

Immobilized *Vibrio cholerae* Sialidase - A practical tool for glycoprotein processing and analysis

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Abstract

Glycans present on proteins can have a profound effect on the protein structure and on biological function. Therefore, the study of glycans is important for protein characterization, whether it be for the investigation of structure, function or as a monitoring and quality control in glycoprotein production. The modification of carbohydrate structures on a protein is a useful strategy to modulate and design its biological function. Enzymes as glycosidases and glycosyl transferases are important tools for both purposes, glycan analysis and processing.

Sialidases are frequently used to investigate the biological role of sialic acid recognition as well as in the analysis of glycoconjugates. However, with this approach several problems remain related to the presence of the enzyme in subsequent steps. To facilitate a broad application of this enzyme in glycoconjugate analysis and engineering the potential of immobilized sialidase was investigated.

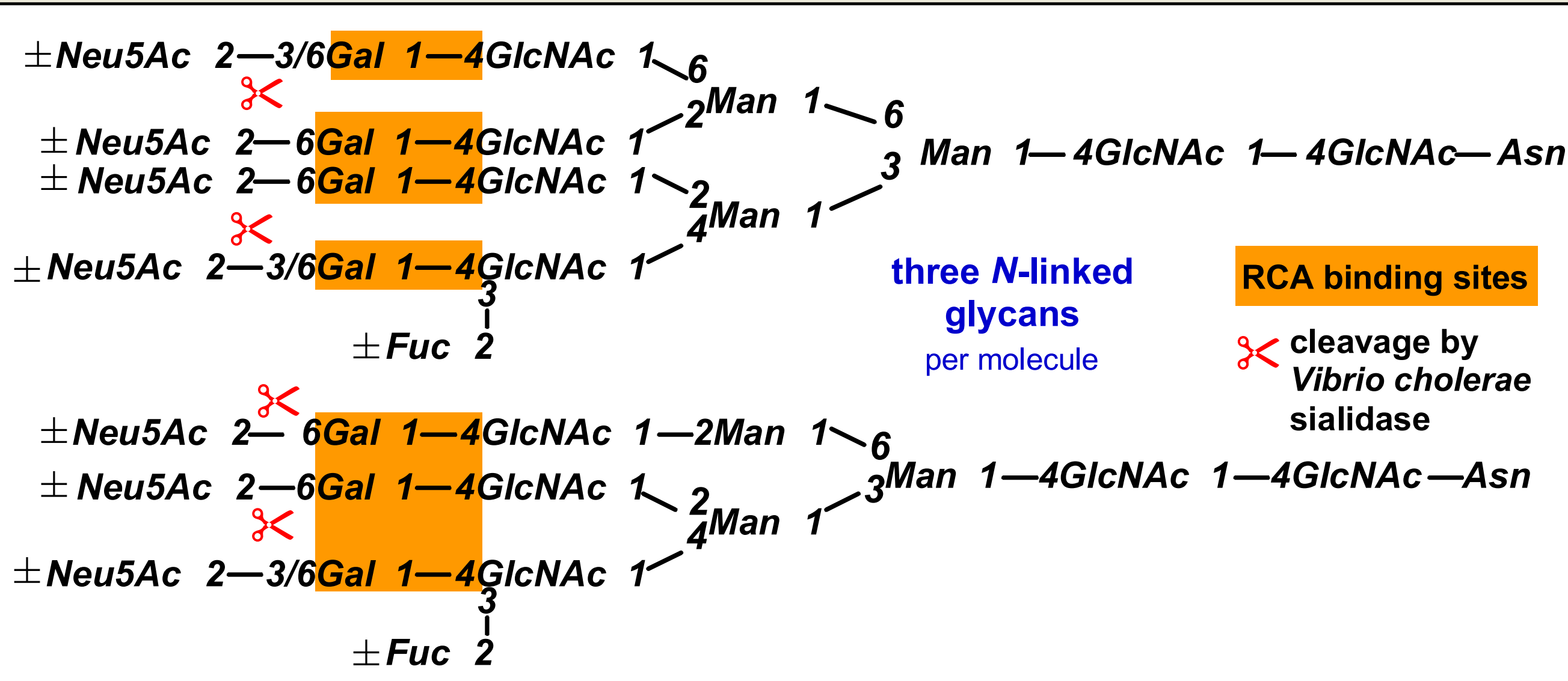
We describe new developments and applications using immobilized *Vibrio cholerae* sialidase, an enzyme cleaving 2-3, 2-6 and 2-8 glycosidic bond of terminal *N*-acetylneuraminic acid in oligosaccharides, polysaccharides, glycoproteins (Corfield 1983) and glycolipids (GlycoCleave[®]; GALAB, Geesthacht). This technique has several advantages compared to the use of soluble enzyme, like easy isolation of the processed glycoprotein after the reaction, the reusability of the valuable enzyme itself and the convenient handling with columns or cartridges. Different approaches in method development have been followed. Results will be shown for the desialylation of model glycoproteins as fetuin and acid-1()-glycoprotein under different conditions. The enzymatically treated products were analysed by SDS PAGE and IEF, sialic acid quantification and high-performance lectin affinity chromatography. The results demonstrate the suitability of the immobilized sialidase for liberation of sialic acids from model glycoproteins. Perspectives for further areas of application will be given.

GlycoCleave[®]; GALAB

The product is a suspension of immobilized sialidase (Sialidase, E.C. 3.2.1.18, from *Vibrio cholerae* culture filtrates). The enzyme is covalently linked to hydrophilic, synthetic polymer particles (particle diameter 65 nm) at a concentration of 1 U/mL. The macroporous material (pore diameters of 100nm) is ideally accessible for proteins. 1 U (unit) liberates 1 mol *N*-acetylneuraminic acid from human acid α -glycoprotein incubated for 1 min at 37°C and pH 5,5 in 50mM sodium acetate buffer containing 1 mM calcium chloride.

II. Monitoring desialylation of bovine α -glycoprotein

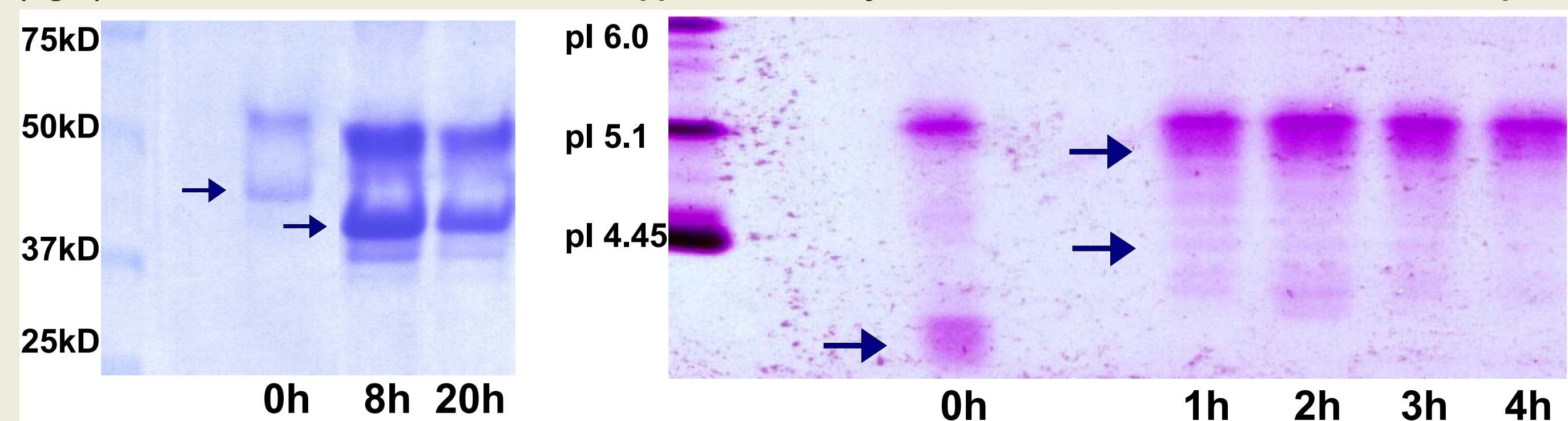
Bovine α -glycoprotein (AGP) is a highly sialylated serum glycoprotein synthesized in the liver. It consists of multiple glycoforms as a result of having three *N*-glycosylation sites occupied by different glycan structures (bi-, tri and tetraantennary glycans). The branching and the number of terminal sialic acid (Neu5Ac) changes (Choe 2000). Cleavage of the terminal Neu5Ac exposes Gal 1-4GlcNAc moieties.



Procedure of α -glycoprotein treatment with GlycoCleave[®]

200 μ L GlycoCleave[®] were transferred to a micro dialysis cartridge (thus liberated sialic acid is continuously exchanged against a buffer reservoir through the dialysis membrane). 1 mg AGP dissolved in 200 μ L 50mM NaAc pH 5.5 / 9 mM CaCl₂ / 154 mM NaCl was added and incubated at 37°C. Protein was separated from GlycoCleave[®] by filtration through a fritted spin column; samples were dialysed against bidest water and lyophilized.

Figure 4 : Desialylation of bovine AGP analyzed by isoelectric focussing and SDS-PAGE
The removal of terminal sialic acids from AGP causes the decrease of molecular weight (left) or a shift in pl (right) of the lower band of AGP. Thus disappears after GlycoCleave[®] treatment and shifted to a basic pl.



α -glycoprotein (AGP) was treated as described above for the times indicated / Coomassie stained SDS-PAGE (left) and IEF gel (right).

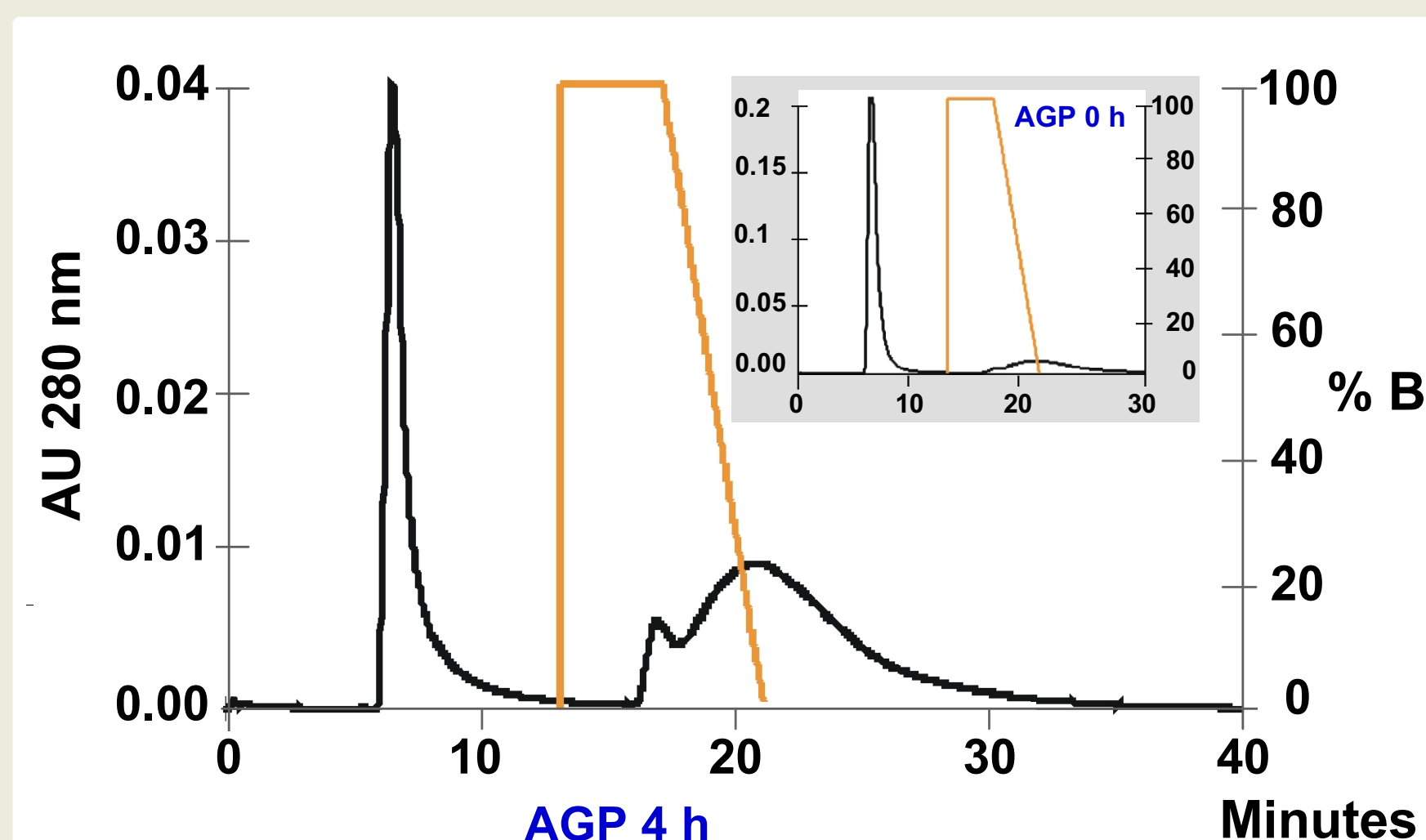
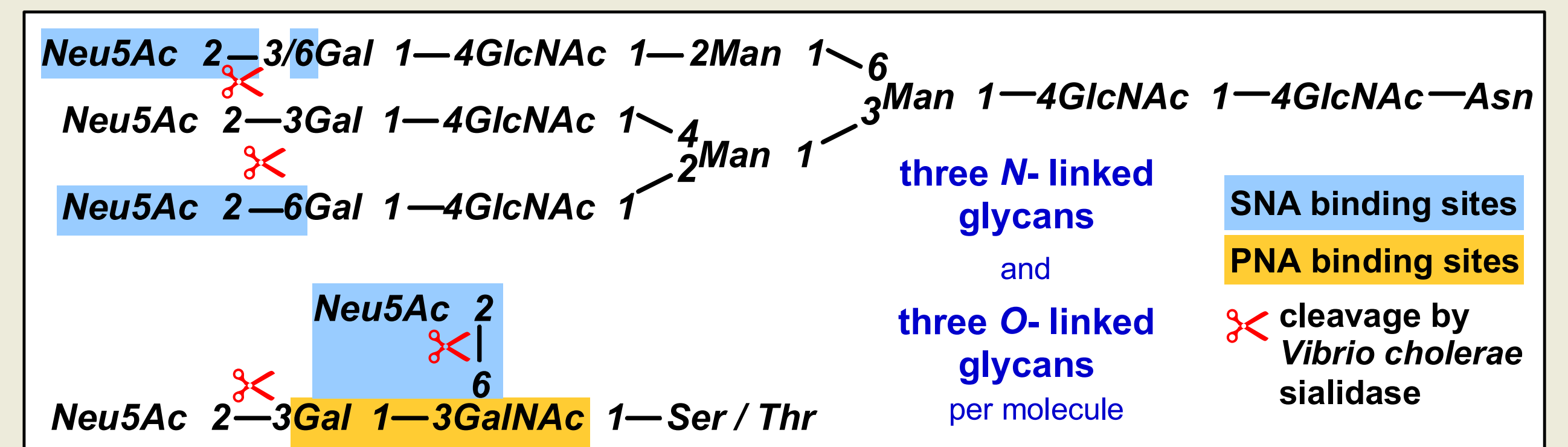


Figure 5 : Affinity separation of AGP on an AffiSep[®]-RCA column.
AGP was treated as described above for the times indicated.

The successful cleavage of Neu5Ac after 4 h treatment can be seen through the binding to RCA to terminal galactose residues.

I. Monitoring desialylation of bovine fetuin

For the determination of the immobilized *Vibrio cholerae* sialidase (GlycoCleave[®]) activity we used bovine fetuin as a ligand, because fetuin is a globular glycoprotein containing three *O*-linked and three *N*-linked glycans with a total carbohydrate content of 22,6% (Green1988). The terminal sugar of each glycan is *N*-acetyl neuraminic acid (sialic acid / Neu5Ac), which is mainly 2-6 linked (Cointe 1998). For complex *N*-glycans shown below cleavage of the terminal Neu5Ac results in terminal Gal 1-4GlcNAc (LacNAc) moieties while desialylation of *O*-glycans exposes the T-antigen Gal 1-3GalNAc (Green 1988).



Procedure of fetuin treatment with GlycoCleave[®]

1mg fetuin was treated with 200 μ L (200mU) GlycoCleave[®] in 2 ml 50mM NaAc pH 5.5 / 9 mM CaCl₂ / 154 mM NaCl for 240 min total and aliquots at different time points were taken. Free sialic acids are separated from fetuin by acetone precipitation. The enzymatic activity of GlycoCleave[®] is determined by measuring the protein bound sialic acid before / after treatment by the following approaches:

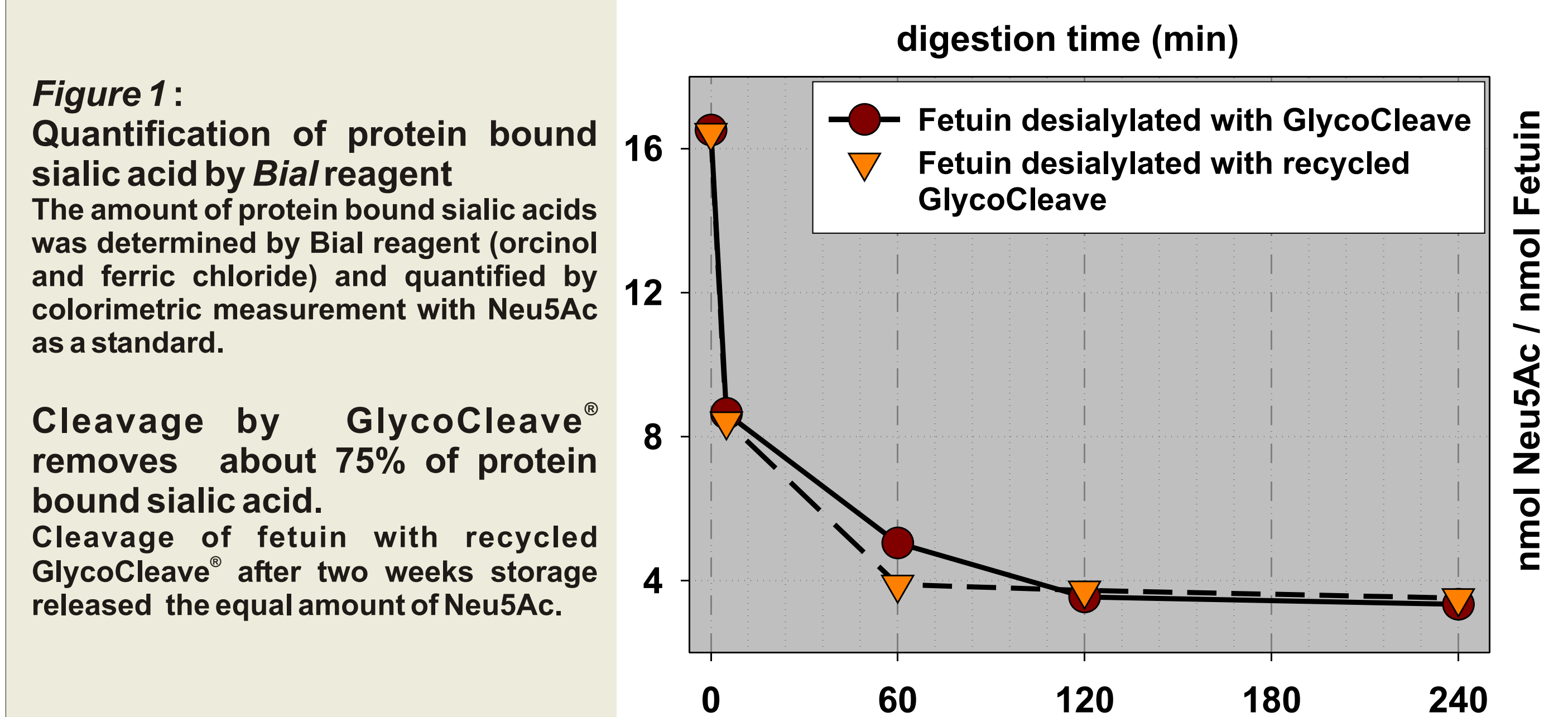


Figure 1 :
Quantification of protein bound sialic acid by Bial reagent
The amount of protein bound sialic acids was determined by Bial reagent (orcinol and ferric chloride) and quantified by colorimetric measurement with Neu5Ac as a standard.

Cleavage by GlycoCleave[®] removes about 75% of protein bound sialic acid.
Cleavage of fetuin with recycled GlycoCleave[®] after two weeks storage released the equal amount of Neu5Ac.

Figure 2 : Progress of fetuin desialylation monitored by SDS-PAGE
The removal of *N*-acetyl-neuraminic acid results in increased mobility.

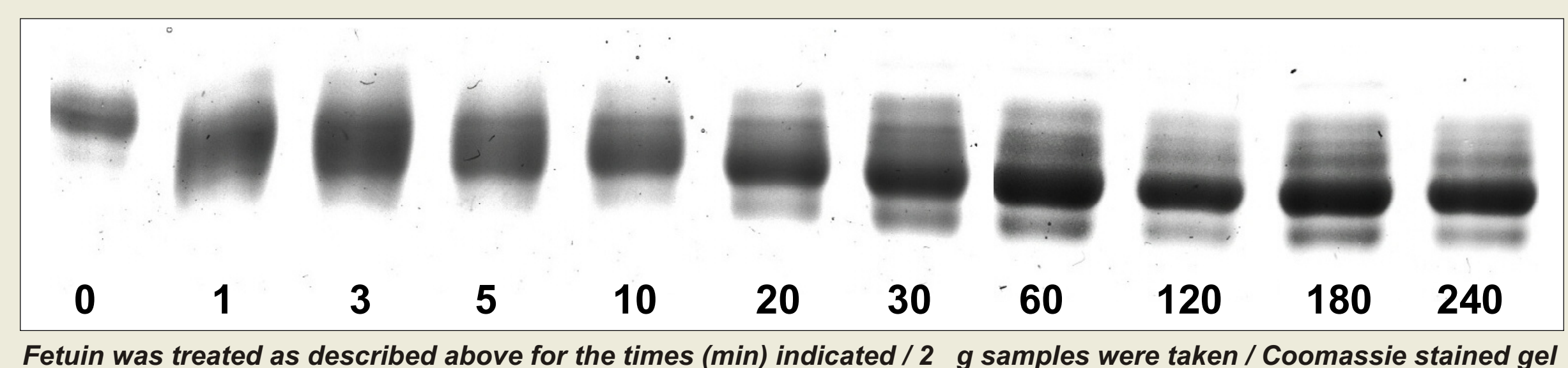
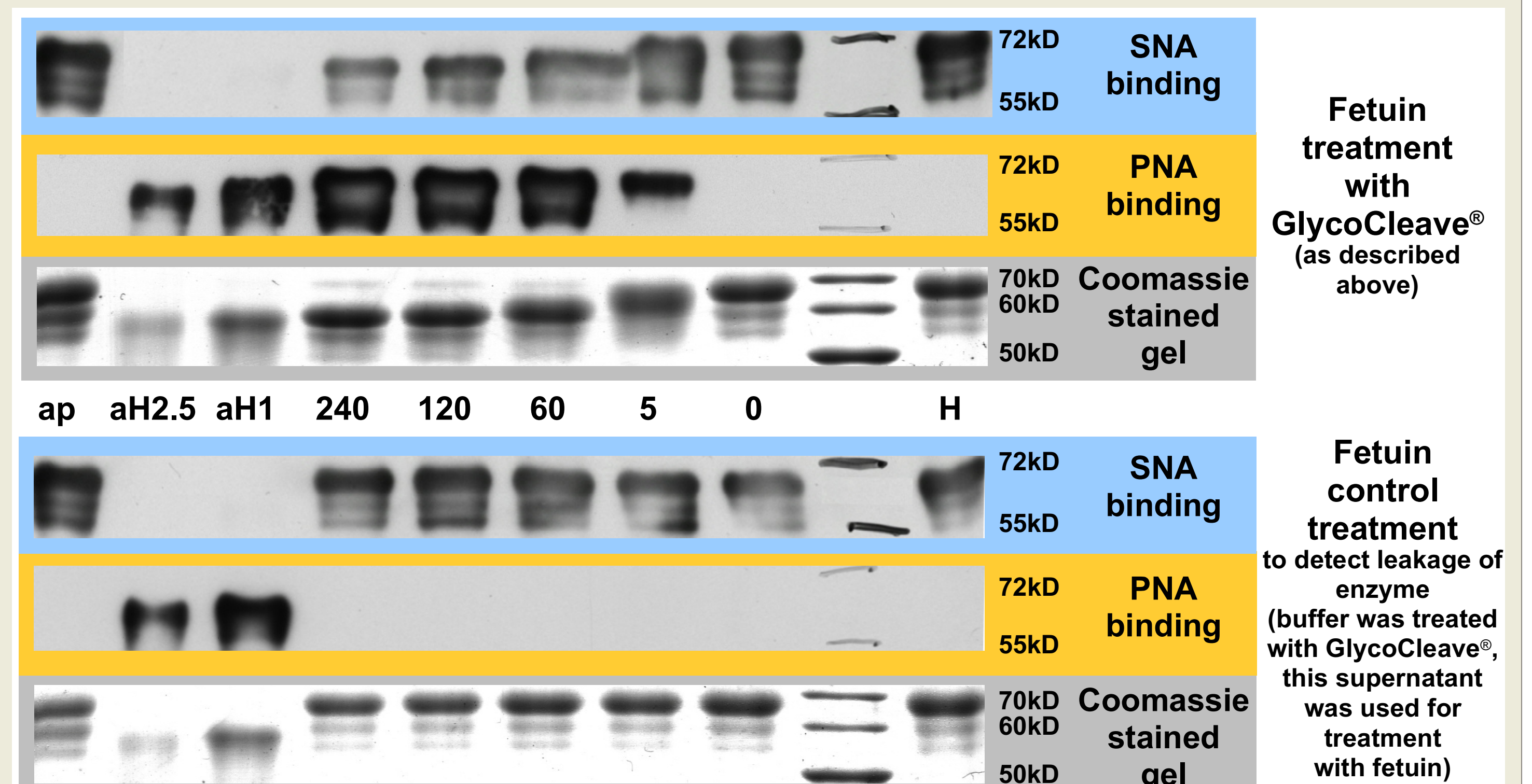


Figure 3: sialic acid determination of fetuin by lectin blotting with PNA and SNA



2 μ g samples were taken : ap acetone precipitated fetuin control / H hydrolysis control without HCl / 240 240 min treatment / 0 untreated / aH2.5 acidic hydrolysis 2.5h 70°C (as a control for completely desialylated fetuin)

SNA binding decreased with the time of GlycoCleave[®] treatment, but is not completely abolished like in the acidic hydrolysed control. PNA does not bind to completely sialylated, untreated fetuin. Already after 5 min treatment with GlycoCleave[®] T-antigen moieties of fetuin were exposed and were recognized by PNA. There seems to be no leakage of immobilized enzyme in GlycoCleave[®] since desialylation was not observed in the control treatment of fetuin clearly visible in the PNA blot.

Conclusions and perspectives

- GlycoCleave[®] is convenient to desialylate glycoproteins.
- GlycoCleave[®], immobilized VC sialidase is as efficient as the soluble enzyme.
- The main desialylation process was already reached after 60 min.
- Recycling of GlycoCleave[®] is easy (washing) and it can be reused.
- Leakage of the immobilized enzyme was not detectable.

References

- Choe, J. et al (2000). Separation of alpha-acid glycoprotein glycoforms using affinity-based reversed micellar extraction and separation. Biotechnol. Bioeng. 70, 484-490
Cointe, D., Leroy, Y. and Chirat, F. (1998) Determination of the sialylation level and of the ratio alpha(2-3)/alpha(2-6) sialyl linkages of N-glycans by methylation and GC/MS analysis. Carbohydr. Res. 311, 51-59
Corfield, A.P., Higa, H., Paulson, J.C. and Schauer, R. (1983) The specificity of viral and bacterial sialidases for alpha(2-3)- and alpha(2-6)-linked sialic acids in glycoproteins. Biochim. Biophys. Acta 744, 121-126
Green, E.D. et al. (1988) The asparagine-linked oligosaccharides on bovine fetuin. J. Biol. Chem. 263, 18253-18268