Purification of the GfsA-3x FLAG Protein Expressed in *Aspergillus nidulans*

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[Abstract] GfsA is a fungal β-galactofuranosyltransferase involved in the biosynthesis of *O*-glycan. To investigate the enzymatic functions of GfsA, we attempted to obtain a recombinant protein of this enzyme from two heterologous host organisms. However, GfsA could not be expressed as a recombinant protein in either *Escherichia coli* (*E. coli*) or *Saccharomyces cerevisiae* (*S. cerevisiae*). Therefore, we decided to employ *Aspergillus nidulans* (*A. nidulans*) as the host organism, and produced a strain that expressed 3x FLAG-tagged GfsA using chromosomal tagging. To confirm its expression, a solubilized protein was prepared from the tagged strain and analyzed with an anti-FLAG antibody. The strain that expressed 3x FLAG-tagged GfsA produced a functional protein with a mass of approximately 67 kDa. The method described in this manuscript allows purification of the GfsA-3xFLAG protein as expressed in *A. nidulans* cells.

Materials and Reagents

1. *Aspergillus nidulans* expressing 3x FLAG-tagged GfsA (Komachi *et al.*, 2013)
2. 3x FLAG-peptide (Sigma-Aldrich, catalog number: F4799)
3. ANTI-FLAG M2-agarose produced from mouse (Sigma-Aldrich, catalog number: A2220)
4. Mouse IgG-agarose (Sigma-Aldrich, catalog number: A0919)
5. 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) (Dojindo Molecular Technologies, catalog number: GB10)
6. Sodium hydroxide (NaOH) (Wako Pure Chemical Industries, catalog number: 198-13765)
7. Sodium chloride (NaCl) (Wako Pure Chemical Industries, catalog number: 191-01665)
8. Potassium chloride (KCl) (Wako Pure Chemical Industries, catalog number: 163-03545)
9. Manganese (II) chloride tetrahydrate ($MnCl_2$) (Wako Pure Chemical Industries, catalog number: 133-00725)
10. Glycerol (Wako Pure Chemical Industries, catalog number: 075-00611)
11. 3-[(3-Cholamidopropyl) dimethylammonio]-2-hydroxypropanesulfonate (CHAPSO) (Dojindo Molecular Technologies, catalog number: C020)

12. Complete™ protease inhibitor cocktail tablets (EDTA-free) (Roche Diagnostics, catalog number: 1873580)
13. Liquid nitrogen
14. Buffer A (see Recipes)
15. Minimal medium (MM) (see Recipes)
16. Hutner's trace elements (see Recipes)

Equipment

1. Spreader
2. 500-ml Sakaguchi flasks
3. Mortar and pestle
4. Aspirator
5. Centrifuge with an angle rotor
6. Centrifuge with a swing rotor
7. Ultracentrifuge
8. Spatula
9. 15-ml plastic centrifuge tube (*e.g.*, Greiner Bio-One GmbH)
10. 4 °C incubator
11. 30 °C incubator
12. Rotator (*e.g.*, TAITEC)
13. Filter paper (Munktell & Filtrak GmbH, catalog number: 113053)

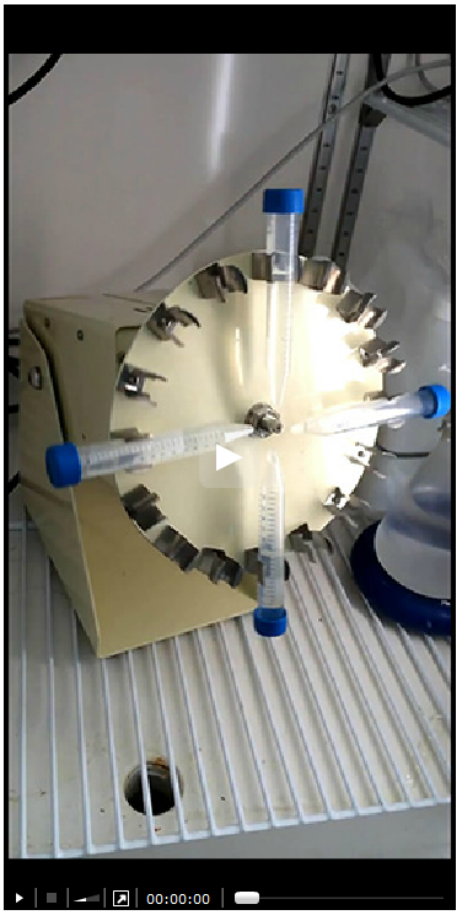
Procedure

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.
   Note: Cells can easily be crushed by wringing wet cells out to dry using a scoopula after this step as much as possible.

8. Grind cells (25 g of wet cells) into a fine powder in liquid nitrogen using a mortar and pestle.
9. Resuspend the lysed cells in 100 ml of buffer A containing Complete™ protease inhibitor cocktail (EDTA-free).
10. Remove cell debris by centrifugation with an angle rotor at 10,000 *x g* for 10 min.
11. Centrifuge the supernatant at 100,000 *x g* for 45 min using an ultracentrifuge.
12. Resuspend the resultant pellet in 10 ml of buffer A containing 0.5% CHAPSO using a spatula.

    Note: Pilot experiments are needed to determine the suitable conditions under which the detergents solubilize the target protein.
13. Gently mix the sample for 1 h using a rotator to obtain solubilized membrane proteins.
14. Centrifuge the sample at 100,000 *x g* for 30 min using an ultracentrifuge.
15. Collect the supernatant (approximately 10 ml) into a 15-ml plastic centrifuge tube.
16. Add mouse-IgG-agarose (100 µl) to the supernatant and gently shake the mixture for 1 h (Video 1).

Video 1. Shaking the mixture using a rotator



17. Remove the mouse-IgG-agarose by centrifugation with a swing rotor at 1,400 *x g* for 10 min.
18. Add 200 µl anti-FLAG M2 affinity gel to the supernatant and gently shake the resultant mixture for 1 h.
19. Collect the Anti-FLAG M2 affinity gel by centrifugation with a swing rotor at 1,400 *x g* for 10 min.
20. Gently remove the supernatant with an aspirator.
21. Resuspend the resultant agarose with 15 ml of buffer A containing 0.1% CHAPSO.
22. Repeat steps 18-20 five times.
23. Elute GfsA protein with 20 µl of buffer A with 0.1% CHAPSO containing 0.5 µg/µl 3x FLAG peptide.

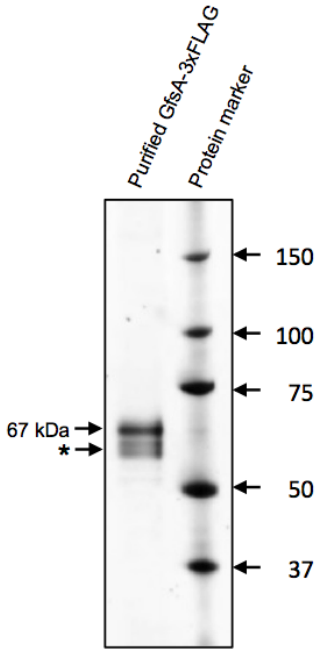


Figure 1. Purification of GfsA-3xFLAG protein. A total of 0.5 mg (silver staining) proteins were separated by 5%-20% SDS-PAGE, and were then assayed by silver staining. GfsA-3xFLAG was detected as a 67 kDa protein. Asterisk indicates a degradation product or insufficiently *N*-glycosylated product of GfsA-3xFLAG.

Notes

1. Perform all manipulations on ice or at 4 °C.

Recipes

1. Buffer A (1 L)

Compounds	Amounts
HEPES	11.9 g
NaCl	5.84 g
KCl	2.24 g
$MnCl_2 \cdot 4H_2O$	0.2 g
Glycerol	50 g

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

Filter sterilize the solution using a 0.45 μm filter

Stored at 4 °C

2. Minimal medium (1 L)

Compounds	Amounts
$NaNO_3$	6.0 g
KCl	0.52 g
$MgSO_4 \cdot 7H_20$	0.52 g
KH_2PO_4	1.52 g
Glucose	10.0 g
Hutner's trace elements	2 ml

Adjust pH to 6.8 using NaOH

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

Autoclave for 20 min

3. Hutner's trace elements

Compounds	Amounts
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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