Determination of D-galactofuranose Content of Galactomannoproteins in *Aspergillus nidulans*

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[Abstract] Galactofuranose (Gal*f*) is a component of several polysaccharides and glycoconjugates in certain species of filamentous fungi. Gal*f* residues are frequently found in *Aspergillus* glycoproteins, including *N*-glycans and *O*-mannose glycans that modify many cell wall proteins and extracellular enzymes. It is known that furanoses, contained in oligosaccharides, are detected as pyranoses after hydrolysis, and that D-galactopyranose is not contained in the galactomannoproteins of *Aspergillus* spp. To determine the levels of D-galactofuranose in galactomannoproteins extracted from *Aspergillus nidulans* (*A. nidulans*), we measured the amount of D-galactopyranose production after galactomannoproteins hydrolysis. The method described in this manuscript allows determination of the D-galactofuranose content of galactomannoproteins in *Aspergillus* spp.

Materials and Reagents

1. *Aspergillus nidulans* conidia
2. Trisodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) (Wako Pure Chemical Industries, catalog number: 191-01785)
3. Citric acid ($C_6H_8O_7$) (Wako Pure Chemical Industries, catalog number: 030-05525)
4. Ethyl-4-aminobenzoate ($C_9H_{11}NO_2$) (ABEE) (Tokyo Chemical Industry, catalog number: A0271)
5. Sodium cyanoborohydride ($NaBH_3CN$) (Tokyo Chemical Industry, catalog number: S0396)
6. Chloroform (Wako Pure Chemical Industries, catalog number: 038-02606)
7. Ethanol (Wako Pure Chemical Industries, catalog number: 050-00446)
8. Trifluoroacetic acid (TFA) (Wako Pure Chemical Industries, catalog number: 206-10731)
9. Glacial acetic acid (Wako Pure Chemical Industries, catalog number: 012-00245)
10. Methanol (Wako Pure Chemical Industries, catalog number: 136-09475)
11. D-galactose (Sigma-Aldrich, catalog number: G0750)

12. ABEE labeling mixture (see Recipes)
13. HPLC solvent A (see Recipes)
14. HPLC solvent B (see Recipes)
15. Minimal medium (see Recipes)
16. Hutner's trace elements (see Recipes)

Equipment

1. 50-ml plastic centrifuge tube (*e.g.*, Greiner Bio-One GmbH)
2. 1.5-ml conical screw cap tube and cap
3. Spreader
4. 500-ml Sakaguchi flasks
5. Centrifuge
6. Rotator (*e.g.*, TAITEC)
7. High-performance liquid chromatography (HPLC) system equipped with a fluorescence detector (Hitachi, LaChrom Elite, model: L-2485) and software for HPLC peak analysis (*e.g.*, Hitachi, model: D-2000 Elite HPLC)
8. Centrifugal evaporator (*e.g.*, SpeedVac®)
9. Heat block or water bath
10. GlycoScope Honenpak C18 column (4.6 mm x 75 mm) (COSMO BIO, catalog number: JOM-J715-1PC)
11. Filter paper (Munktell & Filtrak GmbH, catalog number: 113053)
12. Dialysis membrane (BioDesign Inc., catalog number: D102)

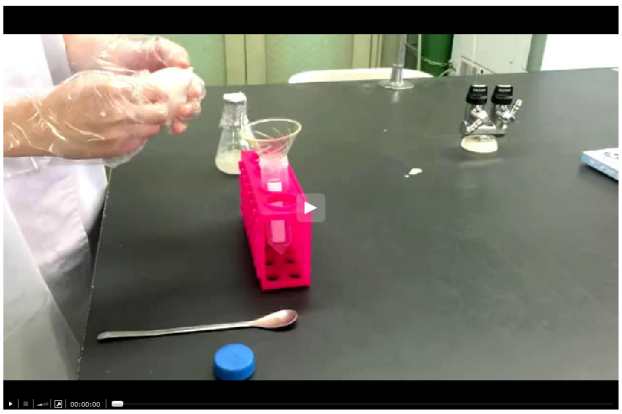
Procedure

1. Streak *Aspergillus nidulans* conidia from frozen stock onto Minimal medium (MM) plate and cultivate for 3 days at 30 °C.
2. Collect the formed conidia with a spreader.
3. Spread *Aspergillus nidulans* conidia (1×10^5) onto MM plates and cultivate for 3 days at 30 °C.
4. Inoculate the collected conidia (2×10^7) into 100 ml of MM in 500-ml Sakaguchi flasks.
5. Shake the flasks at 126 rpm at 30 °C for 24 h.
6. Collect the mycelial cells by paper filtration.
7. Wash the cells twice with approximately 30 ml of distilled water (Video 1).

Video 1. Collection and washing of the mycelial cells

8. Resuspend the cells (approx. 2 g of wet cells) in 10 ml of 100 mM citrate buffer (pH 7.0).
9. Autoclave the sample at 121 °C for 120 min.
10. Remove cell debris by paper filtration (Video 2).

Video 2. Removing cell debris by paper filtration



11. Collect the resultant sample (approx. 10 ml) in a 50-ml plastic centrifuge tube.
12. Add 30 ml of cold 99.5% ethanol and keep the sample on ice for 30 min.
13. Centrifuge at 14,000 *x g* at 4 °C for 10 min.
14. Dry the pellet at room temperature using a centrifugal evaporator at 1,500 *x g*.
15. Resuspend the pellet in 2 ml of distilled H_2O (dH_2O).
16. Dialyze the sample with a dialysis membrane (15.5 mm x 300 mm) against 5 L of dH_2O for 24 h at 4 °C. The resultant sample is designated as the extracted galactomannnoproteins.
17. Incubate part (2.4 µg) of the extracted galactomannoproteins in 500 µl of 4 M trifluoroacetic acid (TFA) at 100 °C for 4 h in a 1.5-ml conical screw cap tube.

18. Dry the resultant sample at room temperature using a centrifugal evaporator at 1,500 *x g*.
19. Resuspend the sample in 10 µl of dH_2O.
20. Label the hydrolysates with fluorescent *p*-aminobenzoic acid ethyl ester (ABEE) using an ABEE labeling mixture.
    a. Add 40 µl of the ABEE labeling mixture (preheating at 70 °C for 5 min) to the sample.
    b. Incubate the sample for 1 h at 80 °C.
    c. Cool the sample to room temperature.
    d. Add 200 µl of dH_2O and 200 µl of chloroform to the sample.
    e. Vigorously vortex the sample and then centrifuge it at 14,000 *x g* for 5 min at room temperature.
    f. Collect the upper aqueous layer.
21. Analyze the ABEE-labeled D-galactopyranose using an HPLC system. Inject 20 µl of the sample into the column at a flow rate of 1.0 ml/min at 45 °C. ABEE-D-galactopyranose can be detected with a fluorescence detector at an excitation wavelength of 360 nm and an emission wavelength of 305 nm.
    Note: Use solvents containing acetonitrile and potassium borate buffer (see the Recipes section for solvents A and B). Set the gradient program at a flow rate of 1.0 ml/min (expressed as a percent of solvent A) and apply the following gradient to analyze the ABEE-D-galactopyranose: 0-45 min, isocratic 100%; 45-50 min, 100-0%; 50-55 min, 0-100%; 55-75 min, isocratic 100%. Use D-galactose as a standard for quantification purposes.

Recipes

1. ABEE labeling mixture
   165 mg ABEE
   35 mg sodium cyanoborohydride
   41 µl glacial acetic acid
   350 µl methanol
   The mixture can be long-term stored at -20 °C
2. HPLC solvent A [0.2 M potassium borate buffer (pH 9.0) containing 6% acetonitrile]
   12.4 g H_3BO_3
   60 ml acetonitrile
   Add water to bring the final solution to 1 L total volume while adjusting pH 9.0 with potassium hydroxide (KOH)
3. HPLC solvent B [0.2 M potassium borate buffer (pH 9.0) containing 50% acetonitrile]
   12.4 g H_3BO_3

500 ml acetonitrile

Add water to bring the final solution to 1 L total volume while adjusting pH 9.0 with potassium hydroxide (KOH)

4. Minimal medium (1 L)

$NaNO_3$	6.0 g
KCl	0.52 g
$MgSO_4 \cdot 7H_2O$	0.52 g
KH_2PO_4	1.52 g
Glucose	10.0 g
Hutner's trace elements	2 ml

Adjust the pH value to 6.8 using NaOH

Add water to bring the final solution to 1 L total volume

Autoclave for 20 min

5. Hutner's trace elements

H_2O (60 ˚C)	100 ml
$ZnSO_4 \cdot 7H_2O$	2.2 g
H_3BO_3	1.1 g
$MnCl_2 \cdot 4H_2O$	0.5 g
$FeSO_4 \cdot 7H_2O$	0.5 g
$CoCl_2 \cdot 6H_2O$	0.16 g
$CuSO_4 \cdot 5H_2O$	0.16 g
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.11 g
EDTA	5.0 g

Adjust the pH value to 6.5-6.8 using KOH

Acknowledgments

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