Enzymatic Deglycosylation of a Protein Containing Core 1 *O*-glycans with *O*-Glycosidase

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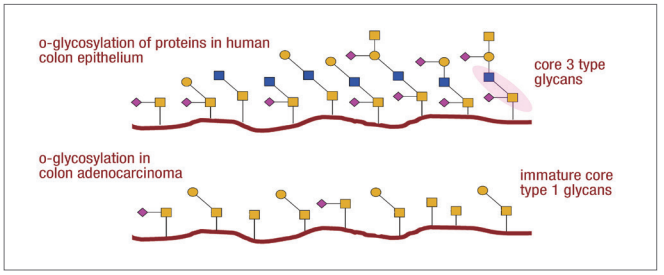
For many cancers, such as colon, ovary, uterus and bladder (mucosas), tumor progression and its poor prognosis strongly correlates with alterations in the patterns of mucin *O*-glycosylation. For instance, β3Gn-T6 (the enzyme responsible for Core 3 *O*-glycan synthesis), is abundant in normal colon tissue while its expression is strongly downregulated in adenocarcinoma (1,2,3). As a result, mucin glycosylation switches from common Core 3 *O*-glycan structures to short Core 1 structures. These Core 1 structures, T and Tn, are hallmark epitopes of cell malignancy (4).

The study of glycan aberrations in cancer opens new avenues in the development of novel therapies. In this regard, the endo-*O*-glycosidase is proving useful to characterize and quantitate a variety of commonly found *O*-glycan forms.

This application note describes the use of a recombinant *O*-Glycosidase that can cleave core 1 *O*-linked disaccharides (5) and immature Tn core (GalNAc). Conditions have been determined that allow this *O*-Glycosidase to be used under the same denaturing conditions used for PNGase F digestion.



Materials

O-Glycosidase & Neuraminidase Bundle (NEB #E0540)

Fetuin (NEB #P6042)

Core 1 disaccharide (Galb1,3GalNAc; Accurate Chemical #BCR20/06)

General Protocol

1. Set up the reaction in 200 µl tubes as follows:

COMPONENTS	DENATURING CONDITIONS	NATIVE CONDITIONS
Fetuin (10 mg/ml) in water	18 µl	18 µl
10X Denaturing Buffer	2 µl	2 µl
Incubate 95°C for 5 minutes. Cool on ice, spin		
10X NP-40	4 µl	–
10X GlycoBuffer 2	4 µl	4 µl
Milli-Q Water	18 µl	24 µl
Neuraminidase	2 µl	2 µl
O-Glycosidase	2 µl	2 µl

2. Incubate at 37°C for 1 to 4 hours. After incubation, add 1 µl of 4 M KCl followed by 150 µl of methanol. Chill overnight at 4°C to precipitate proteins. After the overnight precipitation, spin the sample at 14,000 rpm for 30 minutes, and reserve the supernatant.

3. Concentrate supernatant to dryness with a Speed Vac set at medium heat (Savant; equipped with a high vacuum pump and finger trap immersed in a Dewar containing isopropanol and dry ice). Reconstitute with 400 µl Milli-Q® water.

4. De-ionize the sample from step 4 by gently rocking in 200 µl of prepared mixed bed ion exchange resin AG 501-X8 (Bio-Rad; #142-6424) for 5 minutes. Collect the supernatant with a 1 ml syringe using a 23 gauge needle. **Note:** before use, the resin must be converted to the acetate form by soaking in an equal volume of 1 M acetic acid followed by washing ten times with equal volumes of water.

5. Remove the needle and load the entire sample (400 µl) from Step 5 to an activated Sep-Pak® cartridge (Waters; #WAT051910). Collect the entire flow through (400 µl). Wash the Sep-Pak 2 times with 400 µl of Milli-Q water and pool the washes with the flow through. Concentrate to 70 µl using a Speed Vac. **Note:** before use, the Sep-Paks are activated by washing two times with 400 µl methanol followed by 4 times with 400 µl Milli-Q water.

6. Detect free sugars by HPAEC-PAD Chromatography using the following conditions:

Column: CarboPac 20 with Amino Guard.

Elution: 40 mM NaOH isocratic for 12 minutes, 150 mM regeneration for 10 minutes, flow rate: 0.5 µl/min.

Detection: Pulse electrochemical, Au electrode, quadruple potential.

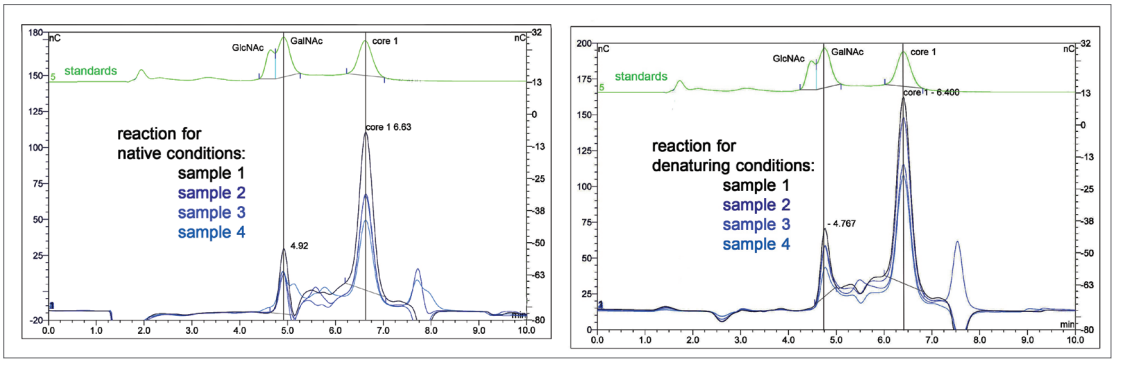
Injection sample: 30 µl, with or without internal Galβ1,3GalNAc standard (30 nanograms).

References:

1. Iwai, T. et al., (2005) *Proceedings of the National Academy of Sciences U.S.A.* 102, 4572-4577.
2. Iwai, T. et al., (2002) *Journal of Biological Chemistry* 277, 12808-12809.
3. Capon, C. et al., (2001) *Biochemical Journal* 358, 657-664.
4. Springer, G.F. et al., (1997) *Journal of Molecular Medicine* 75, 594-602.
5. Koutsioulis, D. et al., (2008) *Glycobiology* 18, 799-805.

Results

Superimposed chromatograms of released sugars with decreasing amounts of enzyme shown in table. The chromatograms represent a 5-fold increase of all materials in Step 2.



		O-GLYCOSIDASE ON NATIVE FETUIN		*O*-GLYCOSIDASE ON DENATURED FETUIN	
Sample #	units	GalNAc Peak Area nC*min	Core 1 Peak Area nC*min	GalNAc Peak Area nC*min	Core 1 Peak Area nC*min
1	200,000	9.55	36.92	9.84	43.29
2	10,000	6.23	21.85	8.02	39.91
3	50,000	6.74	23.01	8.07	27.12
4	12,500	4.51	18.08	5.09	25.11

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