

O-Glycosidase

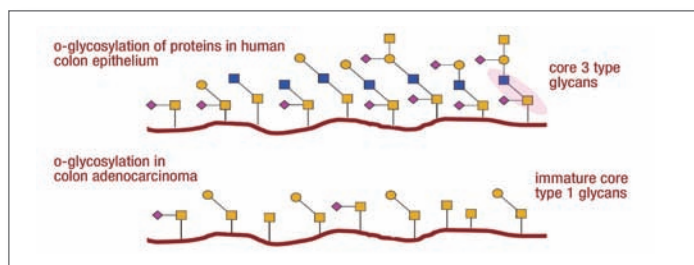
Application Note 1

Enzymatic deglycosylation of a protein containing Core 1 O-glycans. O-Glycosidase (Endo- α -N-Acetylglactosaminidase) removes O-glycans from Bovine fetuin.

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, β 3Gn-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 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.



General Protocol

1. Preparation of substrate: Dissolve 10 mg of Fetuin in 1 ml water.

A) Denaturing Reaction Conditions:

Denaturation Reaction:

Fetuin (10 mg/ml) in water	18 μ l
10X Denaturing Buffer	2 μ l
Heat 95°C for 5 min.; Chill on ice and spin.	

Digestion of substrate:

Denaturing Reaction	20 μ l
10X NP-40	4 μ l
10X G7 Buffer	4 μ l
Milli-Q Water	18 μ l
Neuraminidase (50 u/ μ l)	2 μ l
O-Glycosidase (40,000 u/ μ l)	2 μ l

2. B) Non-Denaturing Reaction Conditions:

Fetuin (10 mg/ml) in water	18 μ l
Digestion of substrate:	
10X G7 Buffer	4 μ l
Milli-Q Water	24 μ l
Neuraminidase (50 u/ μ l)	2 μ l
O-Glycosidase (40,000 u/ μ l)	2 μ l

DNA CLONING

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Materials

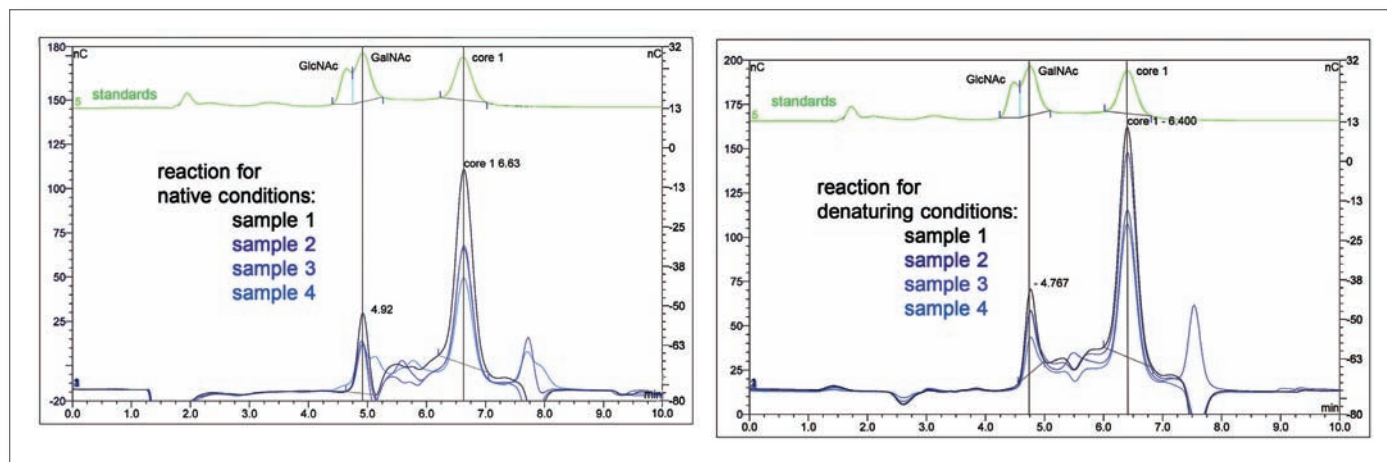
- O-Glycosidase (NEB #P0733)
- Fetuin (Sigma #F2379)
- G7 buffer (NEB #B0701S)
- Denaturing buffer (NEB #B0701S)
- 10X NP-40 (NEB #B0701S)
- Neuraminidase (NEB #P0720S)
- Core 1 disaccharide (Gal β 1,3GalNAc; Accurate Chemical #BCR20/06)

(see other side)

- Incubate at 37°C for 1 to 4 hours. After incubation, add 1 µl of 4M 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.
- 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.
- 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.
- 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.
- 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

- Iwai, T. et al., (2005) *Proceedings of the National Academy of Sciences USA*. 102, 4572-7.
- Iwai, T. et al., (2002) *Journal of Biological Chemistry* 277, 12808-9.
- Capon, C. et al., (2001) *Biochemical Journal* 358, 657-64.
- Springer, G.F. et al., (1997) *Journal of Molecular Medicine* 75, 594-602.
- Koutsoulis, D. et al., (2008) *Glycobiology* 18, 799-805.



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.

Sample #	units	O-GLYCOSIDASE ON NATIVE FETUIN		O-GLYCOSIDASE ON DENATURED FETUIN	
		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

O-Glycosidase

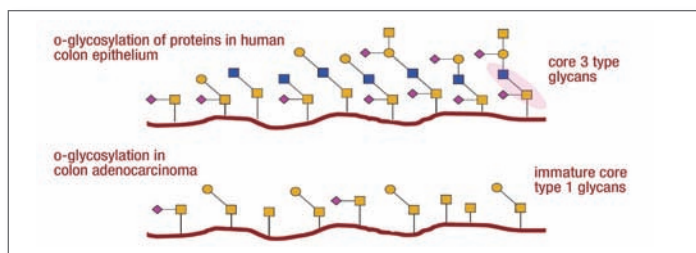
Application Note 2

Enzymatic deglycosylation of a protein containing Core 3 O-glycans. O-Glycosidase (Endo- α -N-Acetylgalactosaminidase) removes O-glycans from Bovine Mucin (submaxillary gland).

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, β 3Gn-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 describes the use of a recombinant O-Glycosidase that can cleave core 3 O-linked disaccharides (5). Conditions have been determined that allow this O-Glycosidase to be used under the same denaturing conditions used for PNGase F digestion.



General Protocol

1. Preparation of substrate: Dissolve 10 mg of Bovine Mucin in 1 ml water.

2. A) Denaturing Reaction Conditions: Denaturation Reaction:

Bovine Mucin (5 mg/ml) in water	18 μ l
10X Denaturing Buffer	2 μ l
Heat 95°C for 5 min.; Chill on ice and spin.	

Digestion of substrate:

Denaturing Reaction	20 μ l
10X NP-40	4 μ l
10X G7 Buffer	4 μ l
Milli-Q Water	18 μ l
Neuraminidase (50 u/ μ l)	2 μ l
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2. B) Non-Denaturing Reaction Conditions:

Bovine Mucin (5 mg/ml) in water	18 μ l
Digestion of substrate:	
10X G7 Buffer	4 μ l
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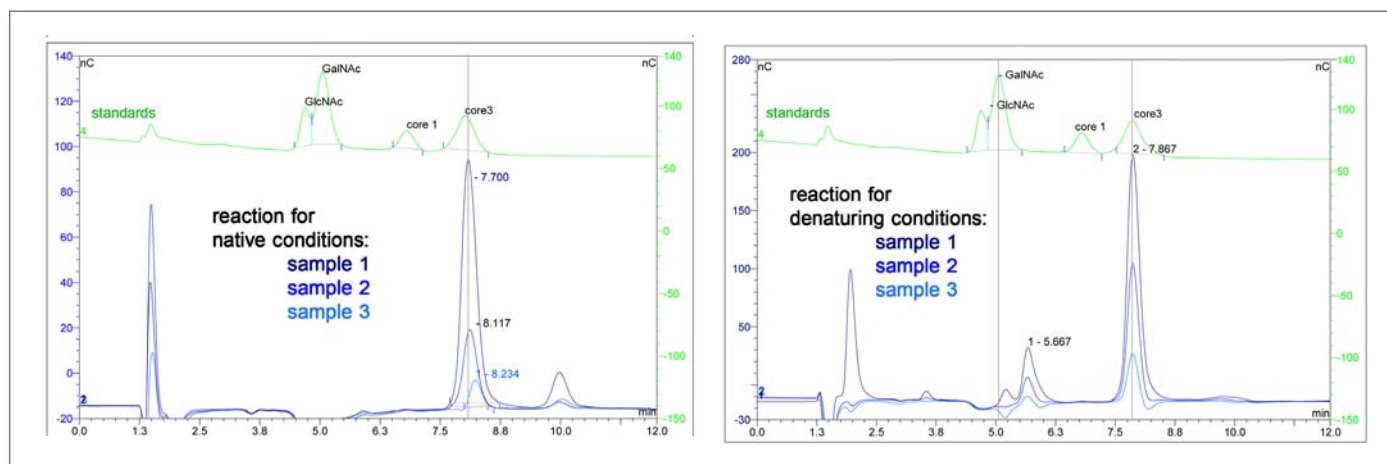
- O-Glycosidase (NEB #P0733)
- Bovine Mucin (Submaxillary gland; Calbiochem #499643)
- G7 buffer (NEB #B0701S)
- Denaturing buffer (NEB #B0701S)
- 10X NP-40 (NEB #B0701S)
- Neuraminidase (NEB #P0720S)
- Core 3 disaccharide (GlcNAc β 1,3GalNAc; Accurate Chemical #BCR20/06).

(see other side)

- Incubate at 37°C for 1 to 4 hours. After incubation, add 1µl of 4M 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.
- 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.
- 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 1ml 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.
- 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 were activated by washing two times with 400 µl methanol followed by 4 times with 400 µl Milli-Q water.
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Column: CarboPac 20 with Amino Guard.
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Detection: Pulse electrochemical, Au electrode, quadruple potential.
Injection sample: 30 µl, with or without internal Galβ1,3GalNAc standard (30 nanograms).

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- Springer G. F., et al., (1997) *Journal of Molecular Medicine* 75, 594-602.
- Koutsoulis D., et al. , (2008) *Glycobiology* 18, 799-805
- Chai W., et al., (2005) *European Journal of Biochemistry* 203, 257-268.



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.

Sample #	units	O-GLYCOSIDASE ON NATIVE FETUIN (SUBMAXILLARY GLAND)		O-GLYCOSIDASE ON DENATURED FETUIN (SUBMAXILLARY GLAND)	
		Unknown Peak Area nC*min	Core 3 Peak Area nC*min	Unknown Peak Area nC*min	Core 3 Peak Area nC*min
1	200,000	0	39.37	13.63	65.06
2	100,000	0	11.63	5.76	33.46
3	12,500	0	2.96	2.48	10.31

Unknown peak is suspected to be core 7 (6). Further analysis will be done.