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A biotechnological tool for glycoprotein desialylation based on immobilized neuraminidase from *Clostridium perfringens*

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ABSTRACT

Background: Sialic acids are widely distributed in nature and have biological relevance owing to their varied structural and functional roles. Immobilized neuraminidase can selectively remove terminal N-acetyl neuraminic acid from glycoproteins without altering the protein backbone while it can be easily removed from the reaction mixture avoiding sample contamination. This enables the evaluation of changes in glycoprotein performance upon desialylation.
Methods: Neuraminidase was immobilized onto agarose activated with cyanate ester groups and further used for desialylation of model glycoproteins, a lysate from tumour cells and tumour cells. Desialylation process was analysed by lectin binding assay, determination of sialyl-Tn or flow cytometry.
Results: Clostridium perfringens neuraminidase was immobilized with 91 % yield and expressed activity yield was of 41%. It was effective in the desialylation of bovine fetal serum fetuin, bovine lactoferrin and ovine submaxilar mucin. A decrease in sialic-specific SNA lectin recognition of 83% and 53 % was observed for fetuin and lactoferrin with a concomitant increase in galactose specific ECA and PNA lectin recognition. Likewise, a decrease in the recognition of a specific antibody (82%) upon mucin desialylation was observed. Moreover, desialylation of a protein lysate from the sialic acid-rich cell line TA3/Ha was also possible leading to a decrease in 47 % in SNA recognition. Immobilized neuraminidase kept 100% of its initial activity upon five desialylation cycles.
Conclusions: Immobilized neuraminidase is an interesting as well as a robust biotechnological tool for enzymatic desialylation purposes.
General significance: Immobilized neuraminidase would contribute to understand the role of sialic acid in biological processes.

1. Introduction

Sialic acids are widely distributed in nature, mainly in vertebrates, but also in higher invertebrates and in some types of bacteria. They can be found in milk oligosaccharides, in blood glycoproteins, in sera of plasma mammals as well as in epithelial and mucus glycoproteins, generally located in the non-reducing terminal position of glycoconjugates. Their varied structural and functional roles give them biological relevance [1]. As they are deprotonated at physiological pH they provide negative charge to the glycoconjugates located in the cell surface, generating repulsion and thus avoiding unwanted interaction among cells for example in blood cell circulation [1,2]. They can also modulate protein turnover as asialo glycoproteins expose underlying monosaccharides such as galactose, which can be recognized by liver receptors leading to their clearance [2]. On the other hand, they can function as ligands for carbohydrate binding proteins, antibodies or enzymes, mediating cell communication both in physiological or pathological states [1,2]. In fact, sialic acids are ligands of selectins, which

Abbreviations: **BCA**, Bicinchonninic acid; **CDAP-BF$_4$**, 1-Cyano-4-dimethylaminopyridinium tetrafluoroborate; **ECA**, *Erythrina cristagalli* lectin; **ELISA-type assay**, Enzyme-linked Immuno Sorbent assay; **FBS**, Fetal bovine serum; **Gal**, Galactose; **GalNAc**, N-acetylgalactosamine; **GlcNAc**, N-acetylglucosamine; **4 MU-NANA**, 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid; **Neu5Ac**, N-Acetyl neuraminic acid; **Neura-agarose**, Neuraminidase immobilized onto agarose; **OPD**, ortho-Phenylendiamine; **OSM**, Ovine submaxilar mucin; **PBS**, Phosphate saline buffer; **PE**, Phycoerythrin; **PNA**, *Arachis hipogaea* lectin; **SNA**, *Sambucus nigra* lectin; **Sialyl-Tn antigen**, Neu 5 Ac-2,6 GalNAc.

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mediate leukocyte rolling along endothelium, and siglecs, which are involved in regulation of the immune response [1,2]. In a different context, sialic acids constitute binding sites for several pathogens and toxins, favouring infectious processes [1]. Moreover, some microbial pathogens are able to decorate themselves with sialic acid, mimicking host cells and thus assisting in the host immune evasion [1,2]. It has also been reported that tumour cells are highly sialylated, and therefore sialic acids are associated with progression and poor prognosis of certain carcinomas [2]. In fact, solid tumours secrete mucins that bear unusual forms of sialylation. These mucins can be detected in the blood stream of cancer patients and are used as diagnostic and prognostic tools [2,3]. Indeed, α2,6 linked sialic acids derived from tumours can bind to the B cell surface molecule DC22/Siglec2 which regulates the immune response favouring tumour cell survival [4,5]. Moreover, recognition of malignant cells by selectins may allow interactions between circulating tumour cells and platelets, leukocytes or endothelium and thereby can facilitate metastasis [2].

Neuraminidases are specific enzymes that catalyses the release of terminal N-acetyl neuraminic acid (Neu5Ac) from glycoconjugates and are already used in glycomic analysis coupled to mass spectrometry [6–11]. Even though this procedure is useful for glycan analysis, it does not provide information regarding sialic acid role in the biological process under study. To achieve this goal, changes in the biological performance of glycoproteins or glycolipids should be evaluated upon sialic acid release, which entails the removal of the neuraminidase upon the desialylation process to prevent interference in the biological assay. This involves an extra purification step that could be avoided by using immobilized neuraminidase. The main advantages of using immobilized glycosidases for glycan analysis are their easy removal from the reaction mixture, which avoids the contamination of the sample and allows enzyme reuse. Besides, enzyme stabilization can be achieved upon immobilization. Among the diversity of immobilization strategies, covalent immobilization has the advantage of preventing enzyme leakage from the support [12–15]. Our research group has a wide experience in covalent enzyme immobilization, particularly in supports activated with cyanate ester groups where exposed amino groups on the enzyme react with cyanate ester groups on the support [16,17]. Neuraminidase from *Clostridium perfringens* is a 71 kDa monomeric enzyme with a range of optimum pH of 5-5.5 and a range of optimal temperature of 51-55 °C. It is specific for the cleavage of Neu5Ac α-2,3; α-2,6 and α-2,8 linked to other sugar such as galactose [18,19].

In this work, we report the immobilization of neuraminidase from *C. perfringens* and its application to the selective cleavage of sialic acids from glycoproteins and more complex biological samples.

2. Materials and methods

2.1. Materials

Neuraminidase form *C. perfringens* (3.2.1.18), fetuin from bovine fetal serum, 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP-BF_4), o-phenylendiamine (OPD) were purchased from Sigma-Aldrich (St. Louis, MO). Bovine lactoferrin was from Murray Golburn Cooperative Co. LTD (Melbourne). Bicinchoninic acid (BCA) for protein determination was purchased from Pierce (Rockford, Illinois, USA). Agarose 4B and NAP-5 (Sephadex G25) columns were from GE Health Care (Buckinghamshire, UK). Biotinylated lectins from *Sambucus nigra* (SNA) *Erythrina cristagalli* (ECA), *Maackia amurensis* (MAL II) and *Arachis hipogaea* (PNA) were from Vector Labs (Burlingame, California, USA). Nunc Maxisorp plates were from Roskilde (Denmark). Purified ovine submaxillary mucin (OSM), kindly given by Professor E. Osinaga, was obtained according to Tettamanti and Pigman (1968) [20]. The monoclonal antibody B72.3 (IgG1), which recognizes the sialyl-Tn determinant [21] was kindly provided by Professor E. Osinaga. The mammary adenocarcinoma cell line TA3/Ha (provided by Professor C. Leclerc, Institut Pasteur, Paris, France) was cultured in complete culture medium, consisting of RPMI-1640 with glutamine (PAA Laboratories, Austria) supplemented with 10% heat-inactivated fetal bovine serum, 50 μM 2-mercaptoethanol, 100 U/ml penicillin (Sigma, St. Louis, MO, USA), 100 mg/ml streptomycin (Sigma), at 37 °C and 5% CO_2. All the other reagents were from analytical grade.

2.2. Enzyme activity assay

15 μL of soluble neuraminidase or 45 μL of immobilized neuraminidase standard suspension were incubated with 11 μL or 33 μL of 2 mM 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid (4 MU-NANA) and 275 μL or 825 μL of 0.15 M potassium acetate buffer pH 4.6, respectively at 37 °C for 30 min. 50 μL aliquots were withdrawn every 5 min and added to 450 μL of 0.2 M glycine-NaOH buffer pH 10.6 to stop the enzymatic reaction. A tenfold dilution of this mixture in 0.2 M glycine-NaOH buffer pH 10.6 was transferred to a black flat bottom ELISA plate and fluorescence was measured at λ 360/460. One enzyme unit (U) was defined as the amount of enzyme hydrolysing 1 μmol of 4-MU-NANA per minute at pH 4.6 and 37 °C.

2.3. Protein quantification

Protein was determined by the bicinchoninic acid (BCA) assay [22].

2.4. Support activation

Agarose activation was carried on as reported by Giacomini et al. [16]. The activated agarose was equilibrated in PBS and immediately used for neuraminidase immobilization.

2.5. Neuraminidase immobilization

500 mg of activated agarose were incubated with 1.7 mL (0.54 U/mL; 0.25 mg/mL) of neuraminidase in PBS for 4 h at room temperature under mild stirring. The immobilized enzyme (Neura-agarose) was filtrated, washed with PBS and stored at 4 °C. Immobilization rate was defined as the difference between the amount of proteins offered to the support and the recovered in the supernatant, expressed as a percentage of the amount of protein offered to the support. Expressed activity yield was defined as the amount of immobilized enzyme, expressed as a percentage of the enzyme units offered to the support. Four immobilization experiments were performed.

2.6. Desialylation of model glycoproteins

Solutions of fetuin (0.23 mg/mL), bovine lactoferrin (0.5 mg/mL) and OSM (0.3 mg/mL) were prepared in potassium acetate buffer 0.15 M pH 4.6 or in PBS. Glycoprotein solutions were gel filtrated in Sephadex G25 columns (NAP-5) or dialysed in order to remove low molecular weight contaminants. Aliquots of 0.6 mL of fetuin, 0.5 mL of lactoferrin or 0.7 mL of OSM were incubated with 250 mg of Neura-agarose (0.7 U/g) at 37 °C for 24 h under mild stirring. Neura-agarose was separated by filtration, washed with PBS and stored at 4 °C. The supernatant was gel filtrated in sephadex G25 columns (NAP-5) previously equilibrated in 50 mM ammonium bicarbonate solution pH 7.8 in order to separate the desialylated glycoprotein from the released sialic acid. Desialylated glycoproteins, which eluted in the void volume, were analysed by lectin binding assay or determination of sialyl-Tn as described in section 2.8. Control experiments were performed incubating the corresponding glycoprotein with non activated agarose. Several desialylating cycles were performed in order to evaluate Neura-agarose re-use.

2.7. Desialylation of lysates from tumour cells

The mammary tumour cell line TA3/Ha was used as it produces abundant mucins expressing Tn (GalNAc-*O*-Thr/Ser) and Sialyl-Tn

Table 1
C. Perfringens neuraminidase immobilization.

Applied Activity [a] (U/g)	Expressed Activity [b] (U/g)	Applied protein [c] (mg/g)	Immobilized protein [d] (mg/g)	Immobilization yield [e] (%)	Expressed Activity yield[f] (%)
1.7 ± 0.3	0.7 ± 0.2	0.86 ± 0.02	0.79 ± 0.04	91 ± 7	41 ± 0.3

[a]Amount of enzyme units offered per g of support; [b]Amount of enzyme units expressed per g of support; [c] Amount of protein offered per g of support; [d] Amount of protein immobilized on the support calculated as (amount of applied protein (mg) – amount of protein in the supernatant (mg))/g of support; [e] Immobilization yield was defined as the difference between the amount of protein offered to the support and recovered in the supernatant expressed as a percentage of the amount of protein offered to the support; [f] Expressed activity yield was defined as the amount of immobilized enzyme units expressed as a percentage the amount of enzyme units offered to the support.

(Neu5Ac-2,6-GalNAc-*O*-Thr/Ser) antigens. The cells were washed with PBS containing 2% of fetal bovine serum (FBS) and centrifuged at 13000 rpm for 7 min at 4 °C. Next, they were re-suspended in PBS, sonicated for 10 min and centrifuged at 200 rpm for 10 min. The supernatant was separated and gel filtrated in Sephadex G25 columns (NAP5) equilibrated in PBS pH 7.4 containing a cocktail of protein inhibitors (Sigma-Aldrich, St. Louis, MO). Aliquots of 0.55 mL of TA3/Ha protein lysate (0.8 mg/mL) were incubated with 250 mg of Neura-agarose (0.7 U/g) for 24 h at 37 °C under mild stirring. Neura-agarose was separated by filtration, washed with PBS and stored at 4 °C, for further use. The supernatant was dialysed against PBS pH 7.4 using membranes with 3.5 kDa cut off, and further lectin binding assay was performed as described in section 2.8. The corresponding controls were performed incubating the lysate with non activated agarose under the same conditions.

2.8. Lectin binding assay

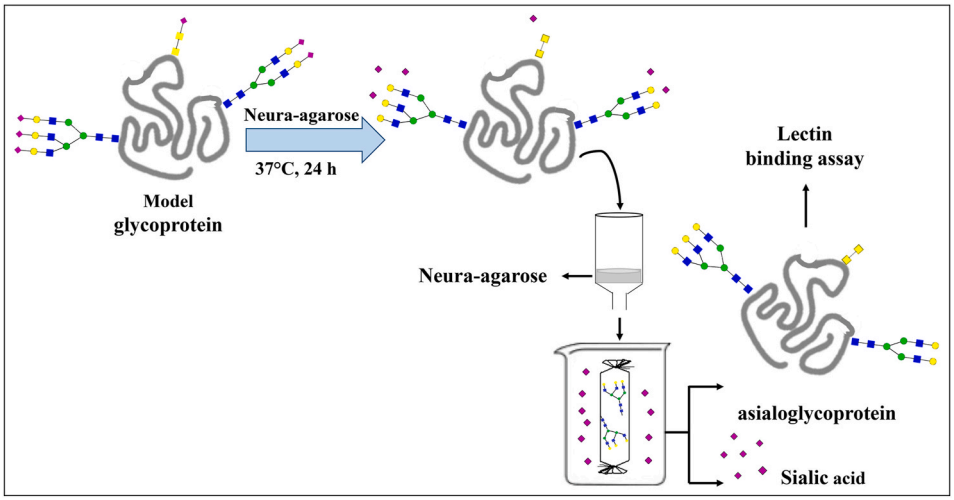
The lectin-reactivity of model glycoproteins and TA3/Ha cell lysate, as well as their desialylated counterparts, was evaluated by an ELISA-type assay (Enzyme-linked Immuno Sorbent assay) as previously described by Rodriguez et al. [17]. Briefly, 0.25 μg/well of glycoproteins or TA3/Ha cell lysates were used for the coating of the microtiter plates and 0.2–0.5 μg/well of the biotinylated lectins. Further incubation with streptavidin-peroxidase was performed and developed with o-phenylendiamine (OPD) in 0.1M citrate-phosphate pH 5.0 and H_2O_2. Absorbance at 492 nm was determined. The lectins from *Sambucus nigra* (SNA), *Maackia amurensis* (MAl II), *Erythrina cristagalli* (ECA) and *Arachis hipogaea* (PNA) were used in this study for the recognition of αNeu5Ac (2–6)Gal, αNeu5Ac(2-3)βGal(1-3)GalNAc, βGal(1–4)GlcNAc and βGal (1–3)GalNAc respectively.

2.9. Determination of sialyl-Tn by ELISA

Anti-sialyl-Tn B72.3 reactivity on OSM as well as its desialylated counterpart was evaluated by ELISA. Briefly, Nunc Maxisorp microtiter plates (Roskilde, Denmark) were coated with OSM and asialo-OSM (0.25 μg/well) in 0.1 M carbonate buffer pH 9.0 during 18 h at 4 °C. After washing three times with PBS containing 0.1% Tween 20, they were incubated with 1% gelatin in PBS for 1 h at 37 °C. After three washes, plates were incubated with culture supernatants containing the B72.3 monoclonal antibody for 1 h at 37 °C, followed by an anti-mouse IgG conjugated to peroxidase for 45 min at 37 °C. Upon three washes, 200 μL of 0.5 mg/mL o-phenilendiamine (OPD) in 0.1 M citrate-phosphate pH 5.0 and H_2O_2 (0.003%) were added. Absorbance at 492 nm was determined.

2.10. Desialylation of tumour cells

Cells from the tumour line TA3/Ha were washed with PBS pH 7.4 containing 2% of FBS, and a cell suspension of 3 x 10^6 cells/mL in the same buffer was prepared. 800 μL of the cell suspension were incubated with 250 mg of neura-agarose for 24 h at 37 °C under mild stirring. Neura-agarose was separated by filtration, washed with PBS pH 7.4 and stored at 4 °C for further use. The cell suspension (1 x 10^6 cell/mL) was incubated with biotinylated SNA (1 μg/well) for 30 min at 4 °C, washed with PBS pH 7.4 containing 2% of FBS and followed by conjugation with streptavidin conjugated to phycoerythrin (PE) (Biolegend, USA) for additional 30 min at 4 °C. Median fluorescence intensity (MFI) on TA3/Ha single cells was determined with a flow cytometer Accuri (BD Bioscience).



Scheme 1. Enzymatic desialylation process.

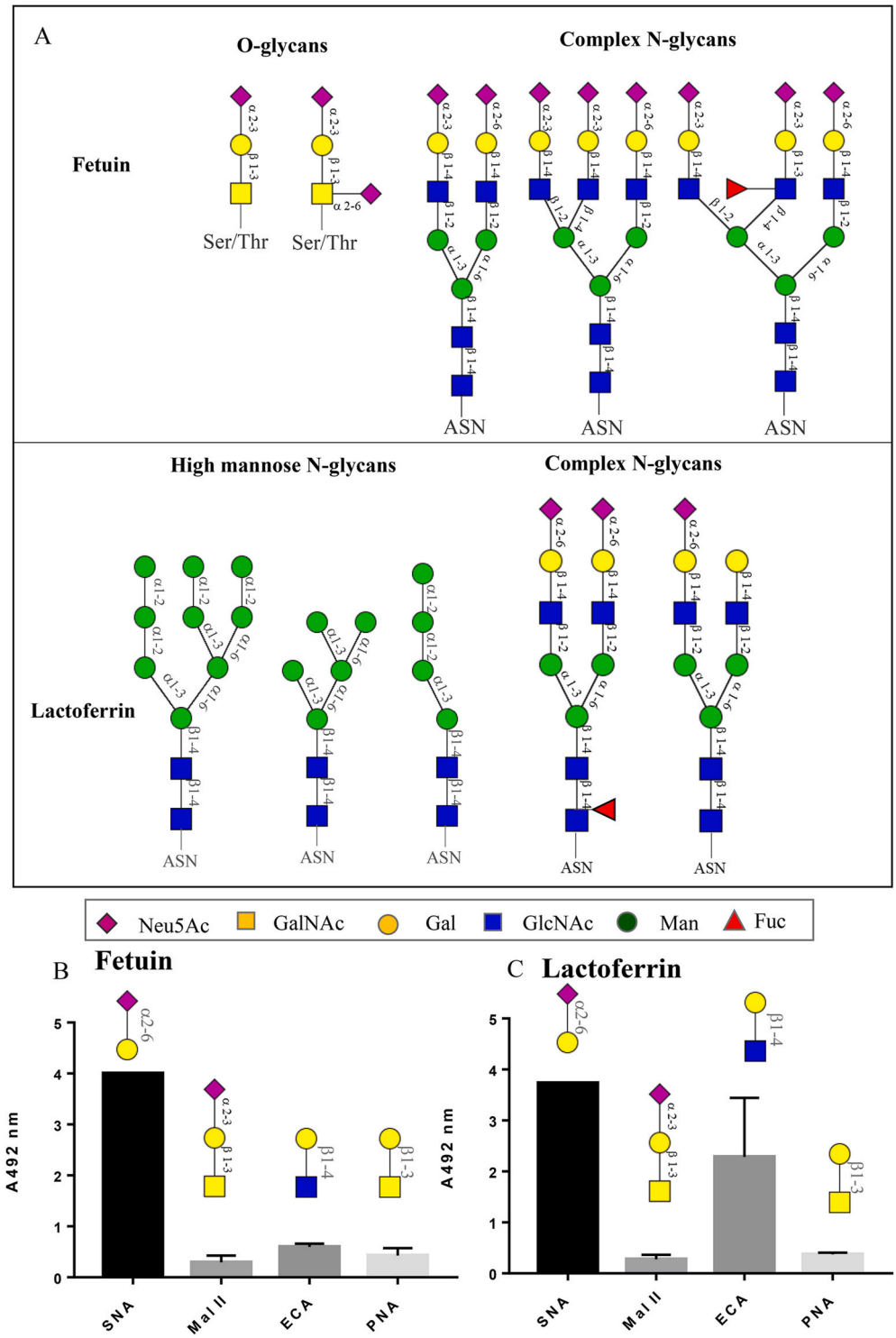


Fig. 1. A) Representative isoforms of fetuin and lactoferrin glycans B-C) Lectin recognition of Fetuin and Lactoferrin. ELISA plates were coated with 1 μg /well of fetuin (A) or Lactoferrin (B) and further incubated with biotinylated lectins SNA (0,25 μg/well); MAL II (0,5 μg/well); ECA (0,5 μg/well) and PNA (1,0 μg/well). Next, it was incubated with streptavidin-peroxidase and developed with o-phenylendiamine and H_2O_2 and absorbance measured at 492 nm.

3. Results

3.1. Neuraminidase immobilization

We achieved the immobilization of *C. Perfringens* neuraminidase onto agarose activated with cyanate ester groups with immobilization yields of 91 % and expressed activity yields of 41 % calculated as described in materials and methods section (Table 1). Even though not all the immobilized enzyme was active, the amount of immobilized enzyme activity was enough for enzymatic desialylation purposes and used in this study.

3.2. Desialylation of model glycoproteins

In order to evaluate the effectiveness of Neura-agarose in enzymatic desialylation of biological samples we assayed the desialylation of the

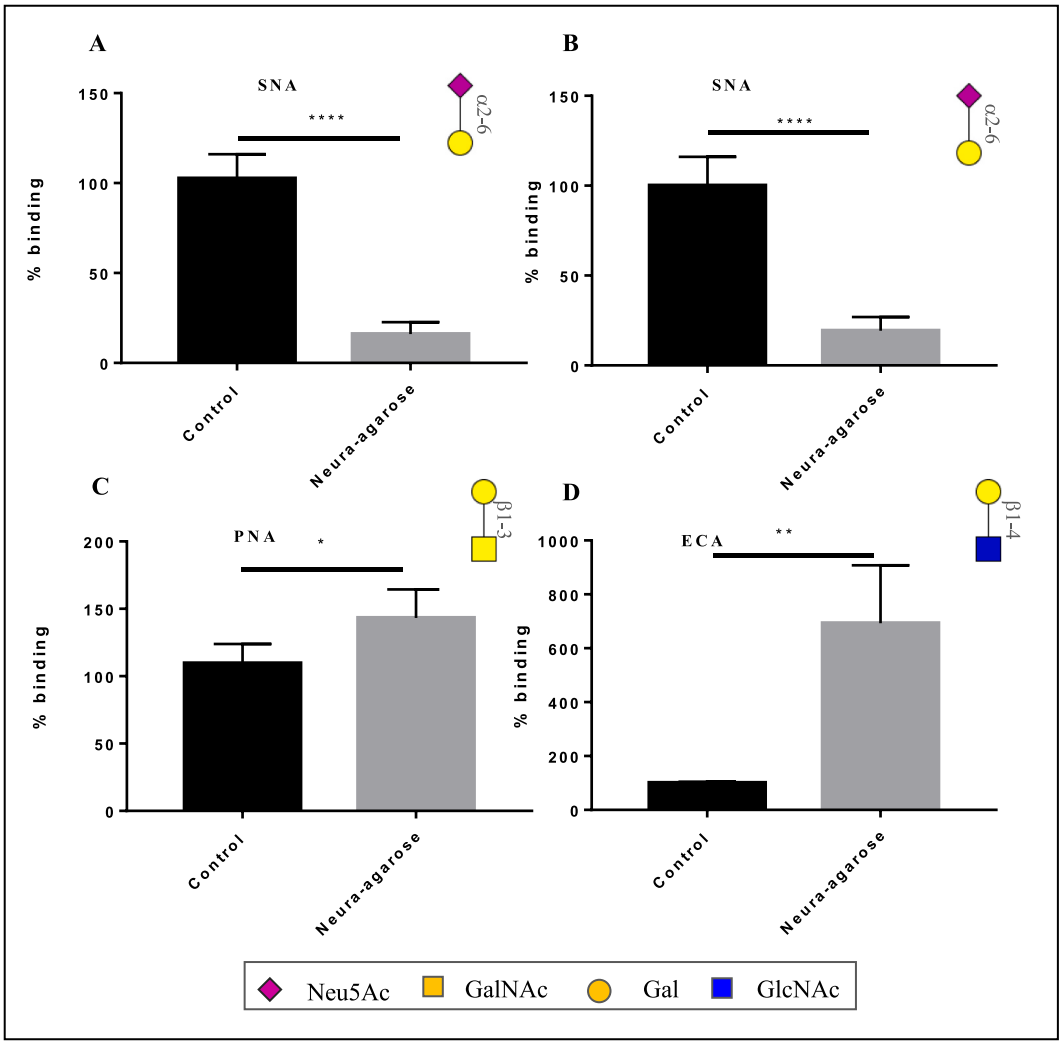


Fig. 2. Lectin binding assay of neuraminidase-treated fetuin. ELISA plates were coated with fetuin enzymatically desialylated with Neura-agarose and the corresponding control (0.25 μg/well) and further incubated with biotinylated lectins (0.2-0.5 μg/well). Next, it was incubated with streptavidin-peroxidase and developed with o-phenylendiamine and H_2O_2 and absorbance meassured at 492 nm. A) Desialylation at pH 4.6, analysed with SNA; B) Desialylation at pH 7.4, analysed with SNA; C) Desialylation at pH 7.4, analysed with PNA; D) Desialylation at pH 7.4, analysed with ECA. **Control:** fetuin incubated with non activated agarose for 24 h at 37°C. **Neura-agarose:** fetuin incubated with Neura-agarose for 24 h at 37 °C. ± SD, indicated by error bars. Asterisks indicate statistically significant differences **** $P < 0.0001$, **$P < 0.01$, * $P < 0.05$.

model glycoproteins fetuin and bovine lactoferrin. Enzymatic desialylation was performed at 37 °C for 24 h under mild stirring. The optimum pH range for *C. perfringens* neuraminidase is near pH 5.0, while most glycoproteins which are potential substrates are stable at physiological pH [18,19]. Thus, the desialylation process was performed both at pH 4.6 and 7.4. After removing Neura-agarose by a simple filtration step, the supernatant was gel filtrated in sephadex G25 columns (NAP-5) previously equilibrated in 50 mM ammonium bicarbonate solution pH 7.8 in order to separate the desialylated glycoprotein from the released sialic acid. Desialylated glycoproteins, which eluted in the void volume, were analysed by lectin binding assay in order to evaluate changes in lectin recognition due to desialylation (Scheme 1).

Fetuin is a glycoprotein which has sialylated N-glycans and O-glycans. At least 23 isoforms of N-glycans have been identified which can be di or tribranched, most of them containing underlying lactosamine units. The most representative isoforms are illustrated in Fig. 1 A [23,24]. On the other hand, bovine lactoferrin has high mannose as well as sialylated complex N-glycans [25]. The most representative structure of bovine lactoferrin complex N-glycans are illustrated in Fig. 1 A. SNA can recognize terminal Neu5Ac α 2,6 linked to Gal or GalNAc, while MAL II is specific for αNeu5Ac (2-3) βGal (1-3) GalNAc,so they can be used for recognition of α 2,6 and α 2,3 sialylated glycans respectively. Moreover, as fetuin and lactoferrin complex N-glycans have underlying lactosamine units, after removal of sialic acid this epitope can be recognized by ECA [26]. On the other hand, the underlying moiety in fetuin O-glycans is βGal (1-3) GalNAc which is a ligand of PNA [26–28]. Fig. 1B and 1C shows the recognition pattern of fetuin and lactoferrin by different lectins using ELLA assays, performed as described under materials and methods section. Specificity of MAL II for αNeu5Ac (2-3)βGal (1-3)GalNAc has been reported using different assays such as lectin blotting assay, affinity chromatography, flow cytometry and molecular modelling [29–32]. Nevertheless, MAL II specificity can vary depending of the experimental conditions used [32]. In fact our results show a very low recognition of fetuin by MAL II when using ELLA Assay (Fig. 1B), even though as have been previously mentioned and illustrated in Fig. 1A it has O-glycans with αNeu5Ac(2-3)βGal(1-3)GalNAc. Moreover, recognition level of lactoferrin (which could be considered a negative control) by MAL II was very similar to the recognition of fetuin. Thus, under this condition MAL II could not be used for evaluation of the desialylation process. This was performed using SNA, ECA and PNA, where a decrease in SNA recognition together with an increase of ECA or PNA recognition was expected after sialic acid removal.

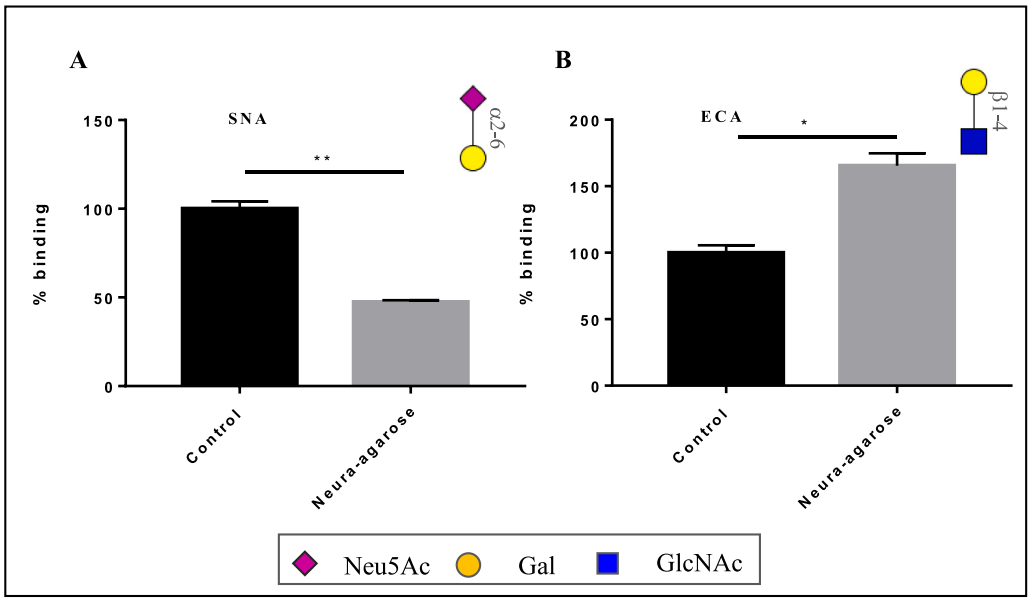


Fig. 3. Lectin binding assay of neuraminidase-treated bovine lactoferrin ELISA plates were coated with lactoferrin enzymatically desialylated with Neura-agarose and the corresponding control (0.25 μg/well) and further incubated with biotinylated lectins (0.2-0.5 μg/well). Next, it was incubated with streptavidin-peroxidase and developed with o-phenylendiamine and H_2O_2 and absorbance measured at 492 nm. A) Desialylation at pH 7.4, analysed with SNA; B) Desialylation at pH 7.4, analysed with ECA. **Control:** Lactoferrin incubated with non activated agarose for 24 h at 37°C. **Neura-agarose:** Lactoferrin incubated with Neura-agarose for 24 h at 37 °C. ± SD, indicated by error bars. Asterisks indicate statistically significant differences ** P<0.01, * P <0.05.

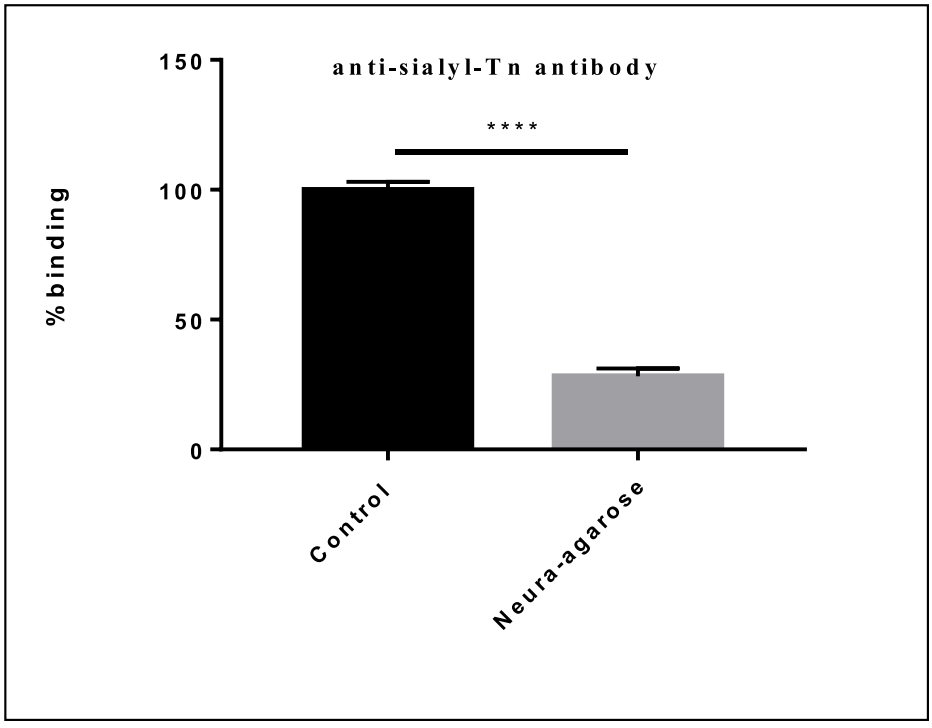


Fig. 4. Anti-Neu5Ac-2,6GalNAc antibody reactivity of neuraminidase-treated ovine submaxilar mucin. ELISA plates were coated with ovine submaxilar mucin (OSM) enzymatically desialylated with Neura-agarose and the corresponding control (2.5 μg/well) and further incubated with anti Neu5Ac-2,6GalNAc antibody. Next, it was incubated with peroxidase conjugated anti-mouse IgG antibody and developed with o-phenylendiamine and H_2O_2 and absorbance measured at 492 nm. **Control:** OSM incubated with non activated agarose for 24 h at 37°C. **Neura-agarose:** OSM incubated with Neura-agarose for 24 h at 37 °C. ± SD, indicated by error bars. Asterisks indicate statistically significant differences **** p< 0.0001.

Indeed, recognition of fetuin by SNA decreased 84 % and 83 % after enzymatic desialylation at pH 4.6 and 7.4 respectively (Fig. 2A–B). This indicated that Neura-agarose was effective at least for removal of α 2,6 Neu5Ac both at acidic and neutral pH. Besides, an increase in PNA recognition of 40% was observed (Fig. 2C). As PNA ligand (βGal(1-3) GalNAc) is present in O-glycans underlying sialic acid linked by α 2,3, this increase in PNA recognition could be an indirect evidence that Neura-agarose was also able to cleave terminal α 2,3 Neu5Ac as reported for the soluble enzyme [18,19]. Even though it has been reported that PNA could also recognize βGal(1-4)GlcNAc (underlying sialic acid linked by α2,6) it has a lower affinity (Kdis=4μM) than for βGal(1-3) GalNAc (Kdis=22 nM) [28,33]. On the other hand, ECA highly recognized the desialylated fetuin at pH 7.4, since the recognition increased in almost 600% (Fig. 2D), confirming the ability of Neura-agarose for desialylation processes. The high increase of ECA recognition after enzymatic desialylation compared to that observed for PNA could be due either to a higher affinity of ECA for βGal(1-4)GlcNAc moieties (underlying α2,6 Neu5Ac) than PNA for βGal(1-3)GalNAc (underlying α2,3 Neu5Ac in O-glycans) or to a higher amount of βGal(1-4)GlcNAc moieties upon desialylation. Kuno et al have previously reported a dissociation constant of 23.7 nM for the complex ECA-asialofetuin similar to the dissociation constant for the complex PNA-βGal(1-3)GalNAc previously mentioned (Kdis= 22 nM) [34]. Thus this results suggest that upon enzymatic desialylation a higher amount of βGal(1-4)GlcNAc moieties than βGal(1-3)GalNAc are exposed, which is in agreement with the low recognition of fetuin by MAL II. In order to check the versatility of

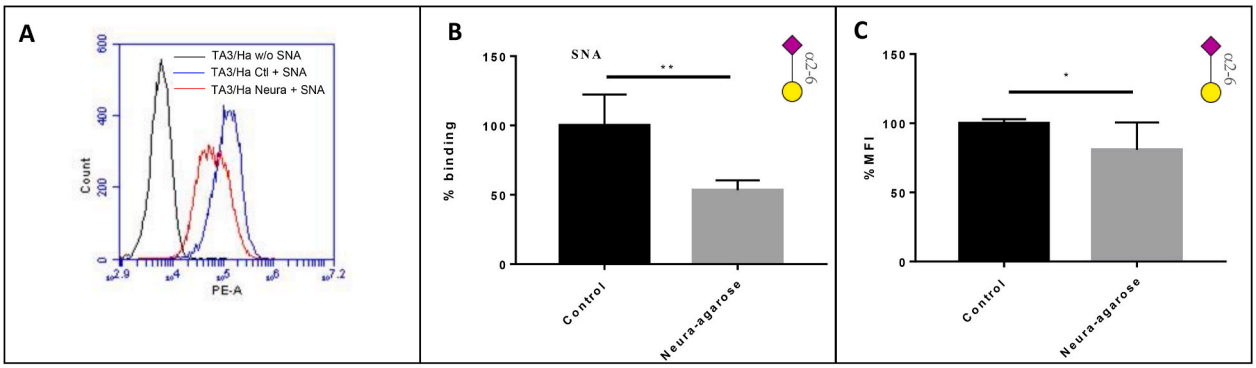


Fig. 5. Performance of Neura-agarose in the desialylation of TA3/Ha cells and TA3/Ha cell lysates. a) Histogram of TA3/Ha cells by flow cytometry; TA3/Ha: TA3/Ha w/o SNA (negative control in absence of SNA); **TA3/Ha Ctl**: Cells incubated with non activated agarose for 24 h at 37°C; **TA3/Ha Neura**: Cells incubated with Neura-agarose for 24 h at 37°C. **b) SNA binding to neuraminidase-treated TA3/Ha cell lysates.** ELISA plates were coated with cell lysates enzymatically desialylated with Neura-agarose and the corresponding control (0.25 ug/well) and further incubated with biotinylated SNA (0.5 ug/well). Next, it was incubated with streptavidin-peroxidase and developed with o-phenylendiamine and H_2O_2 and absorbance measured at 492 nm. **Control**: Cell lysate incubated with non activated agarose for 24 h at 37°C. **Neura-agarose**: Cell lysate incubated with Neura-agarose for 24 h at 37 °C. **c) Evaluation of neuraminidase-treated TA3/Ha cells.** Cells (100,000/well) were incubated with biotinylated SNA (1 ug/well) for 30 min at 4°C, followed by incubation with streptavidin-PE. Results are shown as the percentage of the median fluorescence intensity (MFI) determined in an flow cytometer Accuri. **Control**: Cells incubated with non activated agarose for 24 h at 37°C. **Neura-agarose:** Cells incubated with Neura-agarose for 24 h at 37°C. ± SD, indicated by error bars. Asterisks indicate statistically significant differences **<0.01 ; *P <0.05.

Neura-agarose performance we assayed enzymatic desialylation with bovine lactoferrin, another model glycoprotein. Terminal sialic acid present in lactoferrin complex N-glycans is always linked by α 2,6 to lactosamine (Gal β 1→4 GlcNAc) moiety which can be recognized by ECA [25]. The lectin binding assay showed a decrease of 53 % in SNA recognition with a concomitant increase of 65% in ECA recognition (Fig. 3). These results reinforced the effectiveness of Neura-agarose in enzymatic desialylation.

Another interesting model glycoprotein to evaluate as substrate for enzymatic desialylation is OSM. It is a highly sialylated O-glycoprotein which presents 75% of sialyl-Tn antigen, frequently expressed on tumour cells or mucins secreted by tumour cells [35]. As SNA does not present a good affinity for sialic acid linked to GalNAc [27], the desialylation process was followed using an anti Neu 5Ac-2,6GalNAc (sialyl Tn) antibody. Once again, we observed a high decrease in antibody recognition (82%) due to treatment with Neura-agarose (Fig. 4).

3.3. Re-use of neuraminidase-agarose

Among the advantages of using immobilized enzyme are their easy removal from the reaction mixture as well as the possibility of reusing them. Thus, we evaluated their reuse and observed that Neura-agarose kept 100% of its initial activity upon five re-uses (data not shown).

3.4. Enzymatic desialylation of TA3/Ha cells and TA3/Ha cell lysates

In view of the excellent performance of Neura-agarose in the enzymatic desialylation of diverse model glycoproteins, we went one-step forward and studied Neura-agarose performance in the desialylation of mammalian cancer cells (TA3/Ha) lysates. TA3/Ha expresses sialyl Tn antigen on its surface and secretes mucins which expresses sialyl Tn. Due to the fact that affinity of SNA does not show a high affinity for Neu5Ac linked by α2,6 to GalNAc [27], previously to the preparation of the cell lysates, we checked the recognition of TA3/Ha by SNA by flow cytometry (Fig. 5 A). Binding lectin assay performed with SNA showed a decrease of 47% in recognition upon treatment with Neura-agarose (Fig. 5B), evidencing that the use of this biotechnological tool is feasible for desialylation of more complex biological samples.

The next challenge was to achieve enzymatic desialylation of whole cells which expresses sialil Tn antigen on its surface using Neura-Agarose. After the treatment of TA3/Ha cells with Neura-agarose a decrease in cell recognition by SNA of 20 % was observed (Fig. 5C).

4. Discussion

In this work, we report the successful immobilization of neuraminidase from *C. perfringens* onto agarose activated with cyanate ester groups, with immobilization rates of 91 %. The fact that expressed activity yields were of 41% could be due to partial inactivation during the immobilization process as well as to diffusional problems that lead to underestimation of enzyme activity, which is quite often when working with immobilized enzymes [36]. Nevertheless, the amount of expressed activity was enough for desialylation purposes, with the advantage that the use of immobilized enzymes enables the easy removal from the reaction mixture by a simple filtration step. This allows stopping the enzymatic reaction avoiding the use of drastic conditions such as increase of temperature or pH with the consequent denaturalization of the glycoprotein subjected to deglycosylation. Moreover it avoids further purification steps to remove the glycosidase from the reaction mixture avoiding its interference in further biological assays and allowing the reuse of the neuraminidase. Neura-agarose proved to be effective in the desialylation of several glycoproteins (fetuin from bovine fetal serum, bovine lactoferrin and OSM) both at acidic and neutral pH. A decrease in SNA recognition of 83% and 53 % was observed for fetuin and lactoferrin respectively, with a concomitant increase in recognition by ECA and PNA, lectins which recognized sialic acid underlying moieties, which are exposed after sialic acid removal. Mucin effective desialylation was also achieved using Neura-agarose with a decrease in the recognition by an specific antibody of 82%. Moreover, no decrease in Neura-agarose activity was observed upon at least five re-uses. Results obtained in the desialylation process of TA3/Ha cell lysates evidenced that Neura-agarose can also be used for desialylation of more complex biological samples. Nevertheless, desialylation percentages of whole cells were significantly lower than those obtained for model glycoproteins. Probably this could be due to steric hindrances, which could difficult the access of the sialic acid on the cell surface to the active site of the Neura-agarose, even more if the neuraminidase is immobilized in the inner part of the agarose pores. We should deepen our research in this area, where an interesting alternative could be the immobilization onto nonporous support such as silica. In summary Neura-agarose proved to be an interesting as well a robust biotechnological tool for enzymatic desialylation purposes, which could be used in glycoproteins

as well as in more complex biological samples. It would surely contribute to understand the role of sialic acid in different biological processes.

Author contributions

Lucía Bidondo; Methodology, Formal Analysis, Investigation, **Mercedes Landeira:** Methodology, Formal Analysis, Investigation **Florencia Festari:** Methodology, Formal Analysis, Investigation **Teresa Freire:** Investigation, Conceptualization, Validation, Supervision, Resources, Writing **Cecilia Giacomini:** Conceptualization, Investigation, Supervision, Resources, Writing, review & editing, Supervision, Project administration, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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