Monolith Protocol MO-P-031

CMB3a – Cellulose Nanocrystals

The family 3 carbohydrate-binding module CBM3 is part of the scaffolding subunit of the multienzyme plant cell wall degrading complex termed cellulosome. Cellulosomes are anchored to the external membrane of some lignocellulolytic microorganisms. Several hydrolytic enzymes active on plant polysaccharides are grafted to the cellulosome. However, the CBM3a is a non-catalytic protein able to bind to insoluble cellulose. Its main functionality is to bring the cellulosome in close vicinity to the plant cell wall and enhance the deconstruction of the plant polysaccharides as a source of carbon for the microorganism.

protein – small molecule interaction | cellulose | nanocrystals

---

A1. Target/Fluorescent Molecule

CBM3a

uniprot.org/uniprot/Q06851 | rcsb.org/structure/1NBC

A2. Molecule Class/Organism

Family 3 carbohydrate-binding module (CBM)
Clostridium thermocellum

A3. Sequence/Formula

```
MGVSGNLKVE FYNSNPSDTT NSINPQFKVT NTGSSAIDLS KLTLRYYYTV DGQKDQTFWC DHAAIIGSNG SYNGITSNVK
GTFVKMSSST NNADTYLEIS FTGGTLEPGA HVQIQGRFAK NDWSNYTQSN DYSFKSASQF VEWDQVTAYL NGVLVWGKEP
GLEHHHHHH
```

A4. Purification Strategy/Source

The gene coding for CBM3a was cloned in a pET28a plasmid, resulting in a His_6-tagged protein at the C-terminus. The strain BL21 (DE3) of *Escherichia coli* harboring the plasmid of interest was cultured to mid-exponential phase (A_{600nm} 0.6) in Luria-Bertani broth at 37°C. Recombinant enzyme expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.6 mM at 37°C for 4 h. Cells were harvested by centrifugation at 5,000 × g for 10 min, re-suspended in 10 mL of 50 mM sodium phosphate buffer, pH 7, containing 300 mM NaCl, 20 mM imidazole and a protease inhibitor cocktail (cOmplete™ Protease Inhibitor EDTA-free, Roche) and lysed by sonication on ice for 1 min. The lysate was clarified by centrifugation (30 min at 74,000 × g at 4°C). Proteins were purified by immobilized metal ion affinity chromatography (IMAC) using Talon resin (Clontech) and eluted in 50 mM sodium phosphate buffer, pH 7, containing 300 mM NaCl and 100 mM imidazole. The eluted proteins were desalted with a PD-10 desalting column (GE Healthcare Life Sciences), using 50 mM sodium phosphate buffer, pH 7.0.

A5. Stock Concentration/Stock Buffer

6.37 mg/mL | 339 µM
50 mM phosphate buffer, pH 7.0

A6. Molecular Weight/Extinction Coefficient

18.7 kDa
35,410 $M^{-1}cm^{-1}$ (ε_{280}) | pI_{Th} 6.02

A7. Dilution Buffer

50 mM sodium phosphate buffer, pH 7.0, 0.05% Pluronic® F-127

A8. Labeling Strategy

Monolith Protein Labeling Kit RED – NHS (MO-L001, NanoTemper Technologies GmbH)
1x 10 µg RED-NHS dye

A9. Labeling Procedure

1. Adjust the concentration of CBM3a to 20 µM in 100 µL of 50 mM phosphate buffer, pH 7.0.
2. Add 30 µL of DMSO to 10 µg RED-NHS dye to obtain a ~470 µM solution. Mix the dye thoroughly by vortexing and make sure that all dye is dissolved.
3. Mix 15 µL of the 470 µM dye solution with 85 µL of phosphate buffer, pH 7.0 to obtain 100 µL of a 70 µM dye solution (3.5x protein concentration).
4. Mix CBM3a and dye in a 1:1 volume ratio (200 µL final volume, 7.5% final DMSO concentration).
5. Incubate for 30 minutes at room temperature in the dark.
6. Add 100 µL of TALON® Metal Affinity Resin into the Vivapure Metal Chelate Mini spin column[1], centrifuge at 1500 RCF for 1 min at 10°C to remove storage buffer.
7. Wash the resin with 500 µL of ice cold dH_2O, leave caps open, centrifuge as described in step 6, and discard the flow through.
8. Equilibrate the resin with 400 µL of ice cold 50 mM phosphate buffer, pH 7.0, centrifuge as described in step 6, and discard the flow through. Repeat this step three times.
9. Load 200 µL of labeled protein (see step 4) to the center of the column. Gently mix the protein-dye mixture with resin by shaking and incubate on ice for 15 min. Centrifuge as described in step 6 to remove excess of free dye.
10. Wash with 400 µL of ice cold 50 mM phosphate buffer, pH 7.0, centrifuge as described in step 6, and discard the flow through. Repeat this step two times.
11. Add 200 µL of ice-cold elution buffer (50 mM sodium phosphate, 200 mM imidazole buffer, pH 7.0.), resuspend gently the resin by shaking and incubate on ice for 5 min. Centrifuge at 1500 RCF 3 min at 10°C; collect the flow through as the labeled protein solution #1. Repeat one time and collect the flow through as labeled protein solution #2.
12. Dilute in 50 mM sodium phosphate buffer, pH 7.0 containing 0.05% Pluronic® F-127 to a 50 nM solution.

[1] Gravity Flow Column B from the Labeling Kit **cannot** be used for purification due to interaction of CBM3a with the column.

A10. Labeling Efficiency

After purifying the protein by IMAC, the protein concentration cannot be determined by UV absorption due to the imidazole. In this case, to avoid dialysis dilution, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with labeled CBM3a and a 1:1 serial dilution of CBM3a (non-labeled) as standards. The labeled protein was then quantified using the quantifying tool in the software of Image Lab 5.2.1.

B1. Ligand/Non-Fluorescent Binding Partner

Cellulose nanocrystals

B2. Molecule Class/Organism

Cotton

B3. Sequence/Formula

N/A

B4. Purification Strategy/Source

Cellulose nanocrystals from cotton linters (in a 2% w/w aqueous suspension) were prepared by Laurent Heux (CERMAV, Grenoble, France) using an established method.[2]

B5. Stock Concentration/Stock Buffer

20 mg/mL

B6. Molecular Weight/Extinction Coefficient

N/A

B7. Serial Dilution Preparation

1. Prepare a PCR-rack with 16 PCR tubes. Transfer 20 µL of the 20 mg/mL nanocrystals solution into tube **1**. Then, transfer 10 µL of dilution buffer into tubes **2** to **16**.
2. Prepare a 1:1 serial dilution by transferring 10 µL from tube to tube. Mix carefully by pipetting up and down. Remember to discard 10 µL from tube **16** to get an equal volume of 10 µL for all samples.
3. Add 10 µL of labeled CBM3a (50 nM) to each tube from **16** to **1** and mix by pipetting.
4. Incubate for 5 minutes at room temperature in the dark before loading capillaries.

C. Applied Quality Checks

SDS-Page

[2] See Fumagalli et al., Biomacromolecules 2013, 14, 9, 3246-3255 for further details.

D1. MST System/Capillaries

Monolith NT.115 Red (NanoTemper Technologies GmbH)
Premium Capillaries Monolith NT.115 (MO-K025, NanoTemper Technologies GmbH)

D2. MST Software

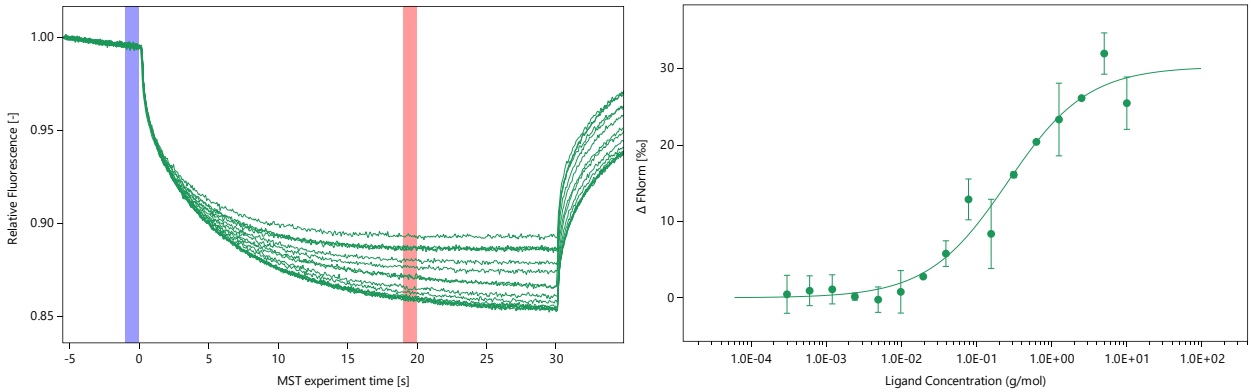
MO.Affinity Analysis v2.3 (NanoTemper Technologies GmbH)

D3. MST Experiment (Assay Buffer/Concentrations/Temperature/MST Power/Excitation Power)

50 mM sodium phosphate buffer, pH 7.0, 0.025% Pluronic® F-127
25 nM CBM3a | 10 mg/mL – 0.3 µg/mL cellulose nanocrystals | 25°C | low MST power | 60% excitation power

D4. MST Results (Capillary Scan/Time Traces/Dose Response)

$K_{d,app}$ = 0.24 mg/mL



D5. Reference Results/Supporting Results

Quantifying CBM Carbohydrate Interactions Using Microscale Thermophoresis
Wu et al., Methods in Molecular Biology 2017, 1588, 129-141

E. Contributors

Haiyang Wu[3], Cédric Y. Montanier[3], Claire Dumon[3], Pierre Soule[4]

[3] Catalysis and Enzyme Molecular Engineering Team, INSA Toulouse, Toulouse, France
[4] NanoTemper Technologies GmbH, München, Germany | nanotempertech.com