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)

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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 x 10^5) onto MM plates and cultivate for 3 days at 30 °C.
4. Inoculate the collected conidia (2 x 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).

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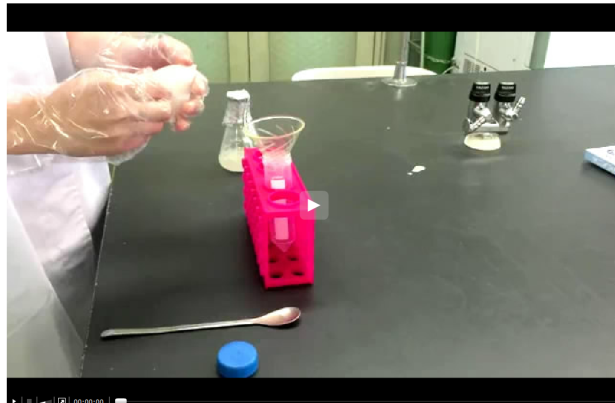
Video 1. Collection and washing of the mycelial cells

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.

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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

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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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References

1. Komachi, Y., Hatakeyama, S., Motomatsu, H., Futagami, T., Kizjakina, K., Sobrado, P., Ekino, K., Takegawa, K., Goto, M., Nomura, Y. and Oka, T. (2013). *GfsA* encodes a novel galactofuranosyltransferase involved in biosynthesis of galactofuranose antigen of

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O-glycan in *Aspergillus nidulans* and *Aspergillus fumigatus*. *Mol Microbiol* 90(5): 1054-1073.

2. Yasuno, S., Murata, T., Kokubo, K., Yamaguchi, T. and Kamei, M. (1997). Two-mode Analysis by High-performance Liquid Chromatography of ρ-Aminobenzoic Ethyl Ester-derivatized Monosaccharides. *Biosci Biotec Biochem* 61(11): 1944-1946.

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