



Master Thesis

A method for the qualitative and quantitative analysis of the glycosylation pattern of mouse soluble intercellular adhesion molecule-1 (sICAM-1)

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A Method for the Qualitative and Quantitative Analysis of the Glycosylation Pattern of Mouse Soluble Intercellular Adhesion Molecule-1 (sICAM-1)

Diploma Thesis
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Summary

Intercellular adhesion molecule-1 occurs in a membrane-bound (ICAM-1) and a soluble form (sICAM-1). ICAM-1 on vascular endothelial cells mediates leukocyte extravasation during inflammatory processes by binding the leukocyte integrins lymphocyte function associated antigen-1 (LFA-1) and macrophage antigen-1 (Mac-1). The function of soluble ICAM-1 is less well understood. Mouse sICAM-1 was found to induce the secretion of the chemokine MIP-2 from mouse astrocytes by a yet unknown mechanism.

(s)ICAM-1 can be used as a model glycoprotein to see how glycosylation can influence biological activity, each of its three functions being differently influenced by the glycosylation of the protein. Binding to LFA-1 is not affected by the glycosylation of human ICAM-1 whereas binding to Mac-1 is hindered by complex-type N-linked glycans. The induction of MIP-2 secretion from mouse astrocytes by mouse sICAM-1 is strongly enhanced by sialylated, galactosylated complex-type N-glycans.

The present study was aimed at analysing the N-glycan profile of a given glycoprotein and in particular of mouse sICAM-1.

Using the well characterized human α_1 -acid glycoprotein (AGP) as a reference protein, we validated and optimized a technique for the qualitative and quantitative analysis of N-glycosylation patterns. The method encompasses the separation of enzymatically released fluorescently labeled N-glycans by anion exchange chromatography according to their charge and MALDI TOF mass spectrometry for the identification of glycans contained in peak fractions.

Using these techniques, the oligosaccharide part of AGP could be characterized correctly, i.e. in agreement with data previously reported by several research groups. The methodology also showed very good reproducibility as well as robustness over time with regard to retention times and relative abundances of glycan species. Despite some drawbacks, including the large amount of glycoprotein needed and the rather complex sample preparation procedure, the technology was found suitable for its intended purpose.

Application of the analytical method to sICAM-1 has not yet brought satisfactory results, probably due to inadequate sample preparation. Strategies to overcome the problems encountered and to further optimize the methodology are presented and discussed.

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Abbreviations

| | |
|--------------|---|
| A1 | 2-AB-labeled monosialylated, galactosylated, biantennary oligosaccharide standard |
| A3 | trisialylated, galactosylated, triantennary oligosaccharide standard |
| 2-AB | 2-aminobenzamide |
| AGP | α_1 -acid glycoprotein |
| BSA | bovine serum albumin |
| CHO | chinese hamster ovary cells |
| CV | coefficient of variation |
| DHB | 2, 5-dihydrobenzoic acid |
| EDTA | ethylene diaminetetraacetate |
| ELISA | enzyme-linked immunosorbent assay |
| Fuc | fucose |
| Gal | galactose |
| GalNAc | N-acetylgalactosamine |
| Glc | glucose |
| GlcNAc | N-acetylglucosamine |
| HPLC | high performance liquid chromatography |
| ICAM-1 | intercellular adhesion molecule-1 |
| IEC | ion exchange chromatography |
| Ig | immunoglobulin |
| LFA-1 | lymphocyte function associated antigen-1 |
| Mac-1 | macrophage antigen-1 |
| MALDI TOF MS | matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry |
| Man | mannose |
| MIP-2 | macrophage inflammatory protein-2 |
| MWCO | molecular weight cut-off |
| NeuAc | N-acetylneuraminic acid |
| NeuGc | N-glycolylneuraminic acid |
| NP-40 | Nonidet [®] P-40 |
| PIC | protease inhibitor cocktail |
| PNGase F | peptide N-glycosidase F |

| | |
|----------|---|
| SD | standard deviation |
| SDS | sodium dodecyl sulfate |
| SDS PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| sICAM-1 | soluble intercellular adhesion molecule-1 |
| TBS | Tris buffered saline |
| TFA | trifluoroacetic acid |
| Tris | tris(hydroxymethyl)amino methane |
| U | unit |

Three letter codes are used for amino acids.

1 Introduction

Aim of the Study

An analytical method to qualitatively and quantitatively analyse the N-glycosylation pattern of recombinant proteins was previously established by S. Ali [1]. In the present study this established technique was validated and optimized with respect to its suitability to distinguish different glycan types and to determine their relative abundances. For this purpose, a model glycoprotein with a well-characterized glycosylation pattern, human α_1 -acid glycoprotein (AGP) was used. The final goal was to apply the procedure to mouse soluble intercellular adhesion molecule-1 (sICAM-1) expressed in CHO cells, the precise glycosylation pattern of which is as yet unknown. We were especially interested in the degree of sialylation, as this seems to be of crucial importance to the induction of macrophage inflammatory protein-2 (MIP-2) secretion from mouse astrocytes by mouse sICAM-1 [2].

In the following, an introduction to glycosylation in general and to the structure and diversity of N-linked glycans is given. Background information on sICAM-1, our glycoprotein of interest, as well as on α_1 -acid glycoprotein, our reference glycoprotein, will be found, followed by an overview of the analytical procedure used in the present study.

Glycosylation

Glycosylation is one of the most common forms of posttranslational modification in eukaryotic proteins. The majority of the biologically important proteins are glycosylated [3].

Glycoproteins are known to be fundamental to many biological processes including fertilization, immune defense, viral replication, parasitic infection, cell growth, cell-cell adhesion, inflammation and more. Glycoproteins and glycolipids are major components of the outer surface of mammalian cells. Oligosaccharide structures change dramatically during development and aberrant glycosylation is characteristic for a number of disease states including rheumatoid arthritis and cancer metastasis [3].

There is no single function for oligosaccharides. The carbohydrate moiety of a glycoprotein may participate directly in recognition events [4], but it may also modify the properties of the protein [5, 6] by altering its stability, solubility, protease resistance and/or quaternary structure. These effects arise mainly from the hydrophilicity and the large size of glycan structures [7, 8], their size allowing glycans to cover functionally important areas of proteins, to modu-

late the interactions of glycoconjugates with other molecules and to affect the rate of processes which involve conformational changes.

Increasing evidence shows that there is a close relationship between the structures of glycans, particularly at their nonreducing end, and the functions of glycoconjugates. It appears that variations of terminal glycosylation, such as fucosylation or sialylation, are crucial and have direct implications in biological activities. For full understanding of the specific functions of the carbohydrate portions of glycoproteins, a detailed characterization of their structures is mandatory [9].

The analysis of protein-linked glycans is of increasing importance, both in basic glyco-biological research and during the production process of glycoprotein pharmaceuticals [10]. The use of recombinant proteins as therapeutic agents has dramatically increased over the past decade [11]. Among those, there are several examples of therapeutic glycoproteins whose biological activity and/or pharmacokinetics depend on glycosylation, which therefore must be monitored closely. For example, human recombinant erythropoietin produced in CHO cells and being glycosylated shows a more prolonged half-life in blood than a preparation having no carbohydrate chains [12].

Because the branched, nonlinear nature of glycans and the variety of intersaccharide linkage types present additional elements of complexity, the analysis of carbohydrates is much more difficult than that of the other two classes of biological polymers, the linear DNA/RNA and protein molecules. Furthermore, in contrast to nucleic acids and proteins, there is no universal methodology for oligosaccharide analysis [13].

Diversity and Structure of N-linked Glycans

The two main classes of glycosidic linkages to proteins involve either oxygen in the side chain of serine, threonine or hydroxylysine (O-linked glycans) or nitrogen in the side chain of asparagine (N-linked glycans). N-glycosylation is much more frequent than O-glycosylation and in the present study we exclusively investigated N-linked glycans. To be glycosylated, an asparagine residue must form part of the tripeptide Asn-X-Ser/Thr where X can be any amino acid apart from proline. However, the presence of this peptide sequence within a protein does not guarantee its glycosylation [14]. It has been estimated that between 10 and 30% of potential glycosylation sites are not occupied [14, 15]. Moreover, site analysis has shown that the distribution of different classes of N-linked oligosaccharide structures is frequently specific for each N-glycosylation site on a protein. For instance, in α_1 -acid glycoprotein, Asn₁₅ never carries a tetraantennary glycan, Asn₃₈ never carries glycans with fucose, Asn₇₅ never carries a

biantennary glycan and only Asn₇₅ and Asn₈₅ carry tetraantennary glycans with more than one fucose [16].

Site specific glycosylation of a protein suggests that the 3D structure of the protein plays a role in determining the extent and type of its own glycosylation, which has been demonstrated in numerous studies of mammalian glycoproteins, e.g. for human erythropoietin, which has three glycosylation sites [17].

Moreover, the cell type has a major role in determining the extent and type of glycosylation, which is both tissue- and species-specific [8]. The inter- and intra-cell line diversity of oligosaccharide structures is believed to be related primarily to the presence, concentration, kinetic characteristics, and compartmentalization of the individual glycosyltransferases and glycosidases found in a given cell [5]. The biosynthesis of N-linked glycans follows a complex, enzymatically controlled pathway, involving many different enzyme reactions (typically eight for the processing of the Man₉GlcNAc₂ N-linked oligosaccharide precursor to form a complex-type N-glycan) [18, 19]. Each individual enzyme reaction may or may not go to completion, giving rise to glycosylated variants or glycoforms of the polypeptide. This also explains why the glycosylation pattern of natural glycoproteins may be influenced by physiological changes and by some diseases, which may affect one or more enzymes in the cell. The glycosylation of recombinant glycoproteins can be sensitive to changes in manufacturing conditions (e.g. culture conditions [20]), which therefore must be controlled carefully.

As their biosynthesis is a well-defined, enzymatically controlled process, only a small subset of all theoretical glycan structures is actually synthesized from the rather limited set of building residues (galactose (Gal), mannose (Man), fucose (Fuc), N-acetylglucosamine (GlcNAc), N-acetylneuraminic acid (NeuAc), N-glycolyl neuraminic acid (NeuGc) etc.).

All N-linked glycans contain the pentasaccharide Man α 1-6(Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc as a common core. On the basis of the structure and the location of glycan residues added to the trimannosyl core, N-linked oligosaccharides can be classified into three main groups [18, 21]: high-mannose- (oligomannose-), hybrid- and complex-type (Fig. 1).

High-mannose glycans contain only α -mannose residues attached to the core whereas complex-type glycans contain no mannose residues other than those in the core. Instead they have branches (antennae) with N-acetylglucosamine residues attached to the core. The number of antennae normally ranges from two to four, but a pentaantennary structure has been reported in hen ovomucoid [21]. Complex type N-glycans show the largest structural variations resulting mainly from the combination of different numbers of antennae and variations of monosaccharides present in the outer chains.

The hybrid type N-glycans have the characteristic features of both complex-type and high-mannose-type glycans. One or two α -mannosyl residues are linked to the Man α 1-6 arm of the trimannosyl core (as in the case of oligomannose-type glycans) and usually one or two antennae (as found in complex type glycans) are linked to the Man α 1-3 arm of the core.

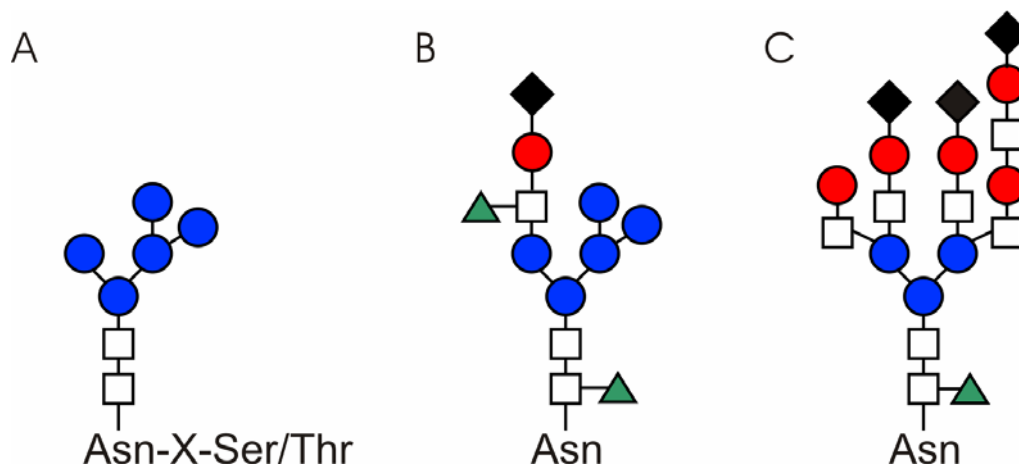


Fig. 1. Major classes of N-glycans. High-mannose-type (A), hybrid-type (B) and complex-type (C). Symbols: White square, GlcNAc; blue circle, Man; green triangle, Fuc; red circle, Gal; black diamond, NeuAc.

A fourth group, actually a subgroup of the complex-type N-glycans, are the poly-N-acetyl-lactosamine N-glycans containing repeating units Gal β 1-4GlcNAc β 1-3- attached to the core. Poly-N-acetyl-lactosamine extensions are most frequently found in tetraantennary glycans.

The structure of N-glycans is defined by a set of critical features that includes (i) the number of antennae, (ii) the degree of sialylation, (iii) the presence of fucosyl residues (on the core and/or on the antennae) and (iv) the number of lactosamine units.

With regard to the physicochemical properties of a glycan, the degree of sialylation is certainly of major importance. N-acetylneuraminic acid or sialic acid is – as its name indicates – an acidic sugar residue which is charged at physiological pH.

Intercellular Adhesion Molecule-1 (ICAM-1) and its Soluble Form (sICAM-1)

We are interested in sICAM-1 because it can be used as a model glycoprotein which is particularly suitable to study how glycosylation can influence biological activity.

Intercellular adhesion molecule-1 occurs as both a membrane bound (ICAM-1) and a soluble, secreted glycoprotein (sICAM-1, [22]).

ICAM-1 is a transmembrane glycoprotein which belongs to the group of immunoglobulin (Ig) like cell adhesion molecules. It contains five Ig-like domains constituting the extracellular

portion of the molecule [23]. The protein is heavily N-glycosylated and has a molecular weight between 85 and 115kDa, depending on the cell type it is expressed in [24-26]. Deglycosylation of ICAM-1 results in a protein of 60kDa [26].

In vivo, ICAM-1 is expressed by several cell types, including leukocytes and endothelial cells [23]. It acts as a binding partner for the leukocyte integrins lymphocyte function associated antigen-1 (LFA-1) [25, 27] and macrophage antigen-1 (Mac-1) [28]. Binding of these β_2 -integrins by ICAM-1 on the surface of vascular endothelial cells mediates firm adhesion and extravasation of leukocytes during inflammatory processes.

N-linked glycosylation of human ICAM-1 has been reported to reduce its affinity for Mac-1, presumably by shielding the ligand binding site. In contrast, glycosylation of human ICAM-1 seems to have no effect on binding of LFA-1 [29].

The soluble, circulating form of ICAM-1, consisting of the extracellular part of ICAM-1, appears to result from proteolytic cleavage of membrane-bound ICAM-1. Native and recombinant soluble forms of ICAM-1 have been reported to inhibit LFA-1/ICAM-1-mediated adhesion *in vitro* and it is conceivable that circulating forms of soluble ICAM-1 are regulators of LFA-1/ICAM-1 mediated cell-cell interaction *in vivo* [30].

Moreover sICAM-1 has some signaling functions. For instance, mouse sICAM-1 induces the secretion of the CXC chemokine macrophage inflammatory protein-2 (MIP-2) from mouse astrocytes by an as yet unknown, LFA-1- and Mac-1-independent mechanism [31, 32]. It was found that sialylated, galactosylated complex type N-glycans strongly enhance the ability of sICAM-1 to induce MIP-2 secretion from primary mouse astrocytes [2].

With glycosylation influencing each of the three functions of human/mouse ICAM-1/sICAM-1 differentially (Fig. 2), it is possible that *in vivo* ICAM-1 functions are specifically regulated by glycosylation.

From a pharmaceutical standpoint, sICAM-1 may offer the unique possibility to study how glycosylation of the same protein may hinder, leave unaffected or enhance biological activity. This fact prompted us to seek methods to analyse the glycosylation pattern of this protein and to assess its biological activity in various functional assays. The present work focusses on the analysis of the glycosylation pattern, especially the degree of sialylation, as sialic acid seems to be of crucial importance to MIP-2 induction.

Murine ICAM-1, in which we are interested in the present study, shows limited homology to the human protein sequence (50%) [33], but, like human ICAM-1, consists of five Ig-like domains. It also is a ligand for LFA-1 and Mac-1 in mice [33, 34].

It is known so far that mouse ICAM-1 contains twelve potential N-glycosylation sites [33], nine of which are found in the extracellular portion representing sICAM-1. The protein is heavily glycosylated with mostly complex-type glycans attached to the N-glycosylation sites. Only recently also some evidence of O-glycans was found [2].

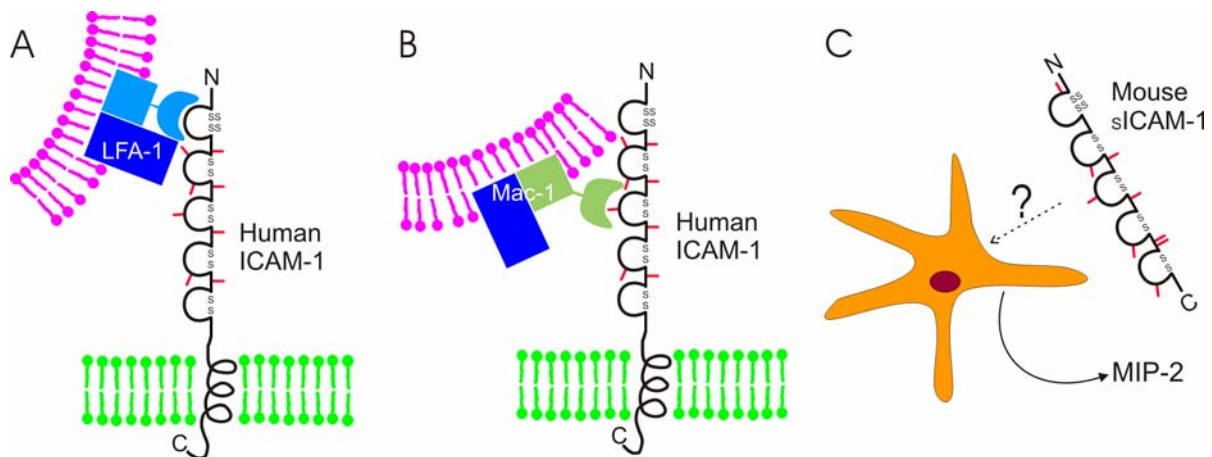


Fig. 2. The three functions of (s)ICAM-1, reported to be differentially influenced by glycosylation. In humans, binding of LFA-1 (A) is unaffected by the glycosylation of ICAM-1 whereas binding of Mac-1 (B) is hindered by complex-type N-glycans [29]. Induction of MIP-2 secretion from mouse astrocytes by mouse sICAM-1 is strongly enhanced by sialylated, galactosylated complex-type N-glycans [2].

α_1 -Acid Glycoprotein (AGP)

As a reference glycoprotein to establish and validate the procedure for glycan profiling, we used human α_1 -acid glycoprotein, one of the major acute phase proteins in human serum. This glycoprotein was first described in 1950 [35, 36] and since then its carbohydrate part has been extensively studied by many research groups [16, 37-42].

α_1 -Acid glycoprotein is heavily glycosylated, its molecular weight of around 43kDa including 45% of carbohydrates [39]. The peptide moiety of the human protein is a single chain of 183 amino acids to which five highly sialylated, complex-type N-glycans are attached [37, 41]. Its carbohydrate part has been thoroughly investigated because it is one of the few serum proteins that contains tetra-antennary as well as tri- and biantennary N-linked glycans.

Because it contains these typical bi-, tri- and tetra-antennary complex-type N-glycans a mixture of glycans derived from human AGP is one of the best libraries of carbohydrates [38].

Some of the tri- and tetra-antennary carbohydrate chains are substituted with a fucose residue to form the sialyl Lewis X type structure (sLe^x), NeuAc α 2-3Gal β 1-4(Fuc α 1-3)GlcNAc-R.

12% of the carbohydrate moiety of α_1 -acid glycoprotein are sialic acid residues, which can be linked in either an α_2 -3 or α_2 -6 linkage to galactose. Due to its high degree of sialylation the glycoprotein is negatively charged, with a very low pI between 2.7 and 3.2 [43], a property which – as well as the type of glycans – it shares with sICAM-1 [2].

The Analytical Procedure

In the analytical procedure validated and optimized in the present study, N-glycans are enzymatically released from the denatured proteins using peptide-N-glycosidase F (PNGase F). This enzyme cleaves the $C_\gamma - N_\delta$ bond of the glycosylated asparagine side chain releasing the intact N-glycans as glycosylamines, which readily hydrolyse to the glycan.

As our approach encompasses the release of the N-glycans from the proteins, no information concerning the glycosylation site a specific oligosaccharide structure is attached to can be derived.

In two of the AGP samples, sialic acid was removed by digestion with neuraminidase prior to the treatment with PNGase F to see how this influences the result of the analysis.

After removal of protein, salts, and detergents with the help of graphitized carbon packings [44] the released oligosaccharide mixtures are reductively aminated with a fluorophore, to enable their detection and quantification in the subsequent procedures. We use the fluorescent compound 2-aminobenzamide (2-AB) [45]. During the labeling reaction a single molecule of 2-AB is attached to the reducing terminus of each oligosaccharide, hence the fluorescence of the labeled glycans is a direct measure for their molar quantities.

After the labeling reaction excess 2-AB is removed and the pool glycans are profiled to determine the types of glycans present and their relative molar abundances. The glycans are first separated by anion exchange chromatography according to their charge, which depends on the number of sialic acid residues attached to the glycan antennae.

A major advantage of all HPLC systems is that they can be used preparatively. This means that the glycans in individual peaks can be collected, the volatile buffers removed, and the glycans identified by mass spectrometry.

In the present study, an additional desalting step is performed prior to mass spectrometric analysis of peak fractions although volatile mobile phases are used, in order to minimize ambiguities in assignments due to different sodium and potassium adducts.

The collected fractions are analysed by matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF MS) to identify the glycan structures contained in the peaks in the chromatogram. MALDI belongs to the mass spectrometric methods which give

only the parent ion for each glycan and produce spectra which are fairly easy to interpret, although laser energy induced desialylation can sometimes be observed. Like in the ion exchange chromatograms, the relative intensities of the peaks in the mass spectra can be used in a comparative manner to provide information on the relative abundances of oligosaccharide structures.

2 Materials and Methods

2.1 Materials

Purified water was prepared using a Milli-Q RG water purification system (Millipore, Billerica, MA).

Human α_1 -acid glycoprotein and protease inhibitor cocktail (PIC) were from Sigma-Aldrich Co. (St. Louis, MO).

Neuraminidase ex. *Vibrio cholerae*, neuraminidase ex. *Arthrobacter ureafaciens* and peptide-N-glycosidase F (PNGase F) were purchased from Roche Diagnostics GmbH (Penzberg, Germany).

The Signal™ 2-AB labeling kit (GKK-404), the GlycoSep™ C HPLC column (I-4721) and GlycoClean™ S cartridges (GKI-4726) were obtained from Glyko Inc. (San Leandro, CA).

Carbograph SPE columns were purchased from Alltech Associates Inc. (Deerfield, IL).

NuTip™ graphitized carbon 10 μ l pipette tips were from Glygen Corp. (Columbia, MD).

Two oligosaccharide standards were used. The trisialylated, galactosylated, triantennary standard (A3) was purchased unlabeled from Dextra Laboratories Ltd. (Reading, UK) and labeled with 2-aminobenzamide using the Signal™ 2-AB labeling kit. The 2-AB-labeled monosialylated, galactosylated, biantennary standard (A1) was obtained from Glyko Inc. (San Leandro, CA).

Acetonitrile HPLC gradient grade was from Scharlau Chemie S.A. (Barcelona, Spain). Glacial acetic acid 99% and ammonium hydroxide solution 25% were obtained from Hanseler AG (Herisau, Switzerland).

Trifluoroacetic acid (TFA), 5-methoxysalicylic acid, bovine serum albumin (BSA) and monoclonal mouse anti-human α_1 -acid glycoprotein antibody (A-5566) were purchased from Sigma-Aldrich Inc. (St. Louis, MO).

Peroxidase-labeled goat anti-mouse IgG antibody was from Kirkegaard & Perry Laboratories (Gaithersburg, MD).

HPC4 hybridoma cell line was obtained from ATCC® (Manassas, VA) and HPC4 monoclonal antibody was affinity purified using the HPC4 epitope peptide coupled to Affi-Gel 15 (BioRad Laboratories, Hercules, CA).

Ultralink® Biosupport Medium was from Pierce Biotechnology (Rockford, IL).

2,5-Dihydroxybenzoic acid (DHB) puriss p.a. for MALDI MS matrix was from Fluka (Buchs, Switzerland).

All other chemicals were of the highest grade commercially available.

2.2 Enzymatic Desialylation of α_1 -Acid Glycoprotein

1nmol (~8.3 μ l) of the native α_1 -acid glycoprotein in Tris buffered saline (TBS; NaCl 150mM, tris(hydroxymethyl)amino methane 20mM, pH 7.4) was desialylated during 60min at 37°C using 5mU of recombinant neuraminidase ex. *Vibrio cholerae* or 10mU of recombinant neuraminidase ex. *Arthrobacter ureafaciens*.

2.3 Preparation of the Glycan Pools

0.2-2nmol of glycoprotein were denatured by boiling for 10min in TBS pH 7.4 containing 0.5% sodium dodecyl sulfate (SDS) and 1% β -mercaptoethanol.

The glycoproteins were deglycosylated by enzymatic digestion with peptide N-glycosidase F (PNGase F) for 16h at 37°C, using 25U of enzyme for 1nmol of α_1 -acid glycoprotein and 450U of enzyme for 1nmol of mouse soluble intercellular adhesion molecule-1 (sICAM-1). Before the addition of enzyme 1 μ l of protease inhibitor cocktail (PIC) and Nonidet[®] P-40 (NP-40) in 7.5x excess of SDS were added.

The release of the oligosaccharides was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and Western blot (see 2.11 SDS-PAGE and Western Blot Analysis).

The glycan pool was separated from the protein and other contaminants (salts, detergents, reagents) using non-porous graphitized carbon black Carbograph SPE columns (column volume 4ml), according to Packer et al. [44]: Columns were conditioned with three column volumes of acetonitrile 80% containing 0.1% trifluoroacetic acid (TFA) followed by three column volumes of water. Samples were applied to the columns and left for 1 to 2min to allow glycans to adsorb to the carbon. Contaminants were washed off with three (for AGP samples) to eight (for sICAM-1 samples) column volumes of water, glycans were eluted with three column volumes of 25% acetonitrile containing 0.05% TFA. Flow was at all times between 0.5 and 1ml per minute (generated by gravity).

The eluate was frozen at -80°C, lyophilized and stored at -20°C.

2.4 Preparation of Fluorescently-Labeled Oligosaccharides

The glycan pool was reductively aminated with 2-aminobenzamide (2-AB) using the Signal[™] 2-AB labeling kit, according to the manufacturer's instructions. Briefly, the dried sample was dissolved in 5 μ l of a solution of 2-AB (0.35M) in dimethyl sulfoxide (DMSO)-glacial acetic acid (30% v/v) containing sodium cyanoborohydride (1M). After incubation at 65°C for 3h the excess of reagents was removed with a GlycoClean[™] S cartridge (post-labeling clean-up),

according to the manufacturer's instructions. The oligosaccharide solution was then filtered through a IC Millex[®]-LG Filter Unit (Millipore, Billerica, MA; pore size 0.2 μ m), frozen at -80°C , lyophilized (protected from light) and stored at -20°C in the dark.

2.5 High Performance Liquid Chromatography (HPLC)

The dried oligosaccharides were dissolved in 250 to 500 μ l water. 50 μ l of this solution, undiluted or diluted up to ten-fold, were injected onto the HPLC column.

HPLC was performed on a Merck Hitachi LaChrom D-7000 system (Merck KgaA, Darmstadt, Germany/Hitachi High Technologies America Inc, San Jose, CA) which consisted of a HPLC pump (Model No. L-7100), an autosampler (No. L-7200), a fluorescence detector (No. L-7480) and an interface (No. D-7000). Merck-Hitachi Model D-7000 Chromatography Data Station software version 4.0 was used for data analysis.

The 2-AB-labeled glycan mixture was separated at room temperature with a GlycoSep[™] C column (0.4ml/min) using an acetonitrile/water/ammonium acetate gradient [composed of water (solvent A), acetonitrile (solvent B) and ammonium acetate 0.5M pH 4.5 (solvent C)], modified from Viseux et al. [13]: In a first step the acetonitrile content was decreased from 80 to 20% while the water proportion was increased accordingly. In a second step, in which acetonitrile was kept constant at 20%, the concentration of ammonium acetate (pH 4.5) was increased from 0 to 160mM in 35min. The eluate was monitored by fluorescence detection (λ_{ex} 330nm, λ_{em} 420nm).

The ammonium acetate buffer was prepared by diluting acetic acid to 0.5M and adjusting the pH to 4.5 with ammonium hydroxide.

All solvents were filtered and sonicated for approximately 10min at room temperature prior to daily chromatographic analyses.

Peak fractions were collected from samples injected undiluted onto the column, frozen at -80°C , lyophilized and stored at -20°C .

2.6 Sample Cleanup for MALDI TOF MS

Samples were desalted prior to mass spectrometric analyses using NuTip[™] graphitized carbon 10 μ l pipette tips (Glygen Corp., Columbia, MD). Tips were conditioned by pipetting five times 10 μ l of 60% acetonitrile containing 0.05% trifluoroacetic acid (TFA) followed by three times 10 μ l of water.

Dried HPLC fractions were dissolved in 5 μ l water. Samples were bound to the carbon by pipetting up and down 50 times 2 μ l volumes (20 times oversampling). Contaminants were washed off by pipetting ten times 10 μ l of water and glycans were eluted with five times 1.5 μ l

of 60% acetonitrile containing 0.05% TFA. Each aliquot was pipetted up and down ten times before collection. Samples were vacuum dried.

2.7 Matrix-assisted Laser-desorption/Ionization Time-of-flight Mass Spectrometry (MALDI TOF MS)

MALDI TOF mass spectra were measured on a 4700 Proteomics Analyzer apparatus (Applied Biosystems, Foster City, CA). A nitrogen laser was used to irradiate samples at 337nm, and an average of 50 shots was taken. The instrument was operated in linear mode using negative polarity, at an accelerating voltage of 20kV. Dried, desalted HPLC fractions were dissolved in 2µl of methanol 70% and mixed with 2µl of matrix solution, consisting of 0.45% 2,5-dihydrobenzoic acid (DHB) and 0.05% 5-methoxysalicylic acid in MeOH 70% containing 10mM ammonium citrate (90µl DHB 1% in MeOH 70% were mixed with 10µl 5-methoxysalicylic acid 1% in MeOH 70% and 100µl ammonium citrate 20mM in MeOH 70%).

Samples (1.4µl each) were applied to a polished stainless steel target, and spots were dried at room temperature.

2.8 Affinity Purification of sICAM-1

Recombinant HPC4 epitope-tagged mouse sICAM-1 was expressed in dihydrofolate reductase-deficient Chinese hamster ovary (CHO) cells as described in [2].

It was purified from the cell supernatants by affinity chromatography, using HPC4 antibody coupled to Ultralink[®] biosupport beads. 2mM CaCl₂ and 0.02% (w/v) sodium azide were added to the culture medium which was incubated overnight with the HPC4-coupled beads (5mg antibody/ml of beads; 1.7µl beads/ml of medium). The next morning the beads were collected by centrifugation (10min, 1200 x g) and transferred to a BioRad Econo Column[®] (BioRad Laboratories, Hercules, CA; bed support pore size 20µm). After washing the beads with 50 to 60 times their volume TBS low salt (NaCl 100mM, tris(hydroxymethyl)amino methane 20mM, pH 7.4) containing 2mM CaCl₂ and 0.02% sodium azide, bound protein was eluted with TBS low salt, containing 5mM EDTA and 0.02% sodium azide. The protein was then dialyzed against three changes of 1lt of TBS (NaCl 150mM, tris(hydroxymethyl)amino methane 20mM, pH 7.4) for at least two hours per buffer change. The protein solution was sterile filtered through a 0.2µm low protein binding filter membrane (Acrodisc Syringe Filter; PALL Gelman Laboratory, Ann Arbor, MI).

sICAM-1 yields ranged from 0.016 to 0.34mg/lt of culture medium.

2.9 Concentration Assessment

UV absorbance of purified sICAM-1 was measured at 280nm after concentrating the sample 13-fold using a Centricon[®] YM-30 concentrator (Amicon Inc., Beverly, MA; MWCO =30,000Da).

For calculation of the concentration the molar absorption coefficient $42200\text{M}^{-1}\text{cm}^{-1}$, calculated using the formula given in [46] for folded proteins in water, was used.

The determined concentration was confirmed by comparison to reference sICAM-1 from earlier batches, which was done by Western blot (see 2.11 SDS-PAGE and Western Blot Analysis) and ELISA, using the commercial mouse sICAM-1 immunoassay kit (R&D Systems Europe, Abingdon, UK; standard curve: 0-20ng/ml; lower detection limit 0.03ng/ml).

2.10 Biological Activity Assay

The ability of purified sICAM-1 to induce MIP-2 secretion from primary mouse astrocytes was assessed. Mouse astrocytes were isolated from the brains of newborn C57BL/6 mice as described in [31] and [47].

Preparation of astrocyte cultures, their stimulation with sICAM-1 and quantification of MIP-2 were done according to [2].

Briefly, the evening before stimulation of the astrocytes the culture medium was replaced with medium containing 1% fetal bovine serum (FBS). The next morning the cells were stimulated with the recombinant mouse sICAM-1 (9 and 37nM). Parallel cell cultures incubated with medium alone were used as controls. After 24h the culture supernatants were collected, centrifuged (2000 x g, 4°C, 10min) and stored at -20°C until analysis by ELISA. The commercial mouse MIP-2 immunoassay kit was used (R&D Systems Europe, Abingdon, UK; standard curve: 0-500pg/ml; lower detection limit: 1.5pg/ml).

2.11 SDS-PAGE and Western Blot Analysis

Proteins were resolved by SDS PAGE (10% polyacrylamide separation gel, 4% stacking gel) in a BioRad Mini PROTEAN[®] 3 cell at 200V during 42min. The prestained broad range BioRad SDS-PAGE standard was used as molecular weight marker (BioRad Laboratories, Hercules, CA).

The proteins were transferred to nitrocellulose membranes (BioTrace[™] NT, Pall Gelman Laboratory, Ann Arbor, MI) using the BioRad TransBlot[®] SD transfer cell (19V, 20min). Blots were blocked with 5% (w/v) non-fat dried milk in either TBS pH 7.4 for sICAM-1 blots, or in TBS pH 7.4 containing 0.1% w/v Tween-20 for AGP blots, for 90min at room

temperature or overnight at 4°C and then washed with TBS or TBS/0.1% Tween-20, respectively. To detect sICAM-1, blots were incubated with HPC4 hybridoma cell supernatant for one hour at room temperature. After washing four times with TBS containing 2mM CaCl₂ (TBS-CaCl₂), the blots were incubated at room temperature for one hour with peroxidase-labeled secondary antibody (goat anti-mouse IgG), diluted 1:10000 in TBS-CaCl₂ containing 1% bovine serum albumin (BSA). After washing four times with TBS-CaCl₂, membranes were soaked for 5min in the enhanced chemiluminescence reagent (ECL plus Western Blotting Detection System, Amersham Biosciences, UK) and exposed to Kodak BioMax ML film (Eastman Kodak Co, Rochester, NY) for a time period of 30s to 7min.

For detection of AGP, blots were incubated with mouse monoclonal anti-human α_1 -acid glycoprotein antibody, diluted 1:1000 in TBS containing 5% (w/v) non-fat dried milk and 0.1% w/v Tween-20. The same secondary antibody as for sICAM-1 was used but diluted 1:10000 in TBS/0.1% Tween-20. AGP blots were washed four times with TBS/0.1% Tween-20 after each antibody incubation. Detection of immunostained bands was performed as described above.

2.12 Silver Staining

The degree of purity of the affinity purified sICAM-1 was assessed using silver staining of the SDS PAGE resolved proteins. Electrophoresis was performed as described above (2.11 SDS-PAGE and Western Blot Analysis). The unstained broad range BioRad SDS-PAGE standard was used as molecular weight marker (BioRad Laboratories, Hercules, CA). Gel slabs were soaked in destaining solution (50% methanol, 10% acetic acid) for 1h, then put in 0.2% aqueous periodic acid and left for 1h at 4°C [48]. Thereafter the gel slabs were incubated in three changes of 50% ethanol for 20min each. After pretreatment with 1.27mM sodium thiosulfate, gel slabs were washed three times for 20sec in ultrapure water. They were then left in impregnating solution (11.8mM silver nitrate containing 750 μ l of formaldehyde solution 35% per litre) for 20min. Three more washing steps (20sec each) were performed before the gels were placed in developer solution (0.6M sodium carbonate with 500 μ l formaldehyde 35% and 20ml pretreatment solution (1.3mM sodium thiosulfate) added per litre) until appropriate bands appeared (1 to 5min). After the desired intensity of staining was achieved, the development was terminated by replacing the developer solution by destaining solution.

3 Results

3.1 Optimization and Validation of the Analytical Method Using Human α_1 -Acid Glycoprotein (AGP)

An analytical method to qualitatively and quantitatively characterize the glycosylation pattern of recombinant proteins was established by Ali [1], using a glycoprotein with a well-characterized glycosylation pattern, α_1 -acid glycoprotein (AGP). In the present study we reproduced this work and further optimized the technique. In particular, we aimed at identifying glycan structures by mass spectrometry after their separation by ion exchange chromatography (IEC).

To test the suitability of the analytical method for our actual purpose, the analysis of the glycosylation of mouse sICAM-1, we validated the chromatographic analysis as well as the overall procedure including the sample preparation. Intra- and inter-assay precision as well as robustness of the overall method over time were assessed. Furthermore the detection and quantitation limit of the IEC for a single, pure oligosaccharide species, the minimal amount of glycoprotein-derived carbohydrate mixture to be injected onto the HPLC column, and the minimal amount of glycoprotein necessary for the analysis were determined.

Like Ali, we used samples of commercial human α_1 -acid glycoprotein (AGP) for these purposes.

3.1.1 Separation and Identification of 2-AB-labeled Glycans by Ion Exchange Chromatography (IEC) in Combination with MALDI TOF MS

As previously done by Ali [1], N-glycans enzymatically released from AGP were separated by ion exchange chromatography, using an amino-bonded polymer-based stationary phase and an acetonitrile/water/ammonium acetate gradient. Our goal was to identify the glycan structures contained in the peaks and compare the findings to previously published data about the glycosylation of AGP to confirm the accuracy of our analytical method.

The carbohydrate chains were eluted according to their charges based on the number of sialic acid residues present, and differently branched structures (bi-, tri- tetraantennary) were also resolved.

The elution positions of the different charge states were based on different sialylated standard oligosaccharides (mono- and trisialylated, for chromatograms see appendix p. 47) and comparison to the chromatogram of AGP glycans given in [13].

By comparing the data to those reported by Viseux et al. [13] and Nakano et al. [38], we could assign the major peaks in the ion exchange chromatogram (Fig. 3) to differentially sialylated glycan classes and confirm the structures by MALDI TOF MS measurement. A list of the carbohydrate chains detected, their molecular masses and their abbreviations is given in Table 1 and Fig. 5.

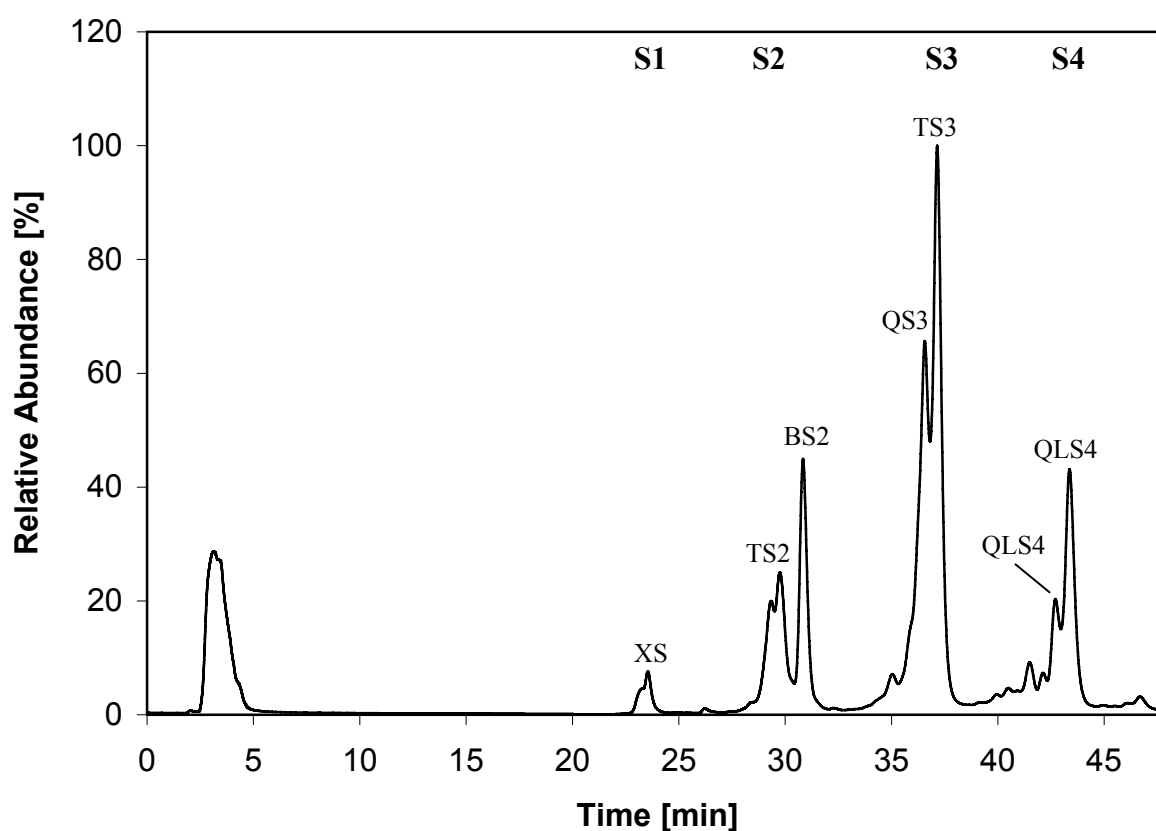


Fig. 3. Ion exchange chromatographic separation of the 2-aminobenzamide-derivatized N-glycans released from human α_1 -acid glycoprotein. Labeled oligosaccharides were injected onto the GlycoSep C HPLC column, which was equilibrated in 80% acetonitrile. The acetonitrile content was linearly decreased to 20% over 13min and then maintained at this level. At 13min, a linear gradient of ammonium acetate pH 4.5 was initiated, going from 0 to 160nM over 35min. S1 to S4 correspond to different charge states, from one to four sialic acids (S). Peak labels refer to the major glycan species contained in the peak. All structures found are summarized in Table 1 and Fig. 5. Symbols: **X** includes biantennary (**B**), triantennary (**T**), and tetraantennary (**Q**) N-glycans; **S** sialic acid residue; **L** lactosamine unit.

The earliest eluting peak appearing at ~23min was due to monosialylated species with different numbers of antennae (S1). The second group of peaks at around 30min was a mixture of disialylated bi-, tri- and tetraantennary carbohydrate chains (S2). The peaks of the third group at around 37min corresponded to trisialylated tri- and tetraantennary oligosaccharides (S3) and those of the fourth group at around 42min were tetrasialylated tetraantennary species (S4).

Trisialylated triantennary glycans were the major species (contained in the largest peak at around 37min). No neutral oligosaccharides were found. Some of the tri- and tetraantennary carbohydrate chains were substituted with one or two fucose residues. Small amounts of lactosamine-extended structures were present, accounting for the low-intensity signals in the last sialylation cluster (S4). Neither fucosylated nor polylactosamine structures were unequivocally resolved by HPLC but they could easily be identified in the mass spectra.

These findings agree with previously published data [13, 38, 42] indicating that our method is accurate.

To illustrate the identification of glycan structures by MALDI TOF MS, the mass spectrum of the most heterogeneous peak, found at about 29min retention time in the ion exchange chromatogram (TS2), is shown in Fig. 4. For the complete set of mass spectra see appendix p. 48ff.

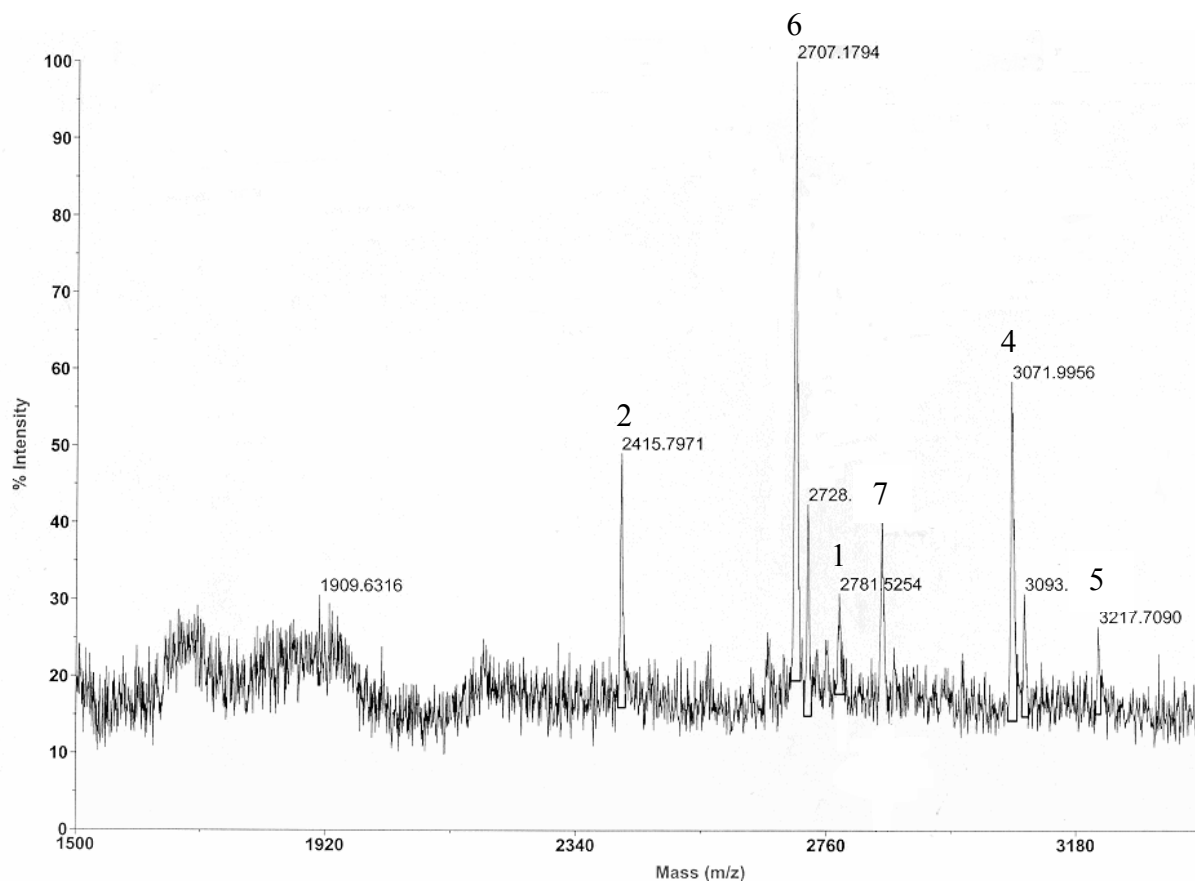


Fig. 4. Mass spectrometric identification of 2-AB-labeled AGP glycans separated by ion exchange chromatography. The MALDI TOF MS spectrum obtained for the HPLC peak at a retention time of about 29min (TS2) is shown, recorded in negative linear mode, as described in Materials and Methods. Peak numbers correspond to Table 1 and Fig. 5.

Disialylated tri- and tetraantennary glycans and their fucosylated counterparts were contained in the HPLC peak at 29min retention time (TS2).

The mass spectrum also shows that some desialylation occurred in the procedure following the collection of HPLC fractions. Monosialylated structures (1 and 2) were found although the peak analysed corresponds to disialylated glycans.

Table 1. List of oligosaccharides and their calculated molecular masses (including 2-AB) found in human AGP samples. Symbols: **B** biantennary, **T** triantennary, **Q** tetraantennary, **F** fucosyl residue, **S** sialic acid residue, **L** lactosamine unit. Peak labels refer to the peak designations in Fig. 3. All glycans contained in a certain peak are listed, including the major ones according to which peaks were labeled. The structures are illustrated in Fig. 5.

| Structure No. | Abbreviation | Molecular Mass (including 2-AB) | Peak Label |
|----------------------|---------------------|--|-------------------|
| 1 | QS | 2781.4 | |
| 2 | TS | 2416.1 | XS |
| 3 | BS | 2050.7 | |
| 4 | QS2 | 3072.7 | |
| 5 | QFS2 | 3218.8 | |
| 6 | TS2 | 2707.3 | TS2 |
| 7 | TFS2 | 2853.5 | |
| 8 | BS2 | 2342.0 | BS2 |
| 9 | QS3 | 3363.9 | |
| 10 | QFS3 | 3510.1 | QS3 |
| 11 | QF2S3 | 3656.2 | |
| 12 | TS3 | 2998.6 | |
| 13 | TFS3 | 3144.7 | TS3 |
| 14 | QLS4 | 4020.5 | |
| 15 | QLFS4 | 4166.7 | QLS4 |
| 16 | QL2S4 | 4385.9 | |
| 17 | QS4 | 3655.2 | |
| 18 | QFS4 | 3801.3 | QS4 |
| 19 | QF2S4 | 3847.5 | |

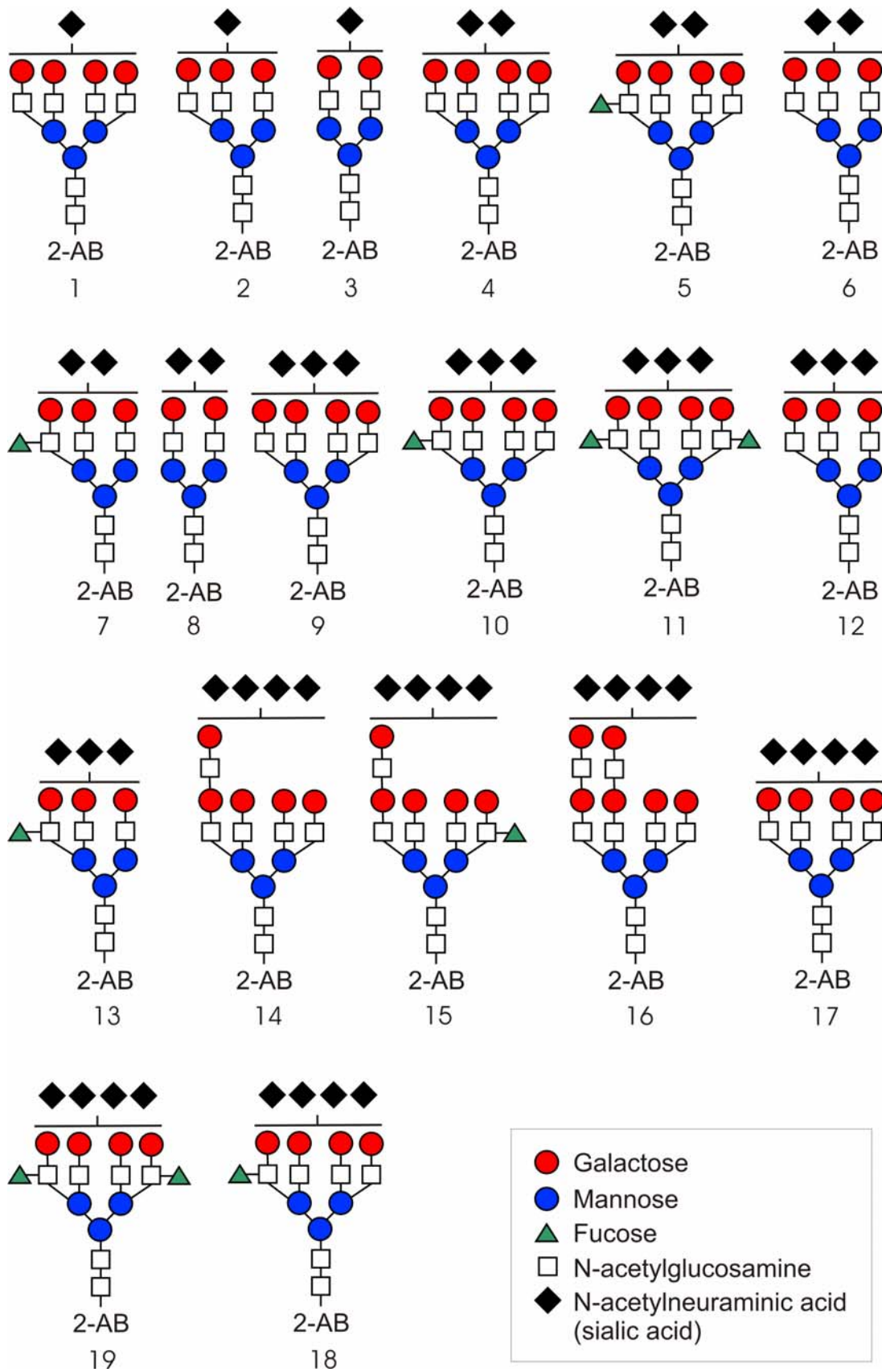


Fig. 5. Structures of oligosaccharides found in human α_1 -acid glycoprotein. Numbers refer to Table 1.

3.1.2 Precision of the Ion Exchange Chromatographic Analysis and the Overall Method Including the Sample Preparation Procedure

We were interested in the repeatability of the chromatographic analysis when the same glycan sample was measured several times (intra-assay precision). Moreover, we wanted to know if the overall method, including deglycosylation, sample cleanup, derivatization and chromatographic analysis, was reproducible (inter-assay precision), also over a long time period (robustness over time).

In addition to the identification of the glycan species, this study aimed at deriving some quantitative information on the relative abundance of the different glycans present in the mixture. Therefore retention times and relative peak areas of major species which are resolved as calculated by the HPLC software were compared. The peak area values were normalized to the most abundant component (the trisialylated, triantennary N-glycan (TS3)), which was set equal to 100%.

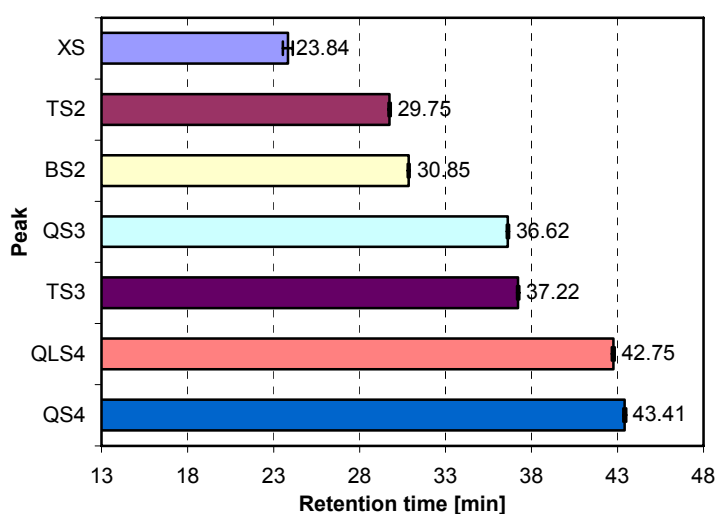
3.1.2.1 *Intra-Assay Precision*

To assess the repeatability of the ion exchange chromatographic analysis with regard to retention times and relative abundances of major glycan species, the same AGP glycan sample was measured three times (intra-assay precision; Fig. 6).

The IEC analysis showed excellent repeatability in retention times, coefficients of variation being well below 1%, except for the peak corresponding to monosialylated species (XS) with a variability in retention time of 1.23%.

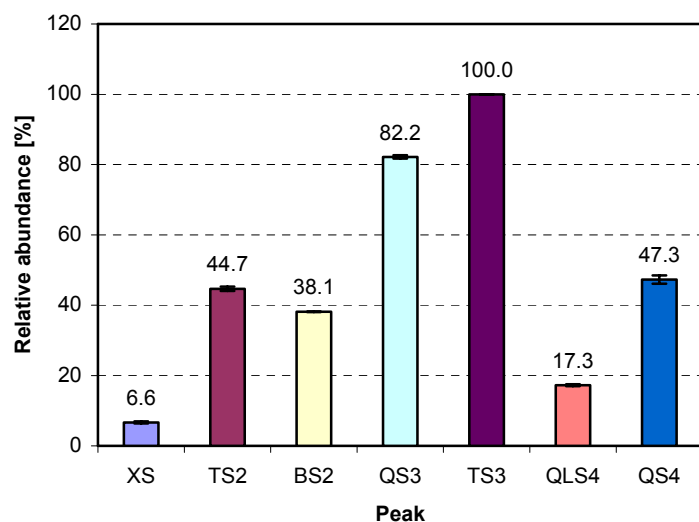
Relative abundances of the major species resolved were also very well repeatable, variability being below 5% for all peaks.

A



| Peak | Average [min] | SD | CV [%] |
|------|---------------|------|--------|
| XS | 23.84 | 0.29 | 1.23 |
| TS2 | 29.75 | 0.06 | 0.20 |
| BS2 | 30.85 | 0.06 | 0.20 |
| QS3 | 36.62 | 0.07 | 0.19 |
| TS3 | 37.22 | 0.07 | 0.18 |
| QLS4 | 42.75 | 0.08 | 0.20 |
| QS4 | 43.41 | 0.09 | 0.20 |

B



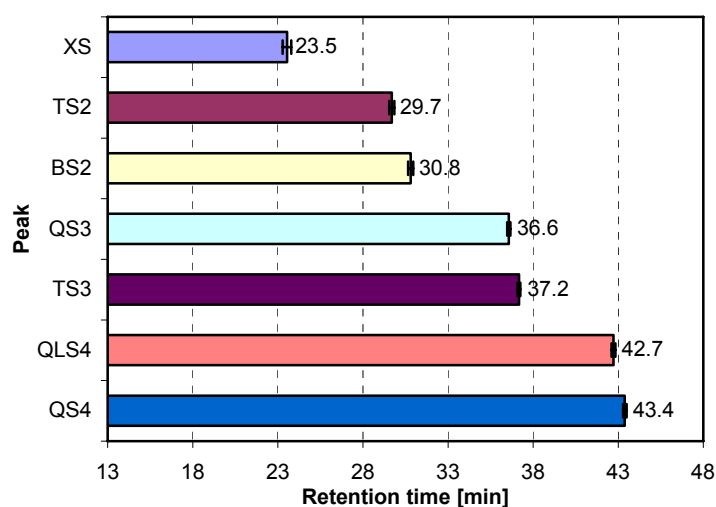
| Peak | Average [%] | SD | CV [%] |
|------|-------------|------|--------|
| XS | 6.6 | 0.30 | 4.52 |
| TS2 | 44.7 | 0.61 | 1.36 |
| BS2 | 38.1 | 0.08 | 0.20 |
| QS3 | 82.2 | 0.47 | 0.57 |
| TS3 | 100.0 | 0.00 | 0.00 |
| QLS4 | 17.3 | 0.28 | 1.60 |
| QS4 | 47.3 | 1.17 | 2.47 |

Fig. 6. Intra-assay precision with regard to retention times (A) and relative abundances (B) of major species. Values indicated represent averages of three analyses of the same sample of 2-AB-labeled glycans from AGP. Symbols: **X** includes biantennary (**B**), triantennary (**T**), and tetraantennary (**Q**) N-glycans; **S** sialic acid residue; **L** lactosamine unit. **SD** standard deviation; **CV** coefficient of variation.

3.1.2.2 Inter-Assay Precision

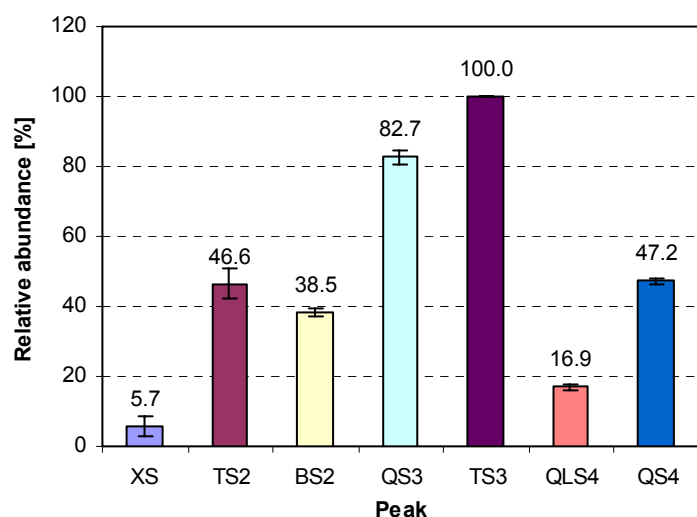
The reproducibility of the overall method, comprising release, labeling and IEC analysis of the glycans, was monitored by comparing chromatograms obtained for five different, individually prepared samples (inter-assay precision; Fig. 7). This series of experiments included all steps, namely, the deglycosylation, the sample cleanup, the derivatization and the IEC.

A



| Peak | Average [min] | SD | CV [%] |
|------|---------------|------|--------|
| XS | 23.5 | 0.25 | 1.06 |
| TS2 | 29.7 | 0.15 | 0.50 |
| BS2 | 30.8 | 0.15 | 0.49 |
| QS3 | 36.6 | 0.09 | 0.26 |
| TS3 | 37.2 | 0.09 | 0.24 |
| QLS4 | 42.7 | 0.11 | 0.26 |
| QS4 | 43.4 | 0.11 | 0.26 |

B



| Peak | Average [%] | SD | CV [%] |
|------|-------------|------|--------|
| XS | 5.7 | 2.82 | 49.94 |
| TS2 | 46.6 | 4.31 | 9.25 |
| BS2 | 38.5 | 1.20 | 3.12 |
| QS3 | 82.7 | 1.99 | 2.41 |
| TS3 | 100.0 | 0.00 | 0.00 |
| QLS4 | 16.9 | 0.62 | 3.68 |
| QS4 | 47.2 | 1.04 | 2.21 |

Fig. 7. Inter-assay precision with regard to retention times (A) and relative abundances (B) of major species. Values indicated represent averages of analyses of five different, individually prepared samples of 2-AB-labeled glycans from AGP. Symbols: **X** includes biantennary (**B**), triantennary (**T**) and tetraantennary (**Q**) N-glycans; **S** sialic acid residue; **L** lactosamine unit. **SD** standard deviation; **CV** coefficient of variation.

The overall method also showed excellent reproducibility in retention times, coefficients of variation being well below 1%, except for the peak corresponding to monosialylated species (XS) with a variability in retention time of 1.06%.

Relative abundances of the major species resolved by HPLC were also well reproducible, despite the rather complex sample preparation. Variability was typically below 10% except for the smallest peak (XS), where variability was high (50%).

3.1.2.3 Robustness over Time

To assess if the method could be used to monitor batch-to-batch consistency of glycoproteins, for instance to compare sICAM-1 samples expressed and purified from cell supernatants at different points in time, its robustness over time was tested.

From the same commercial AGP batch, stored at -20°C , six glycan samples were prepared and analysed at three different points in time over a six month period (Table 2). Experiment 1 was performed by Ali [1] and experiment 2 and 3 three and six months later, respectively. For each experiment all preparation steps (PNGase F digestion, 2-AB-labeling, sample cleanup) and the IEC analysis were carried out in duplicate.

The results in Table 2 demonstrate the robustness of the method over time. Again migration times showed excellent reproducibility with coefficients of variation below 1% for all peaks. Relative abundances of the major species resolved by HPLC were also well reproducible, despite the rather complex sample preparation. Coefficients of variation were typically below 10% except for the smallest peak (XS), where variability was high (22%) and the peak TS2 with a variability of 10.45%.

This method can hence be used to monitor the batch-to-batch consistency of glycoproteins.

Table 2. Variability of the IEC analyses of the AGP glycan pool over a six-month period with regard to retention times (A) and relative abundances (B) of major species. Experiment 2 and 3 were carried out three and six months later than experiment 1. Each experiment was performed with two individually prepared AGP samples, each analysed once by IEC. Values given for each experiment are the averages of two analyses out of one experiment performed in duplicate. Symbols: **X** includes biantennary (**B**), triantennary (**T**) and tetraantennary (**Q**) N-glycans; **S** sialic acid residue; **L** lactosamine unit. **SD** standard deviation; **CV** coefficient of variation.

A

| Glycans | experiments | | | average | SD | CV [%] |
|-------------|-------------|-------|-------|---------|------|--------|
| | 1 | 2 | 3 | | | |
| XS | 23.37 | 23.46 | 23.82 | 23.6 | 0.19 | 0.82 |
| TS2 | 29.90 | 29.65 | 29.74 | 29.8 | 0.10 | 0.35 |
| BS2 | 30.80 | 30.76 | 30.82 | 30.8 | 0.02 | 0.08 |
| QS3 | 36.06 | 36.58 | 36.49 | 36.4 | 0.23 | 0.63 |
| TS3 | 36.62 | 37.17 | 37.08 | 37.0 | 0.24 | 0.65 |
| QLS4 | 41.95 | 42.69 | 42.65 | 42.4 | 0.34 | 0.80 |
| QS4 | 42.58 | 43.36 | 43.32 | 43.1 | 0.36 | 0.83 |

B

| Glycans | experiments | | | average | SD | CV [%] |
|-------------|-------------|--------|--------|---------|------|--------|
| | 1 | 2 | 3 | | | |
| XS | 6.09 | 4.53 | 7.84 | 6.2 | 1.35 | 22.00 |
| TS2 | 50.86 | 45.58 | 58.74 | 51.7 | 5.41 | 10.45 |
| BS2 | 38.85 | 38.26 | 43.20 | 40.1 | 2.20 | 5.49 |
| QS3 | 85.40 | 82.19 | 81.12 | 82.9 | 1.82 | 2.20 |
| TS3 | 100.00 | 100.00 | 100.00 | 100.0 | 0.00 | 0.00 |
| QLS4 | 17.07 | 17.26 | 16.32 | 16.9 | 0.41 | 2.40 |
| QS4 | 47.12 | 47.77 | 46.76 | 47.2 | 0.42 | 0.89 |

3.1.3 Detection and Quantitation Limit

The detection and quantitation limit of the ion exchange chromatography with fluorescence detection was determined using known quantities of the 2-AB-labeled monosialylated, biantennary oligosaccharide standard (A1).

At the same time a calibration curve was generated from which information about the peak area corresponding to a certain quantity of glycan could be derived (Fig. 8).

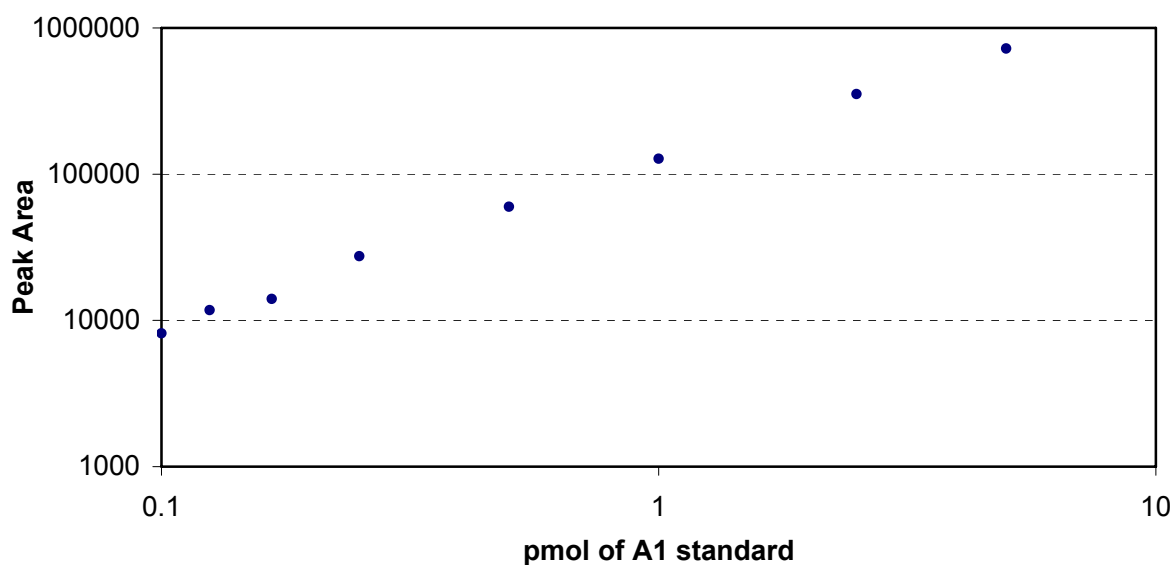


Fig. 8. Detection and quantitation limit of the IEC. Increasing amounts of the 2-AB-labeled oligosaccharide standard A1 (0.1-5pmol) were injected onto the GlycoSep C HPLC column and eluted with the gradient described in Materials and Methods. The fluorescent peak areas measured were plotted against the amount of glycan. Both axes are logarithmic. Values given are single measurements.

The fluorescence signal obtained from varying amounts of A1 between 0.1 and 5pmol was linear over the range tested. The baseline noise was less than 2% of the height of the peak obtained for 0.1pmol of oligosaccharide (not shown). We can therefore say that the detection as well as the quantitation limit of the ion exchange chromatography with fluorescence detection are below 0.1pmol for a single pure glycan species.

3.1.4 Minimal Amount of Glycans Necessary for Reliable Determination of Relative Abundances by Ion Exchange Chromatography

As described above, for a single pure glycan species quantitation by IEC was possible down to 0.1pmol of glycan. However, we observed that with small quantities of AGP glycans, i.e. highly diluted samples, the automatic baseline setting of the HPLC software, on the basis of which peak areas were calculated, was inconsistent, although correct chromatograms were obtained (superimposition showed no differences, data not shown).

To determine the minimal amount of glycan mixture to be injected onto the HPLC column for reliable quantitation, we analysed a dilution series of one of the AGP samples. The sample was injected undiluted (relative concentration 1.0) and diluted 1:2, 1:5, 1:10, 1:20 and 1:40 (relative concentration 0.5, 0.2, 0.1, 0.05 and 0.025 respectively).

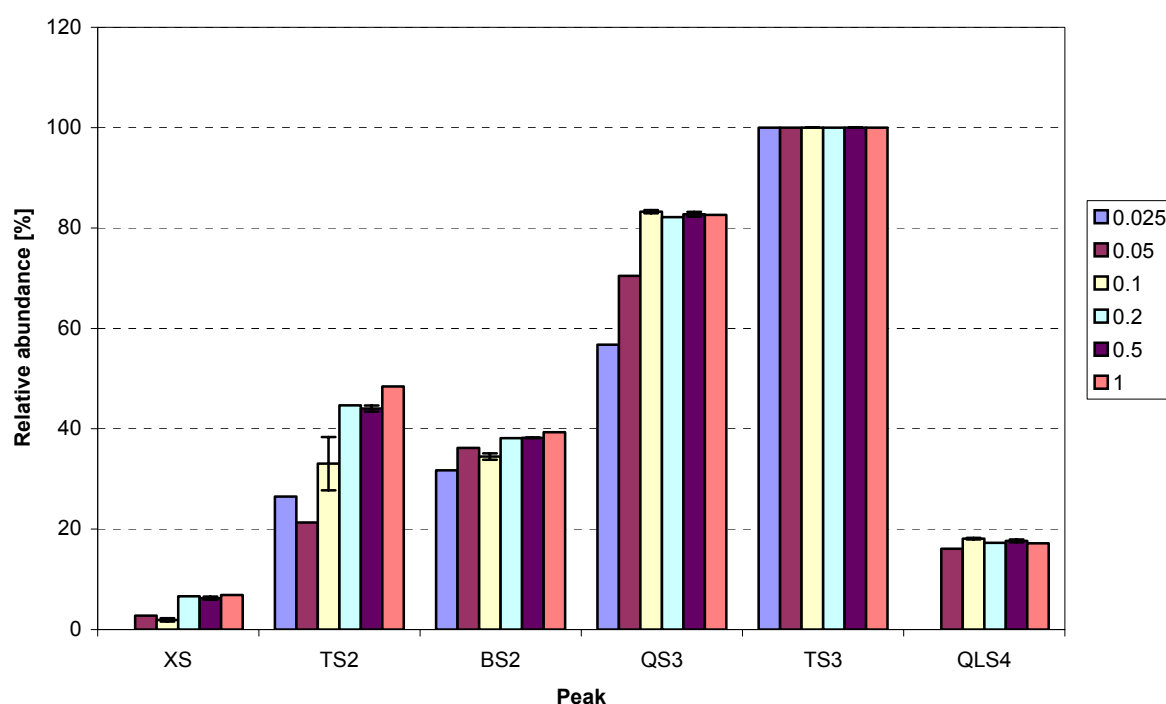


Fig. 9. Minimal amount of AGP glycan mixture to be injected onto the HPLC column for reliable determination of relative abundances. A dilution series of one of the AGP glycan samples was analysed by IEC. Relative abundances of major glycan types, determined on the basis of the peak areas calculated by the HPLC software, are shown. Numbers given in the legend correspond to relative concentrations of the sample which was applied undiluted (relative concentration 1.0), and diluted 1:2, 1:5, 1:10, 1:20 and 1:40 (relative concentrations 0.5, 0.2, 0.1, 0.05 and 0.025 respectively). Values given for 0.025, 0.05, 0.1, 0.2 and 1.0 are single measurements, values given for 0.1 and 0.05 are averages of three measurements and standard deviations are indicated. Symbols: **X** includes biantennary (**B**), triantennary (**T**) and tetraantennary (**Q**) N-glycans; **S** sialic acid residue; **L** lactosamine unit.

The relative abundances of major glycan types, determined on the basis of the peak areas calculated by the HPLC software, are shown in Fig. 9 for varying amounts of AGP glycan mixtures.

As can be seen, at a relative concentration of 0.025, i.e. a dilution of the AGP sample of 1:40, the HPLC software set baselines so as to give a distorted picture of the relative peak sizes compared to more concentrated samples and the smallest peaks (XS and QLS4) were no longer detected as such (blue columns missing).

As estimated using the calibration curve generated with the 2-AB-prelabeled oligosaccharide standard A1 (see Fig. 8) in this sample a total amount of glycans of approximately 1.8pmol was present.

At a relative concentration of 0.05 (corresponding to a total amount of glycans of about 3.6pmol) and a relative concentration of 0.1 (corresponding to a total amount of glycans of about 7.5pmol) some abundances, namely peak XS, TS2 and QS3 for 0.05 and peak XS and TS2 for 0.1, still differ from the corresponding ones determined for more concentrated samples.

The repeatability (intra-assay precision) of the IEC analysis at the relative glycan concentration of 0.1 was assessed by correspondingly diluting and measuring one of the AGP samples three times. The values given in Fig. 9 for sample 0.1 are averages of these three IEC measurements, with standard deviations indicated. Coefficients of variation (not shown in the figure) were below 1% for all peaks, except for XS (17.41%), TS2 (16.03%) and BS2 (1.86%). These results show that the relative abundances determined for small amounts of glycans not only differ from the ones determined for samples containing a higher amount of glycans, but they also vary considerably.

Because no differences were visible when chromatograms were superimposed (data not shown), these results must be due to the inconsistent automatic baseline setting by the HPLC software, on the basis of which peak areas were calculated.

Taken together, we suggest the maximal dilution of the AGP samples submitted to IEC to be 1:5, which corresponds to approximately 15pmol of glycans injected onto the column. As can be seen in Fig. 9 (samples 0.2, 0.5 and 1.0), with this or higher glycan amounts correct relative abundances were determined reliably using the present HPLC software.

The values and standard deviations given in Fig. 9 for the relative concentration 0.5 are the same as shown in Fig. 6. In contrast to the ones of sample 0.1, coefficients of variation were below 5% for all peaks.

3.1.5 Amount of Glycoprotein Necessary for the Analysis

With respect to our final goal, the analysis of sICAM-1, we wanted to determine the minimal starting amount of glycoprotein necessary to obtain correct results in the IEC analysis.

Therefore, AGP samples with different amounts of glycoprotein were submitted to the sample preparation procedure, comprising PNGase F release and 2-AB-labeling of the glycans, and analysed by IEC.

A sufficient amount of glycans, according to the results of the last section (Minimal Amount of Glycans Necessary for Reliable Determination of Relative Abundances by Ion Exchange Chromatography), was injected onto the HPLC column for all samples. Nevertheless, to completely dispel ambiguities due to the inconsistent automatic baseline setting by the HPLC software, which was discussed above, a superimposition of chromatograms rather than a comparison of relative sizes of major peaks is shown (Fig. 10).

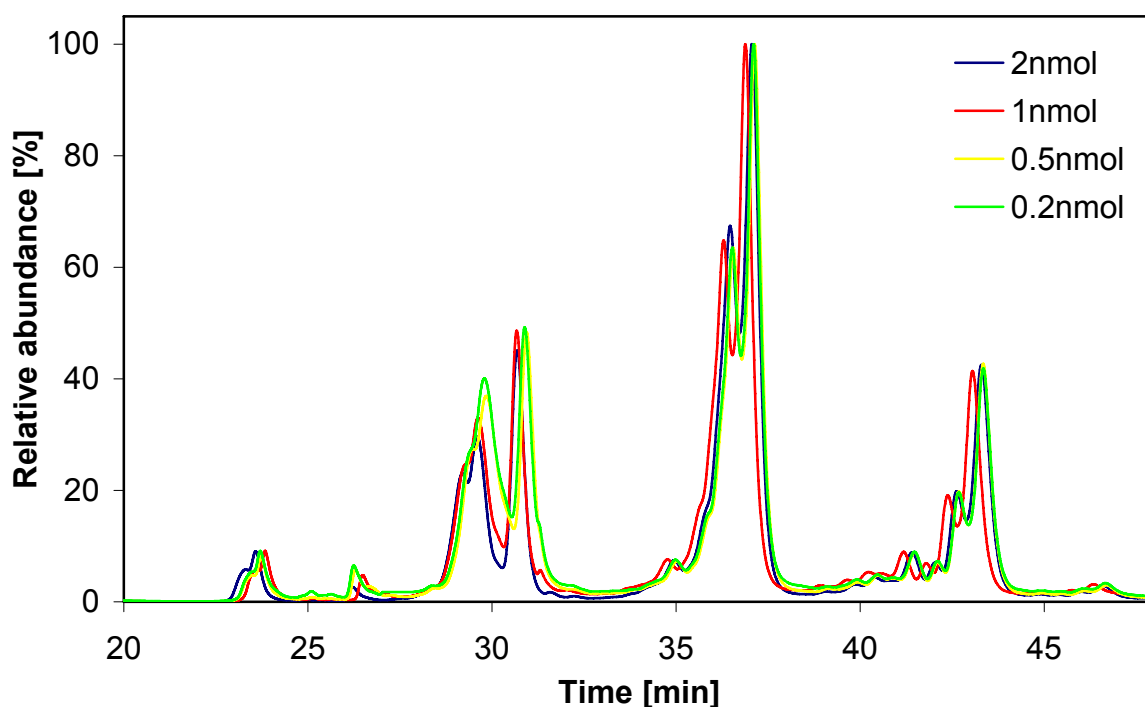


Fig. 10. Amount of glycoprotein necessary for the analysis. HPLC chromatograms obtained for AGP samples differing in the amount of glycoprotein submitted to PNGase F digestion (0.2 to 2nmol) were superimposed. Labeled oligosaccharides were injected onto the GlycoSep C HPLC column, which was equilibrated in 80% acetonitrile. The acetonitrile content was linearly decreased to 20% over 13min and then maintained at this level. At 13min, a linear gradient of ammonium acetate pH 4.5 was initiated, going from 0 to 160nM over 35min.

Correct results could be obtained with as little as 0.2nmol of AGP, as the chromatograms did virtually not differ at all, apart from the peak at around 29min, corresponding to tri- and

tetraantennary disialylated fucosylated and non-fucosylated species, which interestingly became larger relative to the other peaks the less glycoprotein was used for the analysis.

3.2 Removal of Sialic Acid from Complex-type N-Glycans of Human α_1 -Acid Glycoprotein by Treatment with Neuraminidase

To further test whether the degree of sialylation can be easily monitored by IEC, two samples of AGP were treated with neuraminidase prior to deglycosylation by PNGase F. In both cases neuraminidase was applied in great excess (5mU or 10mU per nmol of glycoprotein), but digestion was performed at pH 7.4, which is considerably higher than the pH optimum for neuraminidase activity (around pH 5) but optimal for subsequent PNGase F treatment. For one of the samples neuraminidase ex. *Vibrio cholerae* (5mU/nmol) was used, the other one was treated with the enzyme from *Arthrobacter ureafaciens* (10mU/nmol). Removal of sialic acid was verified by Western blot, where a shift of the protein bands towards lower apparent molecular weights could be observed (data not shown).

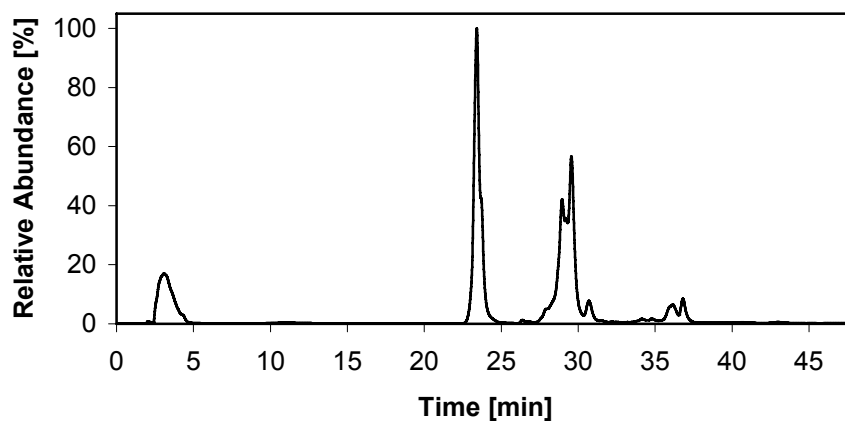
The ion exchange chromatograms obtained for the neuraminidase treated AGP samples, in comparison to that of an untreated sample, are shown in Fig. 11.

The charge profile of both samples changed considerably compared to the fully sialylated sample, and the sample digested with neuraminidase from *Arthrobacter ureafaciens* was desialylated more completely than the one treated with the enzyme from *Vibrio cholerae*.

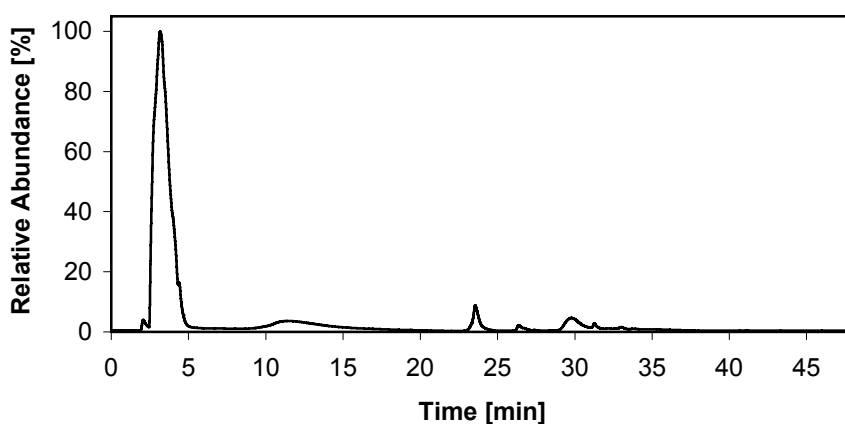
Peaks at approximately 23, 30, 37 and 42min retention time correspond to mono-, di-, tri- and tetrasialylated species respectively. Neutral glycans elute in the flow through peak (~3min), which in these samples was not exclusively due to residual free 2-AB, and in the small bump between 10 and 15min, as confirmed by mass spectrometry (Fig. 21, appendix p. 53).

These data illustrate that the present method is very sensitive in showing changes in sialylation.

A



B



C

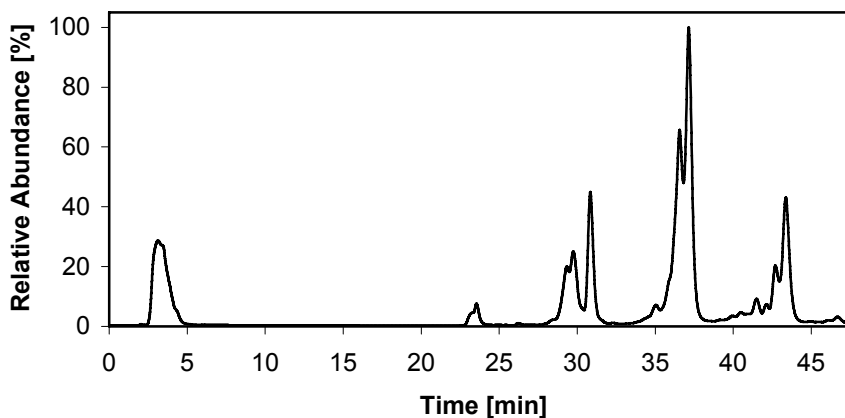


Fig. 11. IEC analysis of neuraminidase-treated complex-type N-glycans of human α_1 -acid glycoprotein. IEC chromatograms of the 2-AB-derivatized N-glycans released from human α_1 -acid glycoprotein pretreated with either neuraminidase ex. *Vibrio cholerae* (A), or neuraminidase ex. *Arthrobacter ureafaciens* (B) and without desialylation (C). Labeled oligosaccharides were injected onto the GlycoSep C HPLC column, which was equilibrated in 80% acetonitrile. The acetonitrile content was linearly decreased to 20% over 13min and then maintained at this level. At 13min, a linear gradient of ammonium acetate pH 4.5 was initiated, going from 0 to 160nM over 35min.

3.3 Preparation of Mouse sICAM-1 for Analysis

The final goal of the present study was to apply the optimized and validated procedure to mouse soluble intercellular adhesion molecule-1 (sICAM-1) expressed in CHO cells, the precise glycosylation pattern of which is as yet unknown.

3.3.1 Purity of sICAM-1 Samples

sICAM-1 to be used for the analysis of its N-glycosylation pattern was affinity purified from conditioned media. To assess how pure sICAM-1 was thereafter, we used SDS-PAGE followed by silver staining.

Since the heavily glycosylated proteins are poorly stained by conventional silver staining a periodic acid-silver stain for 1,2-diol groups of glycoproteins and polysaccharides in polyacrylamide gels [48] was performed. This stain facilitates detection of glycoproteins but still stains non-glycosylated proteins more strongly. Hence bands of glycoproteins must not be compared quantitatively to bands of non-glycosylated proteins. However, the analysis showed that sICAM-1 was quite pure with the main protein contaminant in the sample being bovine serum albumin (BSA), probably derived from the CHO cell culture media containing 10% fetal calf serum. There were only a few faint bands from other proteins (Fig. 12).

BSA is not glycosylated and therefore should not influence the analysis of the glycosylation pattern of sICAM-1.

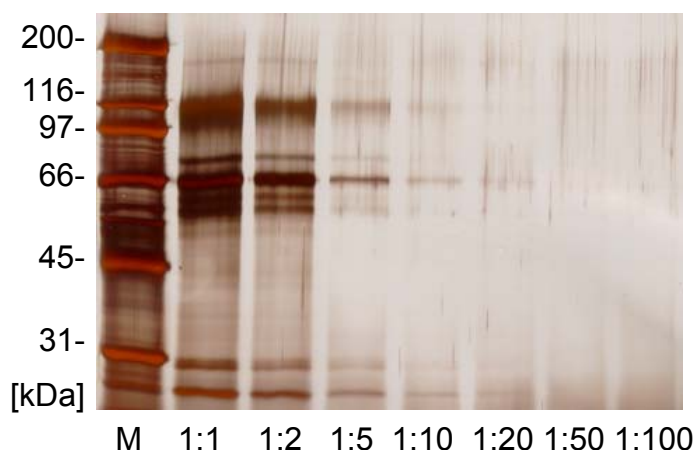


Fig. 12. Purity of sICAM-1 samples. Silver staining of concentrated solution of affinity purified sICAM-1, performed as described in Materials and Methods. sICAM-1 was applied undiluted and diluted 1:2, 1:5, 1:10, 1:20, 1:50 and 1:100 from left to right. **M** marker. Bands at 116kDa correspond to sICAM-1, bands at 66kDa to BSA.

3.3.2 Concentration Assessment

After affinity purification sICAM-1 was concentrated 13-fold. To be able to define the amount of protein solution needed for the analysis, its concentration was determined.

Protein concentration was assessed to be $2.1\mu\text{M}$ by measuring UV absorbance at 280nm. The UV spectrum obtained from the concentrated sICAM-1 solution in the wavelength range of 230 to 400nm is shown in Fig. 13.

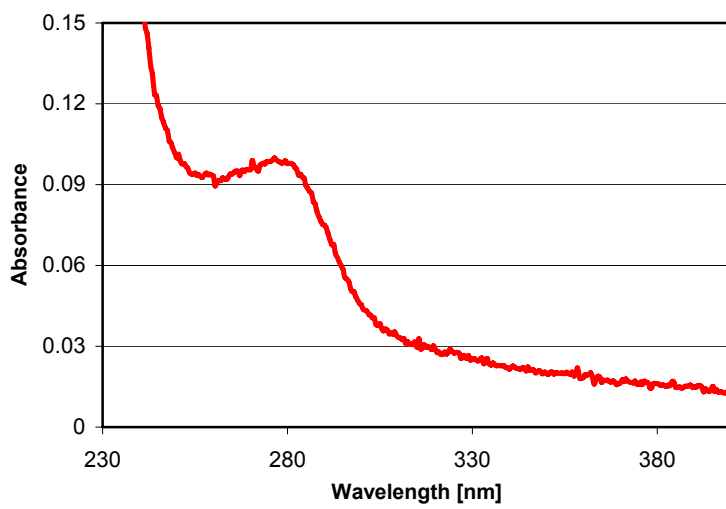


Fig. 13. Concentration assessment. UV spectrum of the affinity purified concentrated sICAM-1, wavelength ranging from 230 to 400nm.

Concentration was also measured by comparison to reference sICAM-1 from earlier batches with known concentrations, using Western blot (Fig. 14) and ELISA (Table 3).

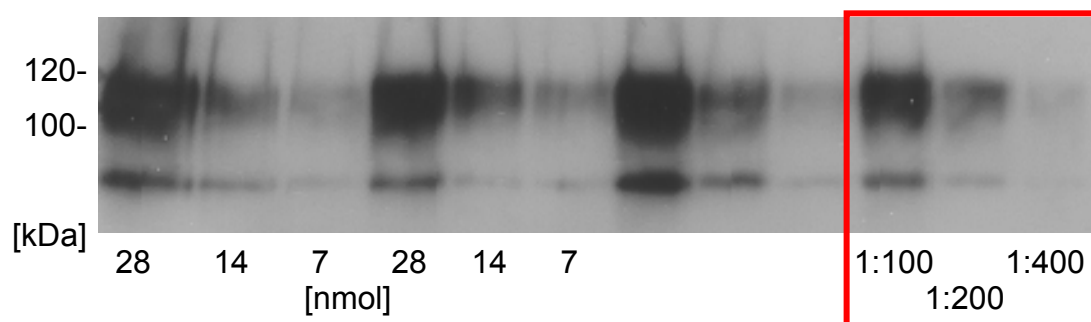


Fig. 14. Comparison of concentrations by Western blot. The first six bands on the left correspond to the indicated amounts of reference sICAM-1. The three bands on the right marked red represent the freshly purified, concentrated sICAM-1, diluted 1:100, 1:200 and 1:400.

Western blot showed that the concentration was in a range of approximately 2 to $3\mu\text{M}$.

The ELISA results are given in [ng/ml]. Since the standard sICAM-1 used for generating the ELISA calibration curve has an unknown molecular weight, no conversion from ng/ml into nM was possible.

Table 3. Comparison of concentrations by ELISA. Two reference proteins with approximately known concentrations were measured in duplicate each and the values given represent averages. The concentrated purified sICAM-1 was measured only once. The measured concentrations are given in [ng/ml] rather than [nM] because the molecular weight of the standard sICAM-1 contained in the ELISA kit was unknown.

| Protein | Approximate known Concentration [nM] | Measured Concentration [ng/ml] |
|-------------------------------|---|---|
| Reference sICAM-1 I | <100 | 2679 |
| Reference sICAM-1 II | 280 | 5798 |
| Concentrated purified sICAM-1 | 2000 | 88914 |

ELISA gave a 15 to 35 times higher concentration for the affinity purified and concentrated sICAM-1 than for the two reference proteins measured in parallel.

Taken together, comparative ELISA and Western blot confirmed that the concentration of the purified concentrated sICAM-1 was approximately 2 μ M as determined by UV spectroscopy, although UV absorbance may to a certain extent be due to other proteins in the solution.

3.3.3 Biological Activity Assay

Next, we tested whether our freshly purified, concentrated sICAM-1 was biologically active with regard to induction of MIP-2 secretion from primary mouse astrocytes.

Two independent experiments were performed in duplicate. Astrocytes were incubated with 9 and 37nM sICAM-1 for 24h. MIP-2 concentrations in the cell supernatants were then measured by ELISA (Fig. 15 and Table 4 (appendix p.54)).

Our freshly purified sICAM-1 strongly induced MIP-2 production in a dose-dependent manner.

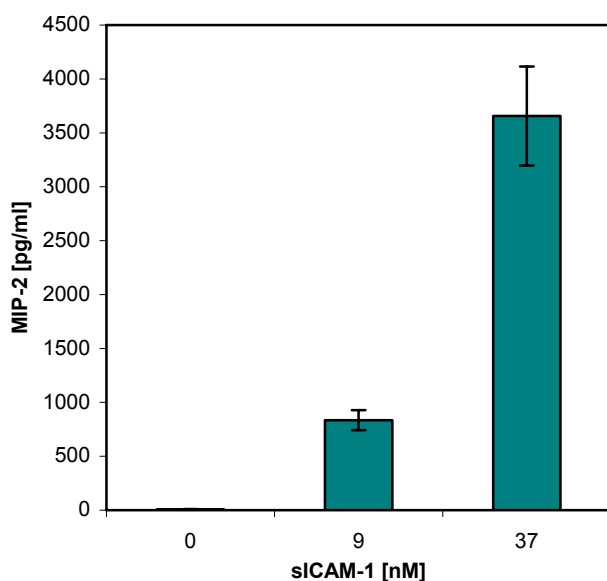


Fig. 15. Biological activity assay: Induction of MIP-2 secretion from primary mouse astrocytes by purified mouse sICAM-1. The cells were incubated for 24h with two concentrations of sICAM-1 (9 and 37nM) and with medium alone as negative control. MIP-2 concentrations were strongly elevated when sICAM-1 was present during the incubation time. The data are presented as average \pm SD of four assays (two independent experiments, each performed in duplicate).

3.4 Analysis of the N-Glycosylation Pattern of Mouse sICAM-1 by Ion Exchange Chromatography and MALDI TOF MS

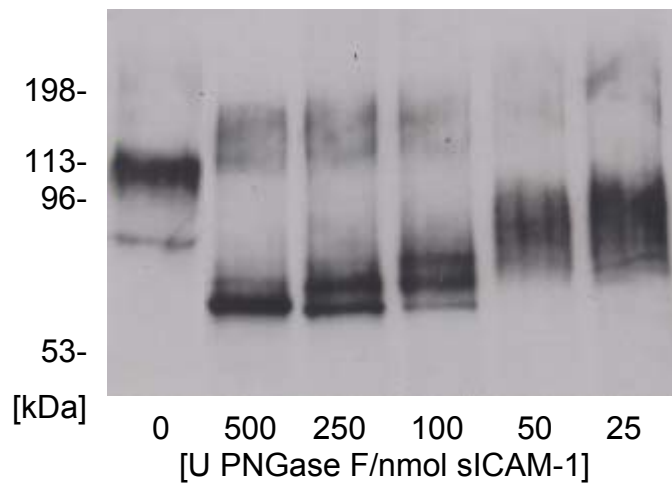
The purified, concentrated and biologically active sICAM-1 was submitted to the analytical procedure described above, starting with the release of N-glycans by digestion with PNGase F.

3.4.1 Release of N-glycans from sICAM-1 by PNGase F Digestion

The amount of PNGase F necessary for complete deglycosylation strongly depends on the glycoprotein. To find out how much PNGase F would be needed to completely deglycosylate sICAM-1, we treated sICAM-1 with increasing amounts of enzyme and monitored the removal of N-glycans from the protein by Western blot.

The minimal amount of enzyme necessary to remove all N-glycans from denatured mouse sICAM-1 within 16h at 37°C was found to be around 450U of PNGase F (Roche Diagnostics GmbH, Penzberg, Germany) per nmol of glycoprotein (Fig. 16).

A



B

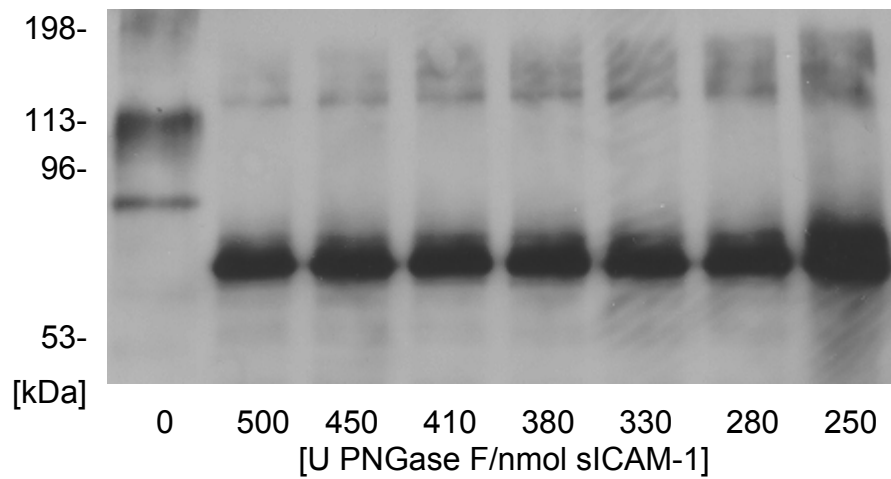


Fig. 16. Enzymatic release of N-glycans from mouse sICAM-1. The glycoprotein was incubated with different amounts of peptide N-glycosidase F (PNGase F) for 16h at 37°C. A) Preliminary estimation of approximate amount of enzyme necessary for complete removal of N-glycans from mouse sICAM-1. B) Exact determination of enzyme amount according to the range established in A.

3.4.2 Ion Exchange Chromatography of sICAM-1

Using the procedure established for AGP we intended to analyse carbohydrate chains of recombinant mouse sICAM-1 expressed in CHO cells. Two samples were individually prepared and analysed by IEC.

For both samples only a single peak was detected at about 3min retention time (Fig. 17).

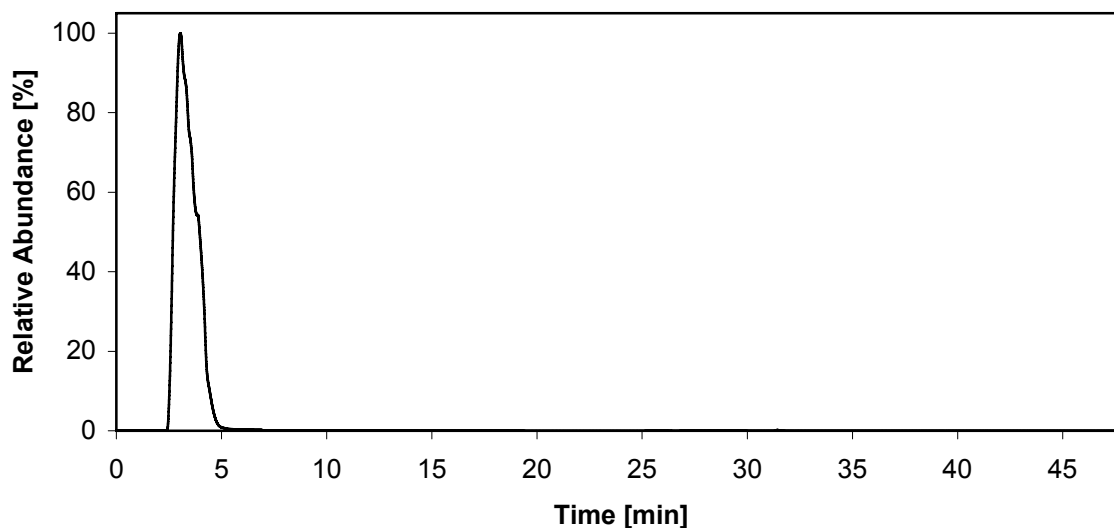


Fig. 17. HPLC chromatogram of the 2-aminobenzamide-derivatized N-glycans released from mouse soluble intercellular adhesion molecule-1. Labeled oligosaccharides were injected onto the GlycoSep C HPLC column, which was equilibrated in 80% acetonitrile. The acetonitrile content was linearly decreased to 20% over 13min and then maintained at this level. At 13min, a linear gradient of ammonium acetate pH 4.5 was initiated, going from 0 to 160nM over 35min.

This chromatogram suggests that either no glycans were present or the labeling reaction with 2-AB failed, thus glycans were not detected although present.

3.4.3 MALDI TOF MS

To test these possibilities we analysed the collected peak fraction as well as the complete sICAM-1 sample by mass spectrometry. No glycans were found (data not shown), suggesting that the peak detected in the ion exchange chromatogram is exclusively due to residual free 2-AB.

4 Discussion

In the present work a robust methodology was established for characterizing the N-glycosylation pattern of proteins, encompassing ion exchange chromatography of released N-glycans and MALDI TOF MS measurement of peak fractions.

We used human α_1 -acid glycoprotein (AGP) as a reference glycoprotein to validate and optimize the technique. This glycoprotein resembles our protein of interest, mouse soluble intercellular adhesion molecule-1 (sICAM-1), with regard to its glycosylation pattern and its acidic pI [2, 43]. It contains five highly sialylated, complex-type N-linked glycans [37, 41].

Analysis of AGP showed that ion exchange chromatography superbly resolved the fluorescently labeled sialic acid-containing oligosaccharides based on the number of sialic acid residues present. Species with the same degree of sialylation but different numbers of antennae were also partly resolved. Assignment of the HPLC elution positions of the different charge states and numbers of antennae was based on different sialylated standard oligosaccharides and comparison to the chromatogram given in [13]. It should however be noted that no direct identification is possible exclusively by IEC.

MALDI TOF MS measurement of the peak fractions from IEC allowed to identify the structures of sialo-oligosaccharides. The results of mass spectrometry are in agreement with the HPLC analysis but give a much more detailed picture as all species contained in the peaks can be identified unambiguously, especially fucosylated and nonfucosylated species as well as lactosamine-extended structures which are not clearly resolved by HPLC.

In agreement with previous reports of several research groups, we found sialylated bi-, tri- and tetraantennary oligosaccharides with trisialylated triantennary glycans being the most abundant species in human AGP. No neutral oligosaccharides were found. Some of the tri- and tetraantennary oligosaccharides contained a fucose residue, most probably to form sialyl Lewis x structures. Minor quantities of difucosylated tetraantennary tri- and tetrasialylated structures were identified and small amounts of lactosamine-extended tetraantennary glycans were present as well.

It should be noted that the combination of IEC and MALDI TOF MS does not allow to determine the positions on the glycans where sialic acid, fucose residues and lactosamine repeats are attached. It has been reported that the fucose in human AGP usually occupies a site

on one of the GlcNAc residues of the antennae of the larger structures rather than on the first GlcNAc of the chitobiose core [37].

Furthermore the method does not resolve linkage isomers such as α 2-3- and α 2-6-linked sialic acid. Earlier work using the α 2-3-specific sialidase from Newcastle disease virus has shown that in human AGP sialic acid is present in both α 2-3- and α 2-6-linkage [49].

The fact that the profile determined by ion exchange chromatography very clearly shows the degree of sialylation of the glycan pool was corroborated by the analysis of partly desialylated samples. Complete desialylation was not achieved even with the very active enzyme from *Arthrobacter ureafaciens*. Complete removal of sialic acid residues not being the principal objective of this study, the pH for neuraminidase digestion was not adjusted to the optimum of around five in order to maintain ideal conditions for the subsequent PNGase F treatment.

Relative intensities of the peaks in the ion exchange chromatograms as well as in the mass spectra can be used in a comparative manner to provide information on the relative abundances of oligosaccharide structures. Here again, mass spectra provide more detailed information on all species present whereas HPLC only reveals the relative abundance of major species which are resolved. Nevertheless, the HPLC step prior to mass spectrometry is important to gather isocharged species because desialylation cannot be completely avoided during the MALDI process or the preceding cleanup step, making identification and quantification exclusively by mass spectrometry unreliable with regard to the sialylation profile.

The results summarized in Fig. 6 and Fig. 7 illustrate the excellent reproducibility of the overall method up to the chromatography step, despite its complexity. Variability in retention times is 1% at most. The elution positions being so exact, the HPLC profile may be interpreted directly to predict the structures of the major glycans which elute in a given peak. This allows a rapid general overview of total sugar prints.

Relative abundances were also very reproducible, making the HPLC approach well-suited for the quantitation of major glycan species contained in a mixture derived from a glycoprotein.

Achieving a low variability of results over a long period of time is particularly important if the method is used for monitoring batch-to-batch consistency. As shown in Table 2, very reproducible results were achieved with six individually prepared AGP samples analysed by two independent investigators at three different time points in a six-month period, which demonstrates the robustness of the method.

Regarding the specificity of the method, errors could possibly occur due to aldehydes other than reducing sugar termini labeled by reductive amination with 2-AB. This source of error is eliminated by the pre- and post-labeling purification of the glycans as well as the unequivocal identification of glycan structures by MALDI TOF MS.

Despite all the advantages, there are some drawbacks of the technique, including in particular the rather large amount of glycoprotein (ideally 1nmol) which is needed to achieve reliable results (see 3.1.5 Amount of Glycoprotein Necessary for the Analysis) as well as the relatively low sample throughput due to the complex sample preparation procedure. However, the suggested 1nmol of starting material is not absolutely mandatory, as variations are in an acceptable range in chromatograms obtained with only 0.2nmol of glycoprotein.

Using the standard curve generated with the prelabeled standard glycan (see p. 26, Fig. 8) it was estimated that about 60 to 90% of the glycans initially contained in the sample were lost during the preparation procedure. Thus starting with 1nmol of AGP, which theoretically contains 5nmol of N-glycans, yields between 500pmol and 2nmol of 2-AB-derivatized glycans ready for profiling.

For the HPLC with fluorescence detection, we determined a detection limit of less than 0.1pmol of glycan, and it was even reported for a similar system, that the detection limit was as low as 10fmol [50]. The simple detection of glycans using the present HPLC technique therefore does not constitute a problem.

However, as outlined under 3.1.4 (Minimal Amount of Glycans Necessary for Reliable Determination of Relative Abundances by Ion Exchange Chromatography, p. 27f.), care must be taken when interpreting peak areas calculated by the HPLC software as baseline setting is often inconsistent, especially when small total amounts of glycans are profiled.

Therefore, using the present instrumentation and software the amount of glycans injected should not be below approximately 15pmol, we even recommend to use 40 to 50pmol, although correct chromatograms can be obtained with less material, as long as there is no need for quantitation on the basis of the peak areas calculated by the software.

Obviously, if peak fractions are to be collected and structures identified by mass spectrometry, it is important to submit a sufficient total amount of glycans to the ion exchange chromatography. Based on our experience with AGP, we recommend 50 to 100pmol.

The detection limit for MALDI TOF mass spectrometry was reported to be about 500fmol in 1998 [51] but great progress in this technique has been made since then. For our MALDI system we very roughly estimated the detection limit to be in the low femtomolar range (see appendix p.55).

Despite the limitations mentioned above and the ones concerning linkage information, the combined analysis by HPLC and MALDI TOF MS as established by Ali [1] and validated and optimized in this study is very valuable as a first step in the analysis of glycoprotein oligosaccharides.

Additional structural information on linkages, such as Gal(1-3)GlcNAc versus Gal(1-4)GlcNAc, NeuAc(2-3)Gal versus NeuAc(2-6)Gal, and (2,4)- versus (2,6)-disubstituted mannose residues could be derived from methylation analysis [52], tandem mass spectrometry experiments (MS/MS) [9, 53, 54] or the combination of exoglycosidase digestions with mass spectrometry [55].

The objective of optimization and validation of our analytical procedure using α_1 -acid glycoprotein was to find out whether it was suitable for its intended purpose, namely glycan profiling of sICAM-1.

A concentrated solution of affinity purified sICAM-1 with the non-glycosylated BSA derived from the CHO cell culture media as the main protein contaminant was used for the analysis. N-linked glycans could successfully be released from the glycoprotein by PNGase F, although a huge amount of enzyme was needed compared to α_1 -acid glycoprotein.

As verified by mass spectrometric analyses of the single peak as well as the complete sICAM-1 samples, all N-linked glycans were lost during the sample preparation procedure.

Most probably the loss occurred during the extensive washing which was necessary to remove the large amounts of buffer salts and, above all, detergents needed for the denaturation of the glycoprotein and the subsequent PNGase F digestion. As the concentration of the sICAM-1 solution was about 60 times lower than the one of the commercial AGP solution, a 60 times larger sample volume was taken for sICAM-1 to start with the same amount of glycoprotein. Therefore, higher amounts of buffer salts were present and – probably most disturbing – larger amounts of SDS and NP-40 had to be added for denaturation of the protein and PNGase F digestion, respectively, which could hardly be removed. It is also conceivable that these even prevented binding of the glycans to the graphitized carbon packing, although no previous reports concerning this problem were found. The mechanism of carbon interaction with carbohydrates is poorly understood and involves both absorption and hydrophobic interaction [44].

To reduce the large volume of the sample in future experiments, the main objective should be to further concentrate the sICAM-1 solution, either with the help of a centrifugal filter device (Centricon[®]) as already done before, by lyophilisation or by protein precipitation. While the use of a Centricon[®] concentrator would result in loss of glycoprotein due to adsorption to the

device and lyophilization in concentration of salts and other contaminants, protein precipitation enables concentration of the protein sample as well as purification from undesirable substances without significant loss of protein. Therefore precedence should be given to the latter approach.

Out of various protein precipitation methods [56] we prefer the one with ice-cold acetone, as it does not involve any chemicals like for instance trichloroacetic acid (TCA) with uncertain effect on the carbohydrate chains and in particular sialic acid content of sICAM-1. A protocol can be found in [56] or on <http://www.piercenet.com> (Technical Resource TR0049.0).

As a less time-consuming alternative the methanol-chloroform precipitation may be considered [57].

To assess the remote possibility of BSA present in the glycoprotein solution interfering with the glycan profiling, an AGP sample with BSA added could be submitted to the procedure. If interference indeed occurs and results differ from the present ones, BSA must be removed from the sICAM-1 solution e.g. by albumin affinity chromatography or size exclusion. However this should constitute the last possibility as it will most probably be associated with considerable losses of sICAM-1.

Once the sample is successfully prepared, the characterization of the glycosylation pattern of mouse sICAM-1 using the present technique possibly requires further optimization concerning the HPLC analysis. In contrast to α_1 -acid glycoprotein, sICAM-1 might contain small amounts of neutral high-mannose-type N-glycans.

The present amine-bonded HPLC method enables a clear distinction between neutral and charged N-linked oligosaccharides. It should be able to separate oligomannosidic type structures according to size in addition to the separation of sialylated oligosaccharides on the basis of charge and size in one chromatographic step.

An advantage of amine-bonded columns is the ability to perform both as a hydrophilic interaction media and as an ion exchange stationary phase [58]. In the initial step of the gradient, neutral oligomannosidic structures could be eluted by increasing the polarity of the mobile phase using water ("normal phase" conditions). This step could be varied by extending it in terms of time (slower decrease of acetonitrile content) as well as in terms of the initial acetonitrile concentration (start with higher acetonitrile content) in order to optimize resolution. As soon as the acetonitrile concentration will have been reduced to 20% the linear ammonium acetate gradient could be initiated as described, to elute mono-, di-, tri- and tetrasialylated species according to their charge.

The ability to resolve neutral and acidic glycans simultaneously is particularly useful where acidic sugars are likely to play a functional role as in the case of the MIP-2 induction from mouse astrocytes by mouse sICAM-1.

In future studies, the technology could be applied to compare the glycosylation of sICAM-1 expressed in CHO cells to the glycosylation of sICAM-1 synthesized in different mouse tissues under different pathophysiological conditions. As glycosylation depends on the cell type expressing a glycoprotein as well as on the disease state the signaling function of sICAM-1 might be favoured in specific cell types and/or under certain pathophysiological conditions [2].

To derive more structural information, the present technique could even be combined with exoglycosidase digestions as described in [50]. For example, sialic acid linkage could be determined using the α 2-3-linkage-specific sialidase from Newcastle disease virus. This might be interesting for sICAM-1 expressed in cell types other than CHO cells. The latter synthesize sialylated glycans with sialic acid occurring in α 2-3-linkage only [59].

5 Acknowledgments

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7 Appendix

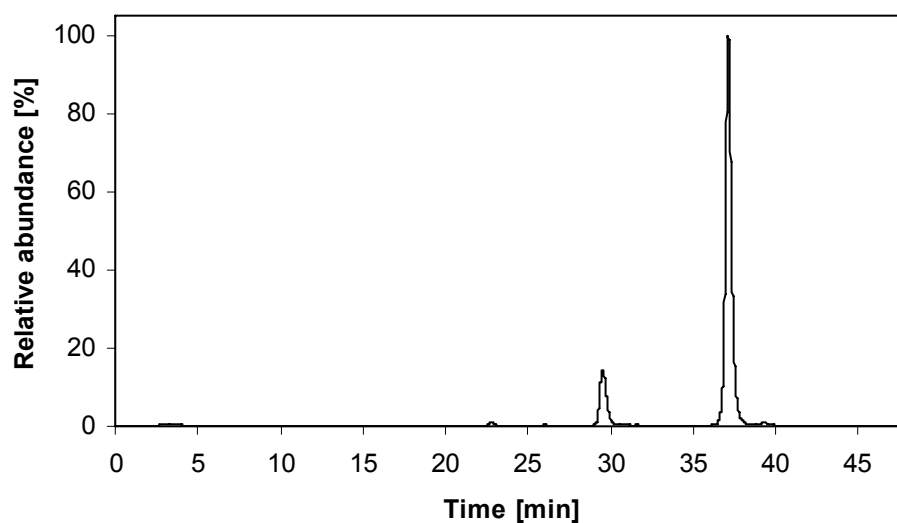


Fig. 18. HPLC chromatogram of the 2-AB-derivatized trisialylated triantennary oligosaccharide standard **A3**. As verified by mass spectrometry, the smaller peaks at retention times around 30min and around 23min correspond to di- and monosialylated species, resulting from partial desialylation of **A3** (retention time approximately 37min).

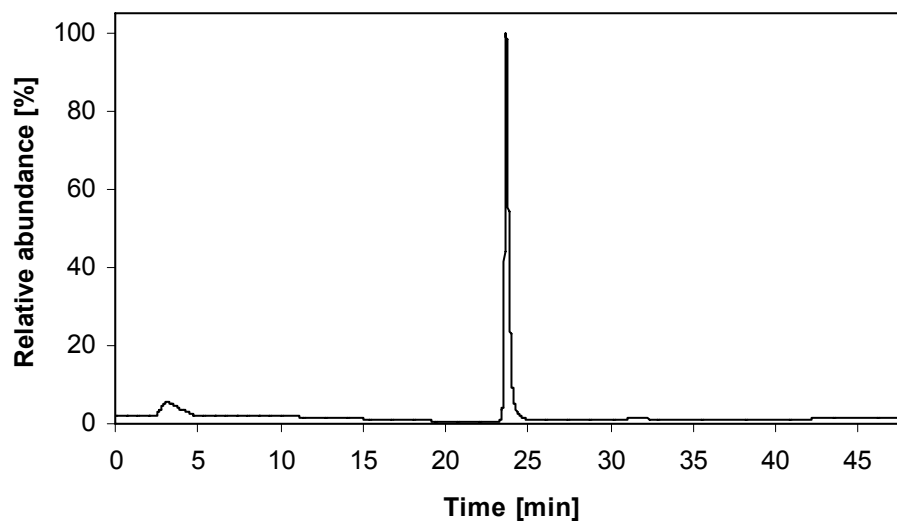
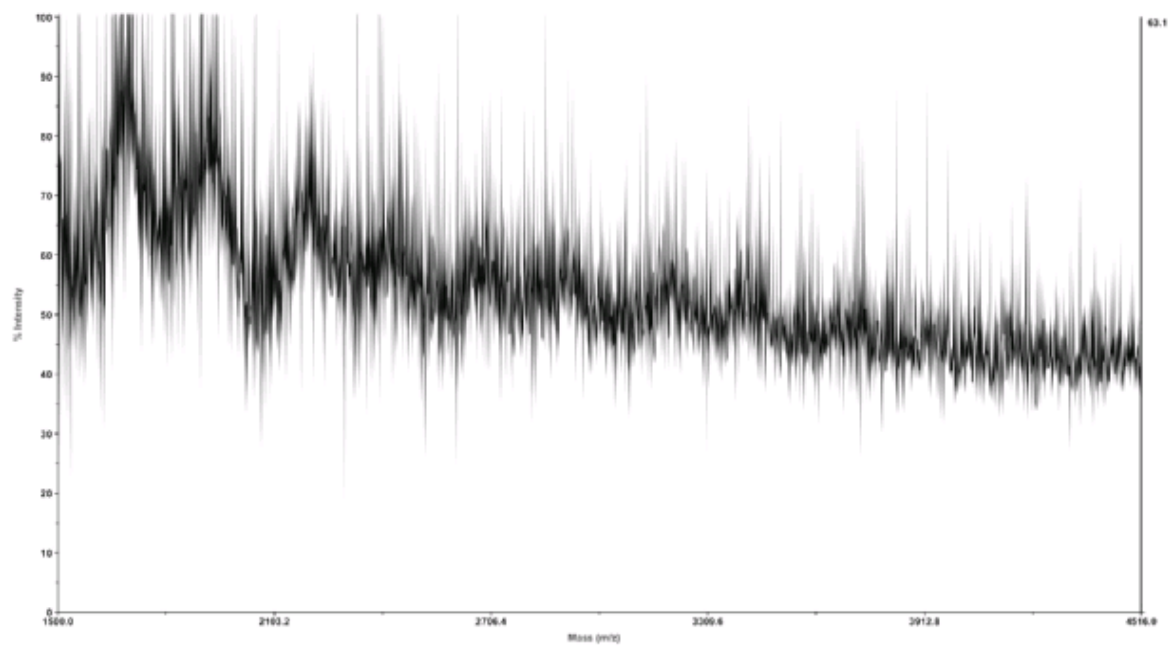
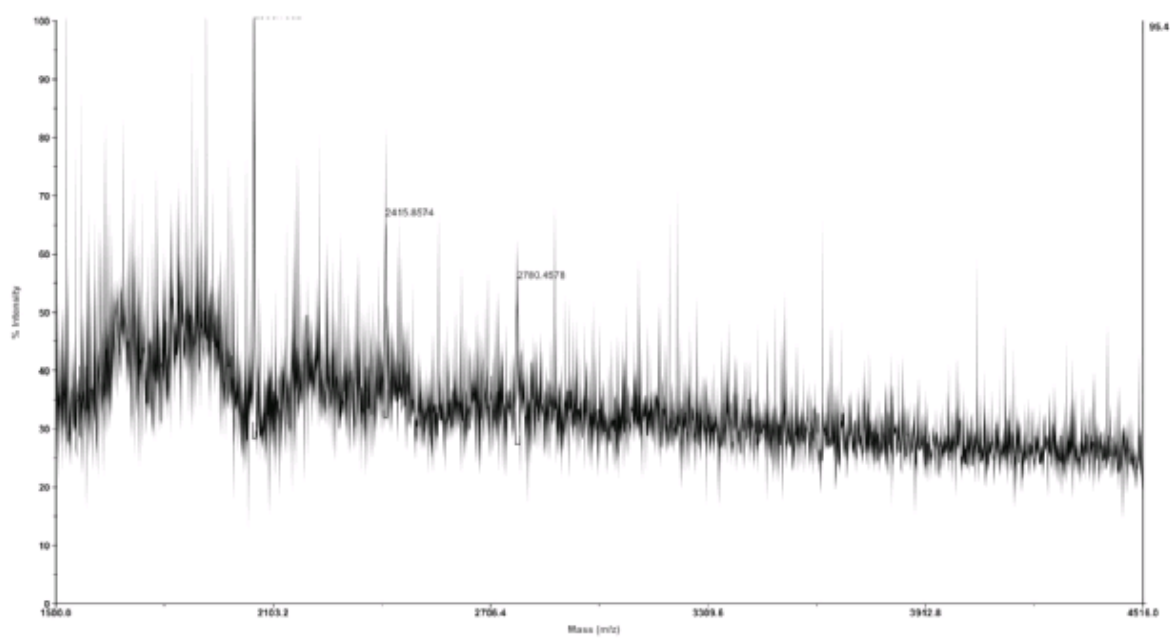


Fig. 19. HPLC chromatogram of the 2-AB-prelabeled monosialylated biantennary oligosaccharide standard **A1**.

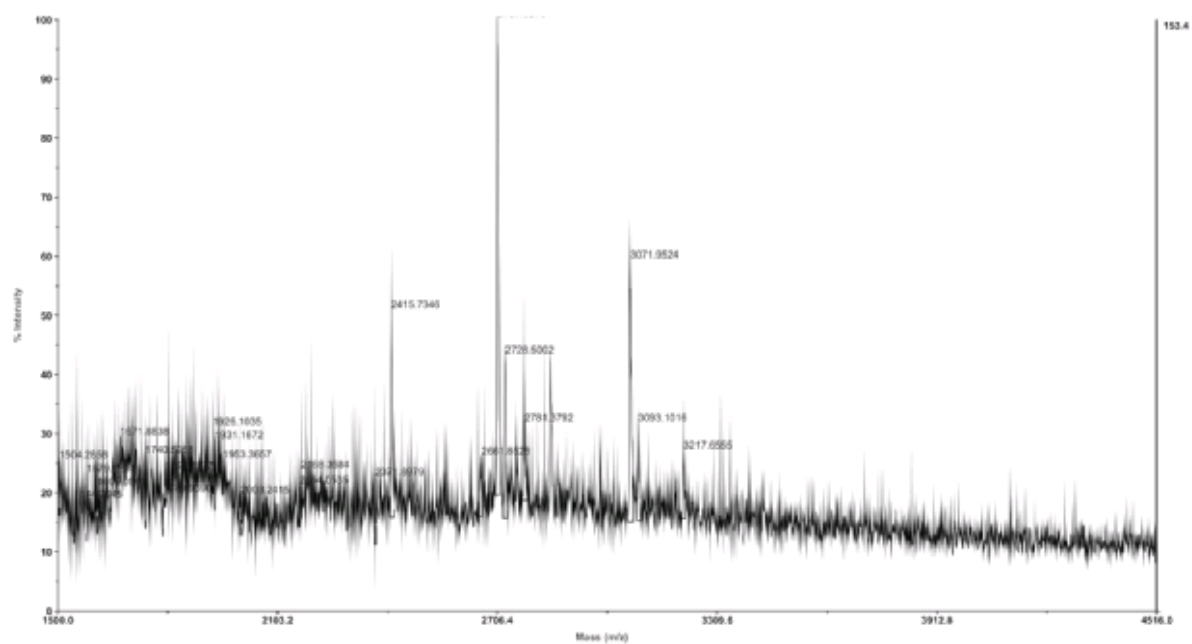
A



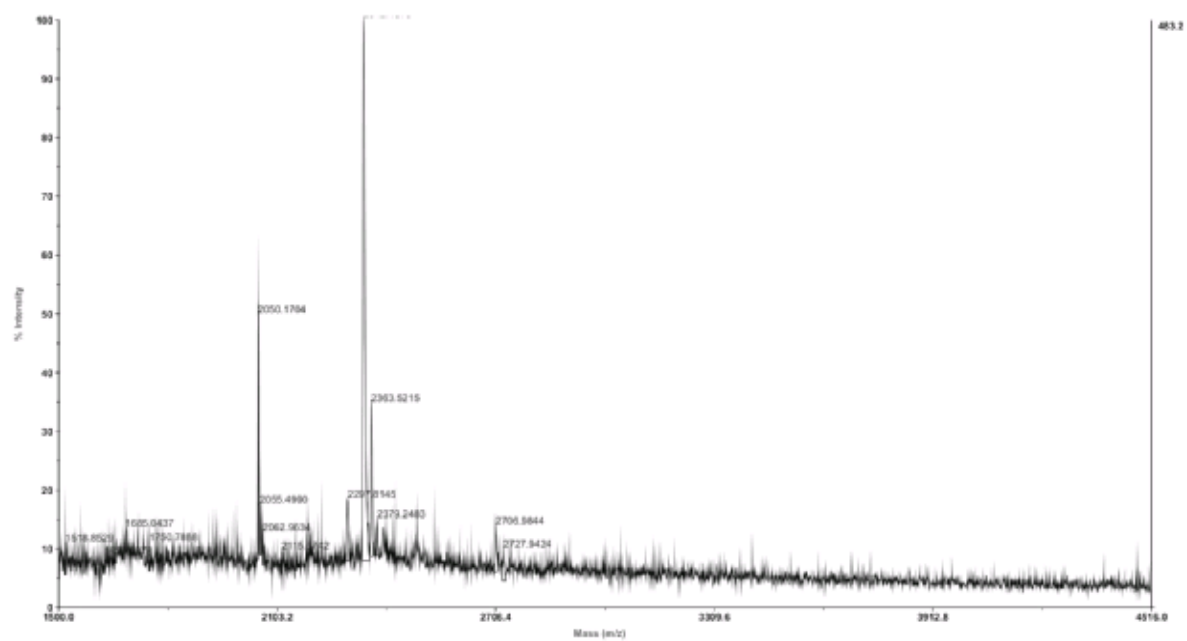
B



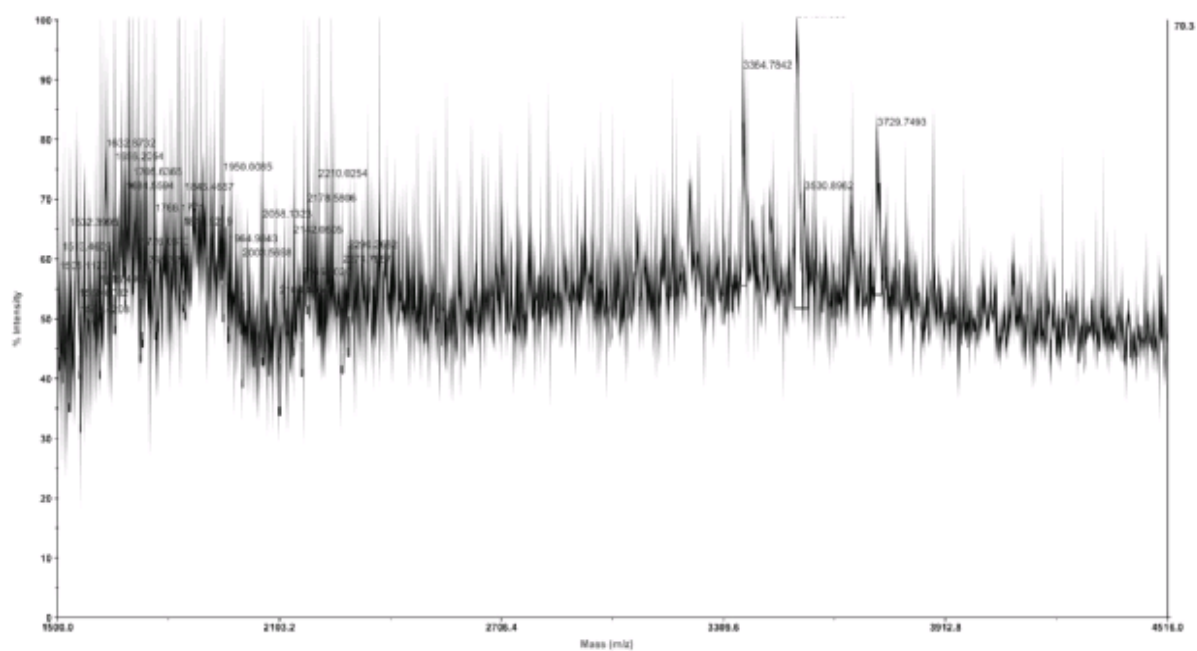
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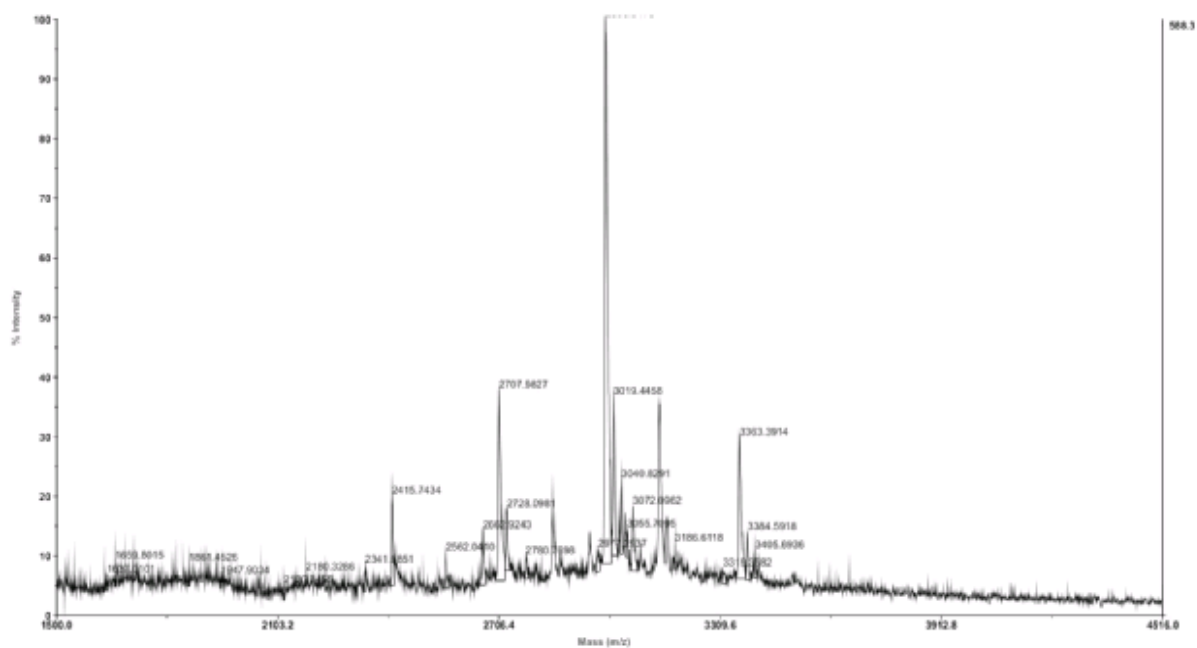
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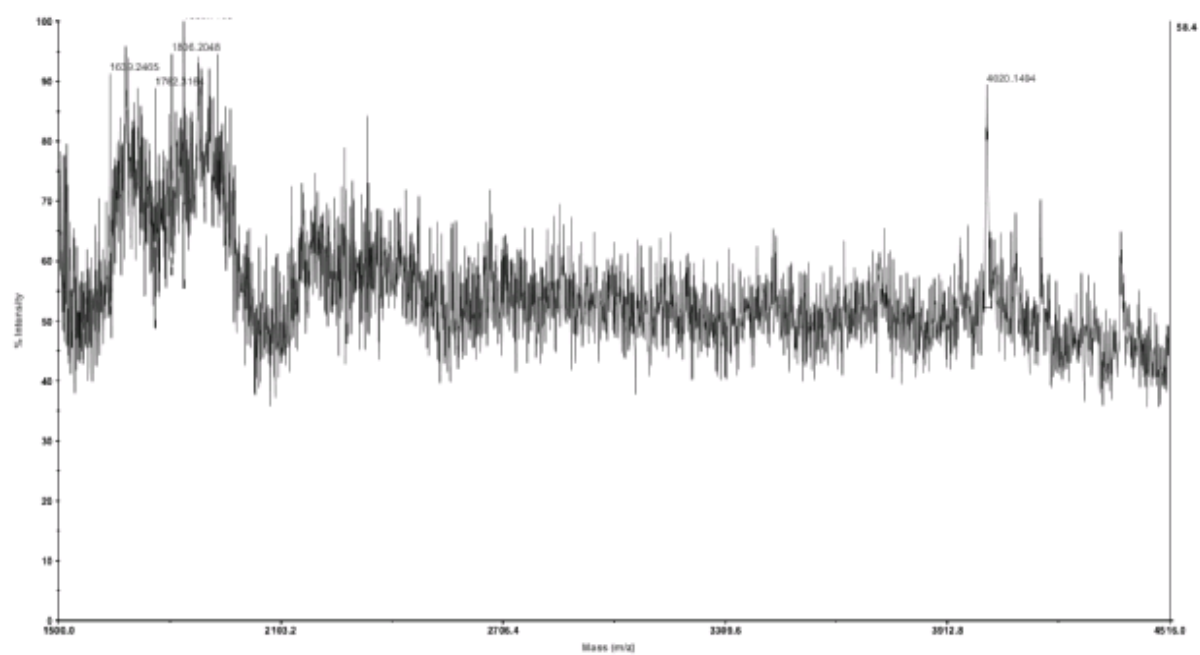
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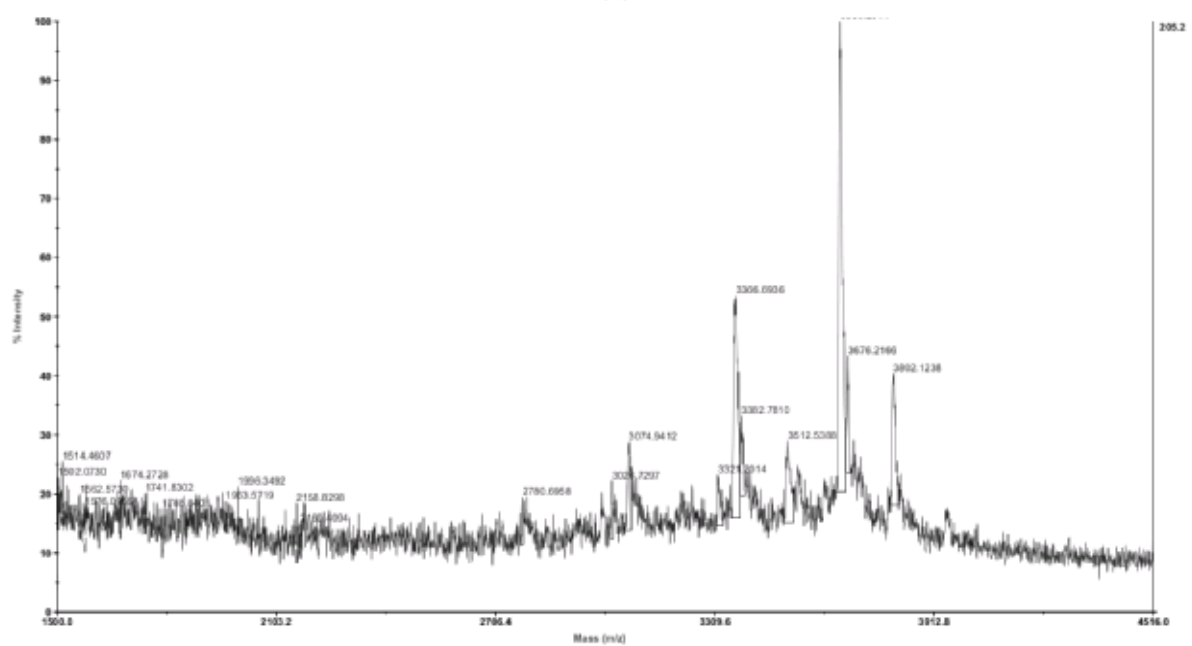
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G



H



I

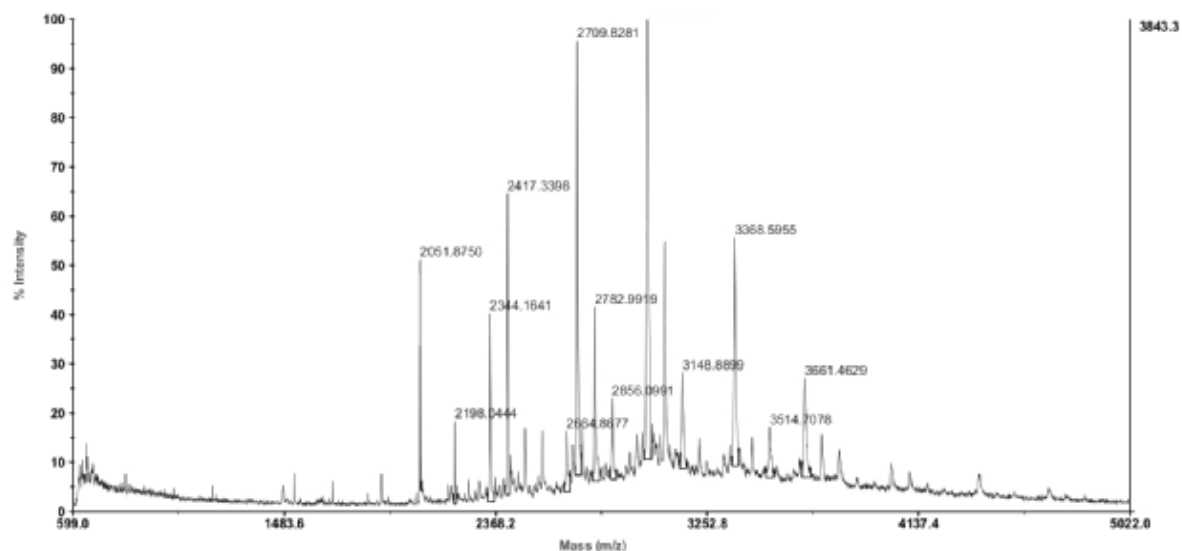
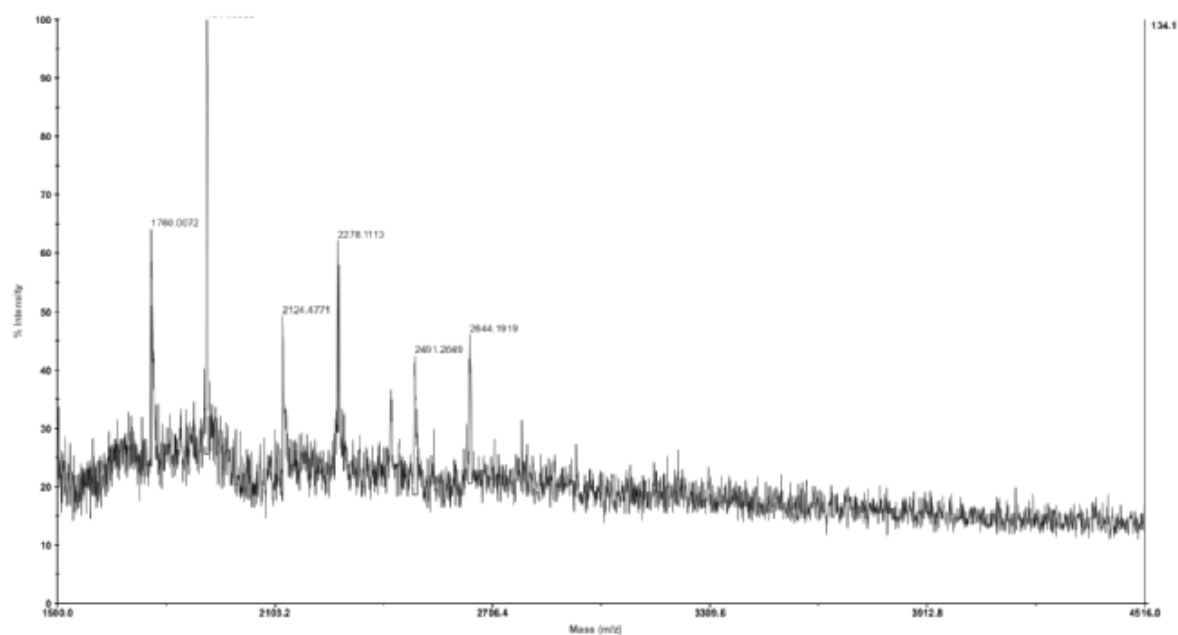


Fig. 20. MALDI TOF mass spectra of HPLC peak fractions of sialylated AGP glycans. Spectra were recorded in negative linear mode, as described in Materials and Methods. A) IEC retention time 2-4.5min, no glycans were detected, indicating residual free 2-AB to be responsible for this peak; B) retention time 22-25min, bi-tri- and tetraantennary monosialylated species were detected; C) retention time 28-30.5min, tri- and tetraantennary disialylated glycans and their corresponding fucosylated structures were detected; D) retention time 30.5-33.5min, biantennary disialylated glycans were detected; E) retention time 33.5-36.5, minor quantities of tetraantennary trisialylated mono- and difucosylated structures were detected; F) retention time 36.5-39, tetraantennary trisialylated structures along with triantennary trisialylated fucosylated and non-fucosylated structures were detected; G) retention time 39.5-42.5, minor quantities of polylactosamine-extended, tetraantennary tetra-sialylated fucosylated and non-fucosylated glycans were detected; H) retention time 42.5-45min, tetraantennary tetra-sialylated mono- and difucosylated and non-fucosylated glycans were detected. Some desialylation occurred in the procedure following the collection of HPLC fractions, therefore in most spectra less sialylated structures than correspond to the collected peak are found in addition to the ones mentioned. I) Mass spectrum of AGP glycans without prior separation by ion exchange chromatography.

A



B

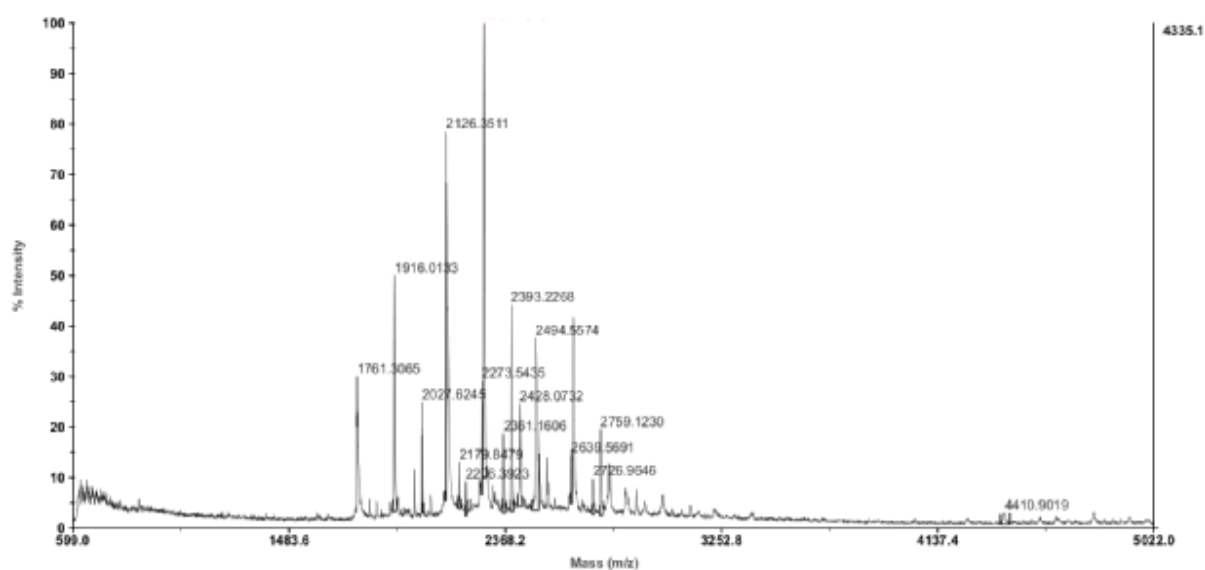


Fig. 21. MALDI TOF mass spectra of desialylated AGP glycans eluted in the HPLC peak at retention time ~3min. Spectra were recorded in negative linear mode, as described in Materials and Methods. Mass spectra of collected peak fractions (retention time ~3min) from samples digested with neuraminidase ex. *Vibrio cholerae* (A), or neuraminidase ex. *Arthrobacter ureafaciens*. For the corresponding chromatograms see Fig. 11 (p. 31). For a mass spectrum of the flow through peak of a sample without neuraminidase treatment see Fig. 20 A.

Table 4. Activity assay. MIP-2 concentrations measured by ELISA in supernatants of primary mouse astrocytes incubated with 0, 9 and 37nM sICAM-1 for 24h. Two independent experiments were performed in duplicate each. **SD** standard deviation; **CV** coefficient of variation.

| sICAM-1 [nM] | 1st experiment | | 2nd experiment | | Average [pg/ml] | SD | CV [%] |
|-----------------|------------------|------------------|------------------|------------------|--------------------|-------|-----------|
| | MIP-2 [pg/ml] | MIP-2 [pg/ml] | MIP-2 [pg/ml] | MIP-2 [pg/ml] | | | |
| 0 | 8.8 | 3.0 | 10.8 | 5.8 | 7 | 3.0 | 41.7 |
| 9 | 919.4 | 697.5 | 801.4 | 921.5 | 835 | 93.1 | 11.1 |
| 37 | 4227.9 | 3923.7 | 3017.8 | 3455.0 | 3656 | 460.0 | 12.6 |

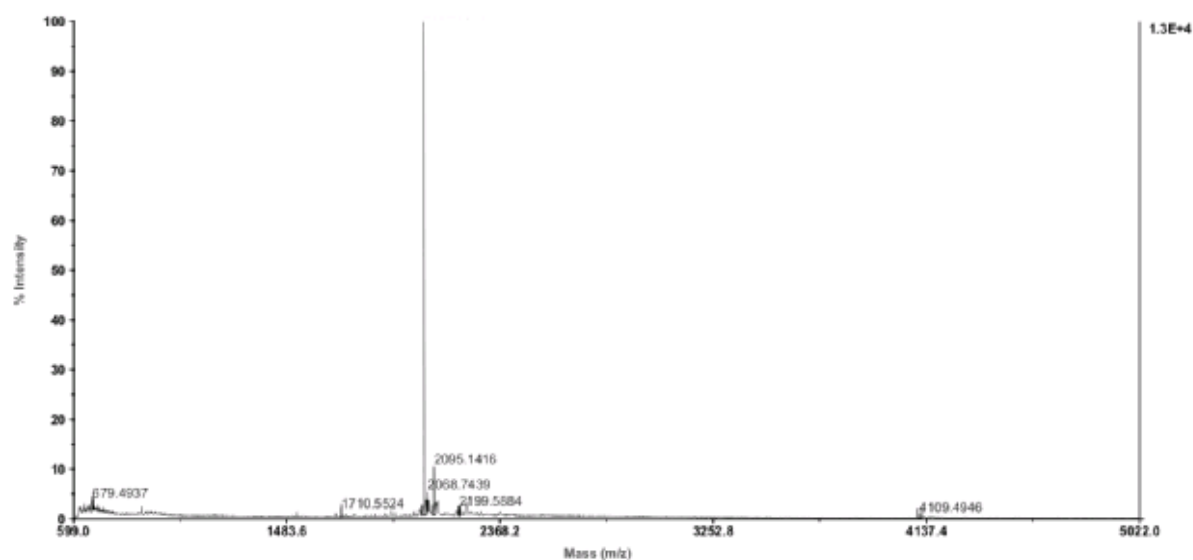


Fig. 22. MALDI TOF mass spectrum of the biantennary, monosialylated oligosaccharide standard A1. The spectrum was recorded in negative linear mode, as described in Materials and Methods. 5pmol of the standard were spotted on the MALDI target.

5pmol of the oligosaccharide standard A1 resulted in a signal of 13000. By comparing this to spectra with signals hardly above baseline noise (e.g. Fig. 20 E, signal height ~50) the detection limit for MALDI TOF MS was very roughly estimated to be around 0.02pmol.