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 Ο-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 (MnCl₂) (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)

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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 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.

    *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 \( \times \) g for 10 min.

11. Centrifuge the supernatant at 100,000 \( \times \) 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 \( \times \) 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)**
   
<table>
<thead>
<tr>
<th>Compounds</th>
<th>Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES</td>
<td>11.9 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.84 g</td>
</tr>
<tr>
<td>KCl</td>
<td>2.24 g</td>
</tr>
<tr>
<td>MnCl$_2$·4H$_2$O</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>50 g</td>
</tr>
</tbody>
</table>

   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)**
   
<table>
<thead>
<tr>
<th>Compounds</th>
<th>Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO$_3$</td>
<td>6.0 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.52 g</td>
</tr>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td>0.52 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>1.52 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Hutner's trace elements</td>
<td>2 ml</td>
</tr>
</tbody>
</table>

   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**
   
<table>
<thead>
<tr>
<th>Compounds</th>
<th>Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O (60 °C)</td>
<td>100 ml</td>
</tr>
<tr>
<td>ZnSO$_4$·7H$_2$O</td>
<td>2.2 g</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>1.1 g</td>
</tr>
<tr>
<td>MnCl$_2$·4H$_2$O</td>
<td>0.5 g</td>
</tr>
<tr>
<td>FeSO$_4$·7H$_2$O</td>
<td>0.5 g</td>
</tr>
<tr>
<td>CoCl$_2$·6H$_2$O</td>
<td>0.16 g</td>
</tr>
<tr>
<td>CuSO$_4$·5H$_2$O</td>
<td>0.16 g</td>
</tr>
<tr>
<td>(NH$_4$)$_6$Mo$<em>7$O$</em>{24}$·4H$_2$O</td>
<td>0.11 g</td>
</tr>
<tr>
<td>EDTA</td>
<td>5.0 g</td>
</tr>
</tbody>
</table>

   Adjust the pH value to 6.5-6.8 using KOH.
Acknowledgments

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References