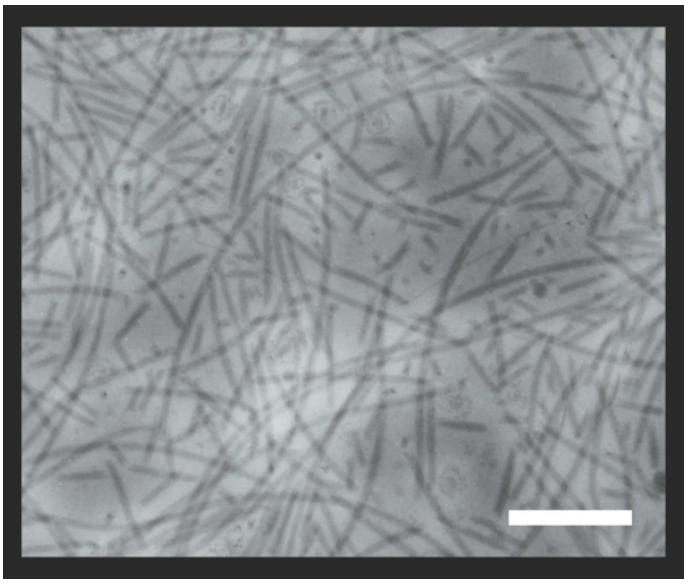

Figure 1. Transmission electron micrograph of flagellar filaments purified from *A. avenae* K1 strain. The purified sample contains the flagellar filaments of various lengths which are formed by mechanical shearing. Scale bar shown at the bottom right corner is 250 nm.

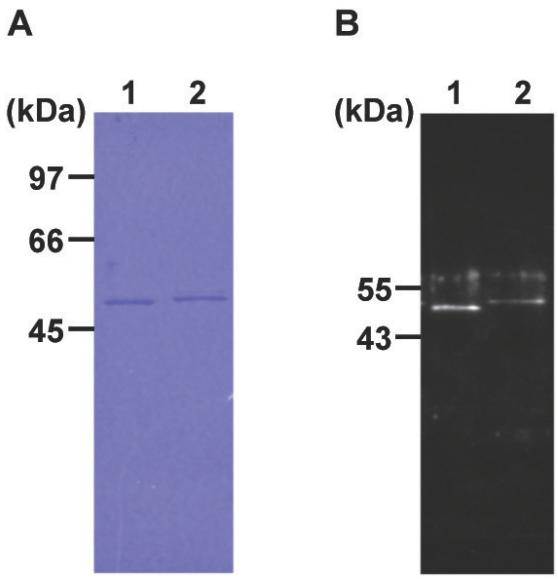

Figure 2. Purity of flagellins in flagellar filaments purified from *A. avenae* N1141 and K1 strains by SDS-PAGE (A) and Western blot analysis (B). A. The proteins were visualized by Coomassie brilliant blue R-250 (CBB) staining. Lane 1, flagellin of N1141 strain (pellet of 20,000 *x g*); lane 2, flagellin of K1 strain (pellet of 20,000 *x g*). Molecular masses of the N1141 and K1 flagellins are 50,820 Da and 51,254 Da, respectively. B. The flagellins were detected by anti-flagellin antibody. Lane 1, flagellin of N1141 strain (pellet of 20,000 *x g*); lane 2, flagellin of K1 strain (pellet of 20,000 *x g*).

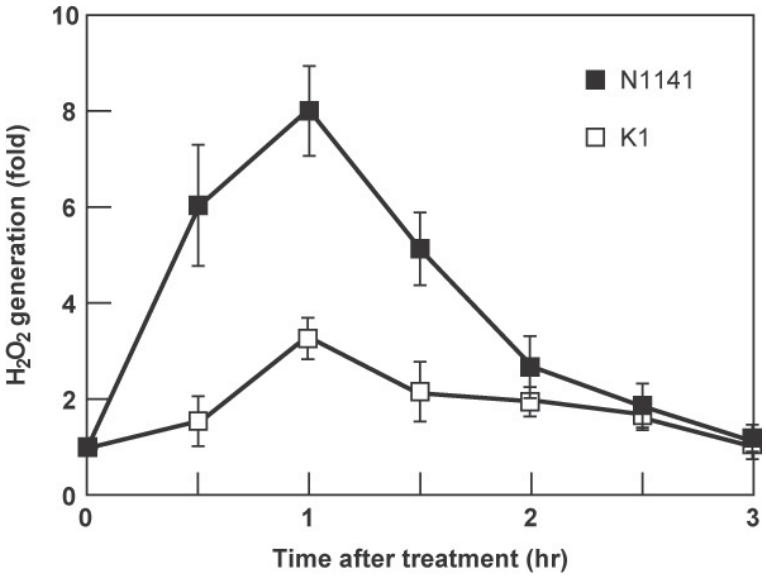

Figure 3. Induction of H_2O_2 generation in cultured rice cells by flagellins purified from *A. avenae*. Time course of H_2O_2 generation in cultured rice cells treated with 100 nM flagellins from the avirulent N1141 strain (solid squares) or virulent K1 strain (open squares). The Y axis represents fold change relative to the amount of H_2O_2 in cultured cells before treatment. Bars indicate the standard deviation of the mean of three experiments.

Recipes

1. Skimmed milk solution
   10% skim milk powder
   80 mM sodium hydrogen L(+)-glutamate monohydrate
   Sterilize the solution by autoclaving
2. *A. avenae* skimmed milk stock solution
   Culture *A. avenae* on LB solid medium for 24 h at 30 °C
   Add 1 ml of skimmed milk solution to the culture solid medium, and harvest
   Freeze immediately in liquid nitrogen, and store at -80 °C
3. 25 mM TBS buffer (pH 7.4)
   25 mM 2-amino-2-hydroxymethyl-1,3-propanediol
   137 mM NaCl
   2.68 mM KCl
   Sterilize the solution using a sterile filter (0.22 μm)
4. 20x R2 Major solution
   800 mM KNO_3
   50.7 mM $(NH4)_2SO_4$
   20.3 mM $MgSO_4 \cdot 7H_2O$
   20.4 mM $CaCl_2 \cdot 2H_2O$
   35 mM $NaH_2PO_4 \cdot 2H_2O$
   Sterilize the solution using a sterile filter (0.22 μm)
5. 1,000x R2 Minor solution
   7.17 mM $MnSO_4 \cdot 5H_2O$
   7.65 mM $ZnSO_4 \cdot 7H_2O$
   0.5 mM $CuSO_4 \cdot 5H_2O$
   48.52 mM H_3BO_3
   0.52 mM $Na_2MoO_4 \cdot 2H_2O$
   Sterilize the solution using a sterile filter (0.22 μm)
6. 500x Fe liquid solution
   10.07 mM $Na_2 \cdot EDTA$
   9.89 mM $FeSO_4 \cdot 7H_2O$
   Sterilize the solution using a sterile filter (0.22 μm)
7. 1,000x MS vitamin solution (10 ml)
   1.03 g MS vitamin powder (1,000x)
   Sterilize the solution using a sterile filter (0.22 μm)
8. 2,4-dichlorophenoxyacetic acid solution
   181 mM 2, 4-dichlorophenoxyacetic acid
   Dissolve 2, 4-dichlorophenoxyacetic acid in1 ml of dimethyl sulfoxide

9. R2S medium (pH 5.6) (1,000 ml)
   50 ml 20x R2 major solution
   1 ml 1,000x R2 minor solution
   2 ml 500x Fe liquid solution
   1 ml 1,000x vitamin solution
   0.1 ml 2, 4-dichlorophenoxyacetic acid solution
   30 g sucrose
   Adjust pH to 5.6 with KOH. Fill up to 1,000 ml with distilled water, and sterilize by autoclaving.
10. 2x sample buffer (pH 6.8)
    125 mM Tris
    139 mM SDS
    12% (v/v) 3-mercapto-1,2-propanediol
    20% (v/v) glycerol
    Adjust pH to 6.8 with HCl, and add to 1% BPB
11. Electrophoresis buffer
    3.5 mM SDS
    24.8 mM Tris
    192 mM glycine
12. CBB staining solution
    0.25% (w/v) CBB
    50% (v/v) methanol
    5% (v/v) acetic acid
13. Destaining solution
    50% (v/v) methanol
    10% (v/v) acetic acid
14. Transfer buffer
    25 mM Tris
    192 mM glycine
    1.7 mM SDS
    20% (v/v) methanol
15. TBST buffer
    25 mM Tris
    13.7 mM NaCl
    0.1% (v/v) Tween-20
16. Blocking buffer
    5% (w/v) skim milk powder
    Dissolve skim milk powder in TBST buffer
17. 50 mM potassium phosphate buffer (pH 7.9)
    50 mM KH_2PO_4

Adjust pH to 7.9 with KOH

18. Luminol solution

    1.1 mM luminol

    Dissolve luminol in 50 mM potassium phosphate buffer (pH 7.9)

19. Potassium ferricyanide solution

    14 mM potassium ferricyanide

    Dissolve potassium ferricyanide in 50 mM potassium phosphate buffer (pH 7.9)

Acknowledgments

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References

1. Che, F. S., Nakajima, Y., Tanaka, N., Iwano, M., Yoshida, T., Takayama, S., Kadota, I. and Isogai, A. (2000). Flagellin from an incompatible strain of *Pseudomonas avenae* induces a resistance response in cultured rice cells. *J Biol Chem* 275(41): 32347-32356.
2. Hirai, H., Takai, R., Iwano, M., Nakai, M., Kondo, M., Takayama, S., Isogai, A. and Che, F. S. (2011). Glycosylation regulates specific induction of rice immune responses by *Acidovorax avenae* flagellin. *J Biol Chem* 286(29): 25519-25530.
3. Katsuragi, Y., Takai, R., Furukawa, T., Hirai, H., Morimoto, T., Katayama, T., Murakami, T. and Che, F. S. (2015). CD2-1, the C-terminal region of flagellin, modulates the induction of immune responses in rice. *Mol Plant Microbe Interact* 28(6): 648-658.
4. Schwacke, R. and Hager, A. (1992). Fungal elicitors induce a transient release of active oxygen species from cultured spruce cells that is dependent on $Ca^{(2+)}$ and protein-kinase activity. *Planta* 187(1): 136-141.

