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Influence of lactation parameters on the N-glycosylation of recombinant human C1 inhibitor isolated from the milk of transgenic rabbits

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The large-scale production of recombinant biopharmaceutical glycoproteins in the milk of transgenic animals is becoming more widespread. However, in comparison with bacterial, plant cell, or cell culture production systems, little is known about the glycosylation machinery of the mammary gland, and hence on the glycosylation of recombinant glycoproteins produced in transgenic animals. Here the influence is presented of several lactation parameters on the N-glycosylation of recombinant C1 inhibitor (rhC1INH), a human serum glycoprotein, expressed in the milk of transgenic rabbits. Enzymatically released N-glycans of series of rhC1INH samples were fluorescently labeled and fractionated by HPLC. The major N-glycan structures on rhC1INH of pooled rabbit milk were similar to those on native human C1 inhibitor and recombinant human C1 inhibitor produced in transgenic mouse milk, with only the degree of sialylation and core fucosylation being lower. Analyses of individual animals furthermore showed slight interindividual differences; a decrease in the extent of sialylation, core fucosylation, and oligomannose-type glycosylation with the progress of lactation; and a positive correlation between expression level and oligomannose-type N-glycan content. However, when large quantities of rhC1INH were isolated for preclinical and clinical studies, highly consistent N-linked glycan profiles and monosaccharide compositions were found.

Key words: C1 inhibitor/glycosylation/lactation/transgenic rabbit milk

Introduction

With new developments in recombinant DNA technology, there has been a surge of innovative approaches for the large-scale production of recombinant proteins, for example, bacterial (Panda, 2003; Swartz, 2001) and baculovirus transfected insect cell expression systems (Altmann *et al*., 1999), yeasts (Cregg *et al*., 2000; Frenken *et al*., 1998; Lin Cereghino *et al*., 2002), fungi (Archer, 1994; Punt *et al*., 2002), transgenic plants (Bakker *et al*., 2001; Fischer and Emans, 2000; Ma *et al*., 2003), transgenic animals (Houdebine, 2000; Niemann and Kues, 2003), and mammalian cells (Molowa and Mazanet, 2003; Werner, 1998). Each method has distinct advantages but also drawbacks (Larrick and Thomas, 2001). Except for mammalian cell lines and transgenic animals (i.e., milk), a major limitation is the lack of elaborate, eukaryotic N- and O-glycosylation machineries, often required for correct protein folding, conformation, intracellular transport, biological activity, tissue targeting, or serum half-life (Jenkins and Curling, 1994; Jenkins *et al*., 1996).

Recombinant glycoprotein production in the mammary gland of transgenic animals is very promising for large-scale economic production, especially for recombinant proteins that require mammalian glycosylation as provided by the host's mammary gland. Recent examples that have reached clinical trials include antithrombin III and tissue plasminogen activator from transgenic goat milk, α-glucosidase from transgenic rabbit milk, and α-antitrypsin from sheep milk (Niemann and Kues, 2003).

The ultimate requirements for any therapeutic glycoprotein are safety, efficacy, and consistency in therapeutic potential. For glycosylation this implies consistency across recombinant glycoprotein batches, with nonimmunogenic glycans that facilitate the required efficacy *in vivo*. As opposed to cell culture approaches (Werner, 1998), little is known about the glycosylation machinery of the mammary gland in the various animal species used for recombinant glycoprotein production. In view of the hormonal regulation of protein glycosylation throughout lactation (Vijay, 1998), it is important to investigate whether glycosylation of a transgenic glycoprotein remains consistent throughout lactation, even at the level of the individual animal. Subtle variations in glycan profiles might arise due to differences between individual animals that are used to breed the transgenic colony, reflecting their unique genetic make-up (e.g., transgene copy number, genomic integration site).

Human serum C1 inhibitor (hC1INH) has six N- and seven O-glycosylation sites (Perkins, 1993). Recently, we have analyzed in detail the N- and O-glycosylation pattern of recombinant human C1 inhibitor (rhC1INH) expressed in the milk of transgenic rabbits using a pooled milk sample. The carbohydrate content of rhC1INH was found to be 14%, and Fuc, Man, Gal, GalNAc, GlcNAc, and Neu5Ac were present in the molar ratio of 0.2:3.0:2.4:1.7:2.6:1.3.

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Neu5Gc did not occur. An ensemble of at least 20 different N-glycans, comprising oligomannose-, hybrid-, and [(α2-6)-sialylated, fucosylated] diantennary complex-type structures, was found. The manno-oligosaccharide pattern of part of the hybrid- and oligomannose-type structures indicated that besides the usual N-glycan processing route the alternative *endo*-mannosidase pathway is also followed. For the O-glycans, four core 1 type structures were established (Koles *et al.*, 2004).

The aim of our present study was to systematically explore the N-glycosylation pattern of rhC1INH throughout lactation in low- and high-expressor lines and to compare these with the glycan profiles observed on seven large-scale batches of rhC1INH produced for (pre)clinical evaluation of the biopharmaceutical. In addition, to demonstrate glycosylation consistency, the monosaccharide composition of 20 additional batches was investigated. For comparison, attention was also paid to differences in N-glycan profiles of hC1INH, rhC1INH, and hC1INH expressed in the mammary glands of mice (rm-hC1INH).

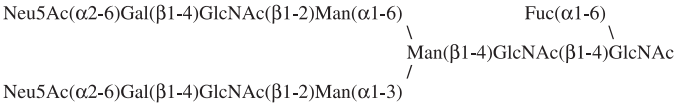
Results

Samples of rhC1INH

rhC1INH-expressing rabbits were grouped into low- and high-expressor lines, on average 2 g/L versus 12 g/L expression level of rhC1INH, respectively. N-Glycosylation profiles of rhC1INH samples were analyzed during the entire lactation for six individual transgenic rabbits in each group (high-expressor lines: rabbits A–F; low-expressor lines: rabbits G–L). Two consecutive lactations were followed for each rabbit, unless mentioned otherwise. Expression levels remained constant over the entire lactation period (data not shown).

N-Glycosylation profiles of hC1INH, rm-hC1INH, and rhC1INH

In Figure 1 the high-performance liquid chromatography (HPLC) profiles of the peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGase F) released and 2-aminobenzamide (2-AB)-labeled N-glycans of hC1INH, rm-hC1INH, and rhC1INH (rabbit J, samples of early and late stages of lactation) are depicted. Fractionation on weak anion-exchange phase GlycoSepC (Figure 1A) and on normal phase GlycoSepN (Figure 1B) columns showed that the degree of sialylation is higher for the N-glycans of hC1INH and rm-hC1INH than for those of rhC1INH. The occurrence of trisialylated components on hC1INH (Figure 1A) is in agreement with earlier findings (Perkins *et al.*, 1990), but these structures were absent on rm-hC1INH and rhC1INH. In Figure 1B, the four major N-glycans have been indicated: mono- and di-(α2-6)-sialylated partially core-fucosylated complex-type structures (codes: 1–4). They were verified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and sequential exoglycosidase digestions (data not shown; see also Koles *et al.*, 2004). The linkage details for the most complete structure is illustrated below.



As can be seen in Figure 1B, the (α2-6)-disialylated N-glycans of rm-hC1INH have a higher degree of core

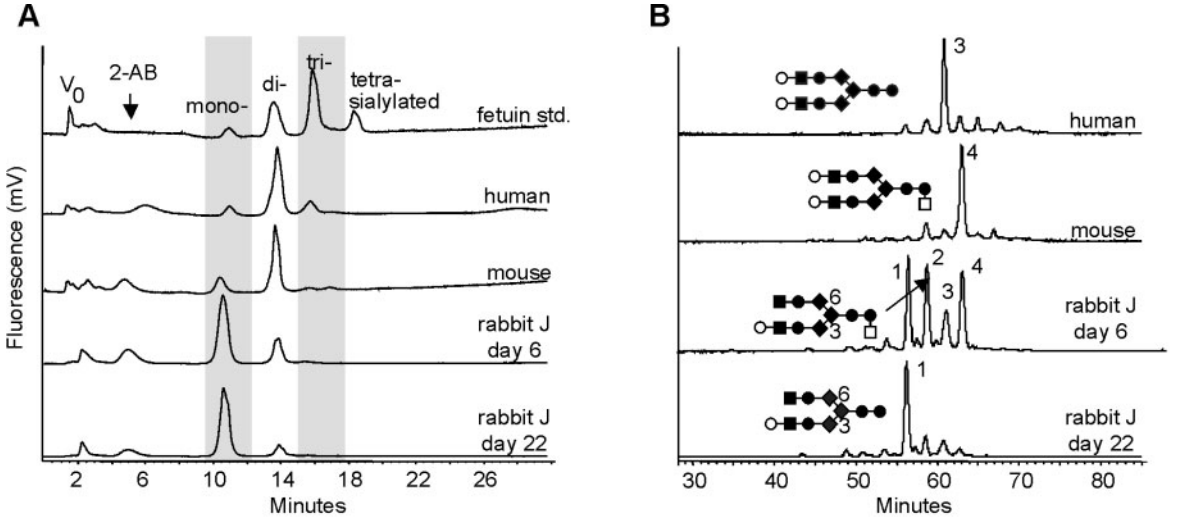


Fig. 1. Species-specific differences in the N-glycosylation profile of C1 inhibitor. (**A**) From top to bottom: GlycoSepC profiles of standard bovine fetuin (elution position of mono-, di-, tri-, and tetrasialylated N-glycans), native serum hC1INH, rm-hC1INH, rhC1INH (low-expressor rabbit J, day 6), and rhC1INH (low-expressor rabbit J, day 22). (**B**) From top to bottom: GlycoSepN profiles of the same glycoproteins, except bovine fetuin; the major peaks 1 to 4 represent mono- and di-(α2-6)-sialylated partially core fucosylated complex-type N-glycans as indicated by short-hand symbolic notation (open circles, Neu5Ac; open squares, Fuc; closed squares, Gal; closed circles, GlcNAc; diamonds, Man). For complete linkage details, see text.

fucosylation than those of hC1INH. Importantly, N-glycan profiles of rhC1INH of rabbit J showed that the degree of sialylation on the 6th day of lactation is higher than on the 22nd day of lactation. A full lactation cycle in rabbits lasts ~30 days.

Sialylation throughout lactation

To further explore the details of sialylation throughout lactation, the GlycoSepC N-glycan profiles of rhC1INH from two different lactations of the high-expressor rabbits A–F and the low-expressor rabbits G–L were compared. Typical N-glycan profiles of rhC1INH from a low-expressor line (rabbit L, 1st lactation) and a high-expressor line (rabbit F, 3rd lactation) are shown in Figures 2A and 2B, respectively. Comparison of these profiles shows that the N-glycans from high-expressor rabbits contain a relatively higher portion of neutral glycans (eluting near the void volume, V_o). For both groups, with the progress of lactation, a steady decrease in the ratio of di- to monosialylated N-glycans was observed (Figures 2C and 2D; circles). Total sialic acid analysis of the various rhC1INH samples revealed a similar pattern (Figures 2C and 2D, triangles), supporting that the overall sialic acid content decreased with the progress of lactation in both groups. It should be noted that the total sialic acid analysis comprises sialic acids on both the N- and O-linked glycans of rhC1INH.

Individual glycosylation profiles during successive lactations of low- and high-expressor rabbits

For a more detailed insight into the structures of carbohydrate chains throughout lactation, the profiles of rhC1INH N-glycans taken from two consecutive lactations from each of the 12 rabbits were compared using normal-phase chromatography on GlycoSepN, eventually combined with MALDI-TOF MS and exoglycosidase digestions (Guile *et al*., 1996). In Figure 3 the typical GlycoSepN profiles of two low-expressor rabbits are shown (Figures 3A and 3B, rabbit J; Figures 3C and 3D, rabbit K), whereas in Figure 4 such analyses are depicted for two high-expressor animals (Figures 4A and 4B, rabbit C; Figures 4C and 4D, rabbit F).

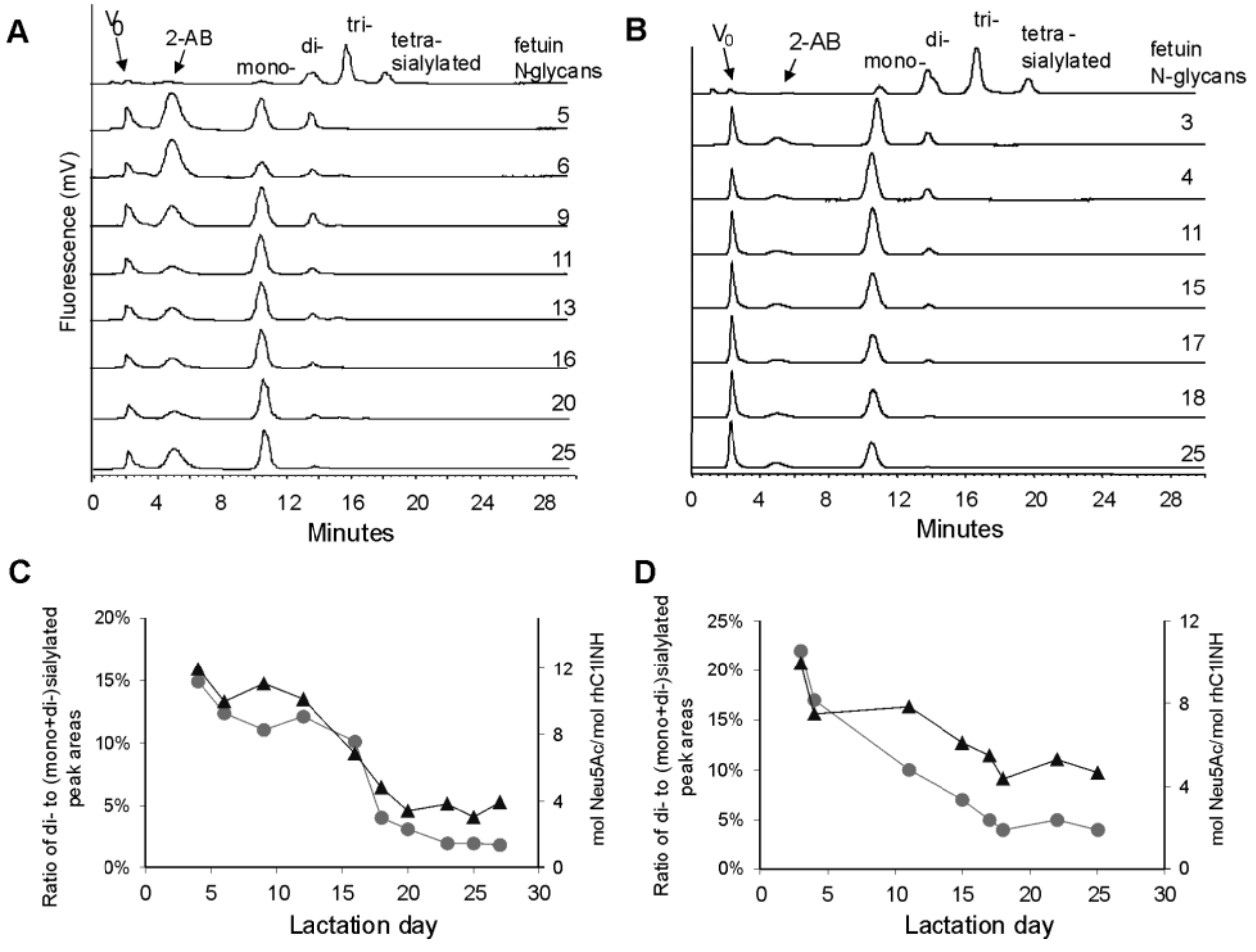


Fig. 2. Changes in sialylated N-glycan profiles of rhC1INH throughout lactation. GlycoSepC profiles of rhC1INH from (**A**) a low-expressor line (rabbit L, 1st lactation) and (**B**) a high-expressor line (rabbit F, 3rd lactation). For comparison, a profile of bovine fetuin has been included; V_o, void volume (neutral glycans). The number on each chromatogram refers to the day of lactation. Molar ratios of di- to (mono- plus di-)sialylated N-glycans (circles) and overall sialic acid content (triangles) throughout lactation for (**C**) low-expressor rabbit H (3rd lactation) and (**D**) high-expressor rabbit F (3rd lactation).

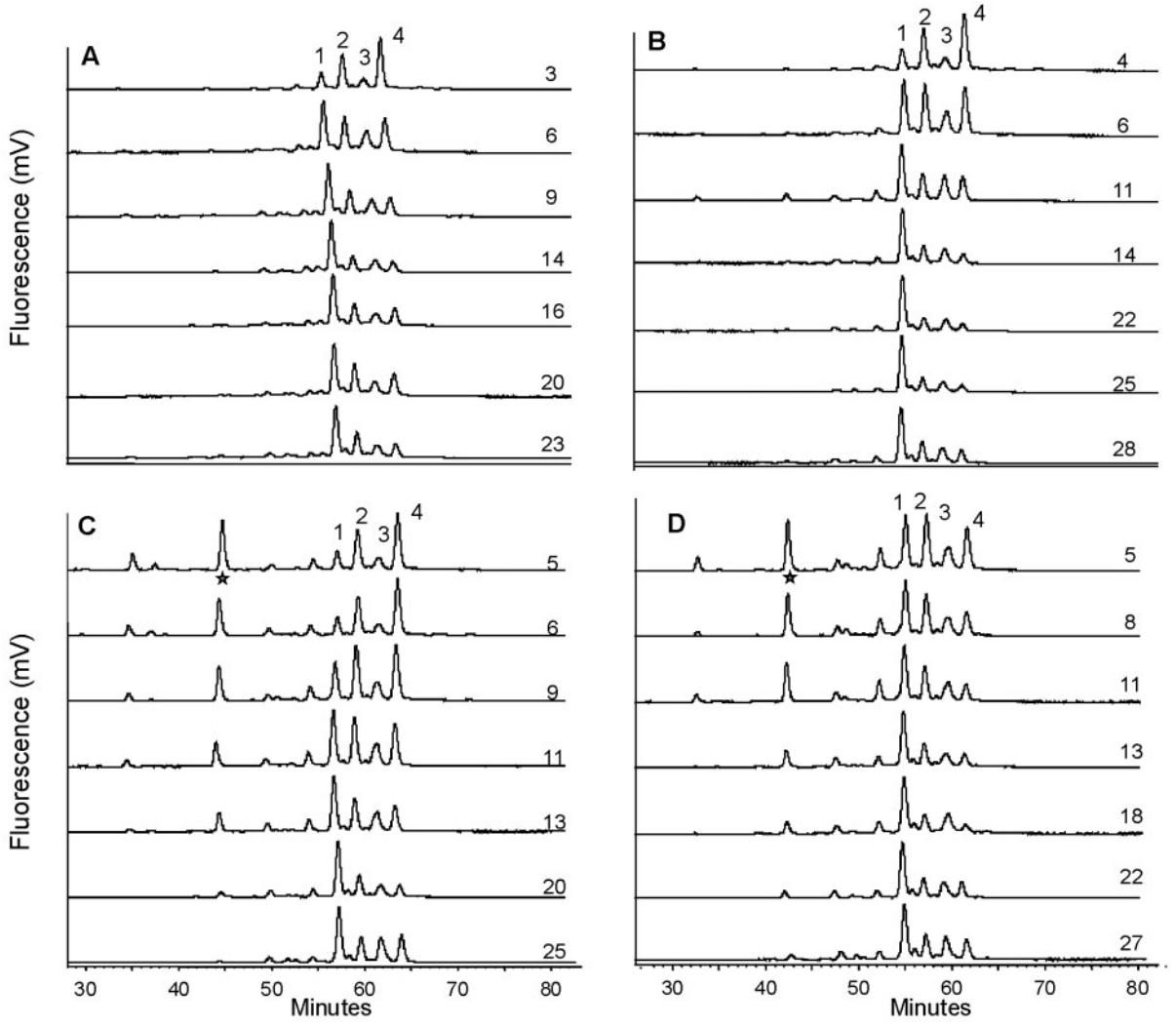


Fig. 3. N-glycan profiles on GlycoSepN of rhC1INH of low-expressor lines. (**A**) Rabbit J, 2nd lactation. (**B**) Rabbit J, 3rd lactation. (**C**) Rabbit K, 1st lactation. (**D**) Rabbit K, 2nd lactation. The number on each chromatogram refers to the day of lactation. The major peaks 1 to 4 represent mono- and di-(α2-6)-sialylated partially core fucosylated complex-type N-glycans, as explained in Figure 1. The peak marked with an asterisk corresponds to $Man_5GlcNAc_2$. To trace the recently reported Lewis x structures N0.5, N1.2, and N1.1 (Koles *et al*., 2004), the glucose unit values of their 2-AB derivatives have been calculated using GlycoBase. Using these predictions, N0.5 will elute together with the major compound 1 (N1.5A), N1.2 will elute in the region between compounds 3 (N2.2) and 4 (N2.1), and N1.1 will come after compound 4. The latter two regions do not show intense peaks, suggesting no great changes in Lewis x profiles. Using the same database, it is predicted that the peak eluting in front of compound 1 stems from hybrid structure N1.6A (see also profiles in Figure 4).

The major peak, marked with an asterisk, observed in addition to the four peaks mentioned before, was assigned to $Man_5GlcNAc_2$ (based on sensitivity to jack bean α-mannosidase and MALDI-TOF MS analysis; data not shown). Variations in the molar ratio of the five major N-glycans were observed between the individual rabbits, both for low- and high-expressor animals. Most pronounced was the variation in the $Man_5GlcNAc_2$ peak between rabbits J and K, both being low expressors.

It appeared that the glycosylation heterogeneity strongly depends on the individual rabbits and that to a certain extent, each animal displays a unique N-linked profile. In addition, the glycosylation profiles also varied slightly during subsequent lactations of the same animal, and these differences were most pronounced at the early stages of lactation. Furthermore, the GlycoSepN profiles confirmed that the level of sialylation decreased during lactation (i.e., peaks 3 and 4 decreased, whereas peaks 1 and 2 increased). Moreover, the profiles clearly showed that the amount of oligomannose-type N-glycans (designated with the asterisk) as well as the core fucosylation strongly decreased with the progress of lactation (peak ratios of 1 versus 2, and 3 versus 4 changed in favor of the nonfucosylated structure).

High-expressor lines produce rhC1INH with higher amounts of oligomannose-type N-glycans

Evaluation of all GlycoSepN N-glycan profiles of the 12 individual rabbits indicated a trend for higher amounts of $Man_5GlcNAc_2$ in the rhC1INH samples of the

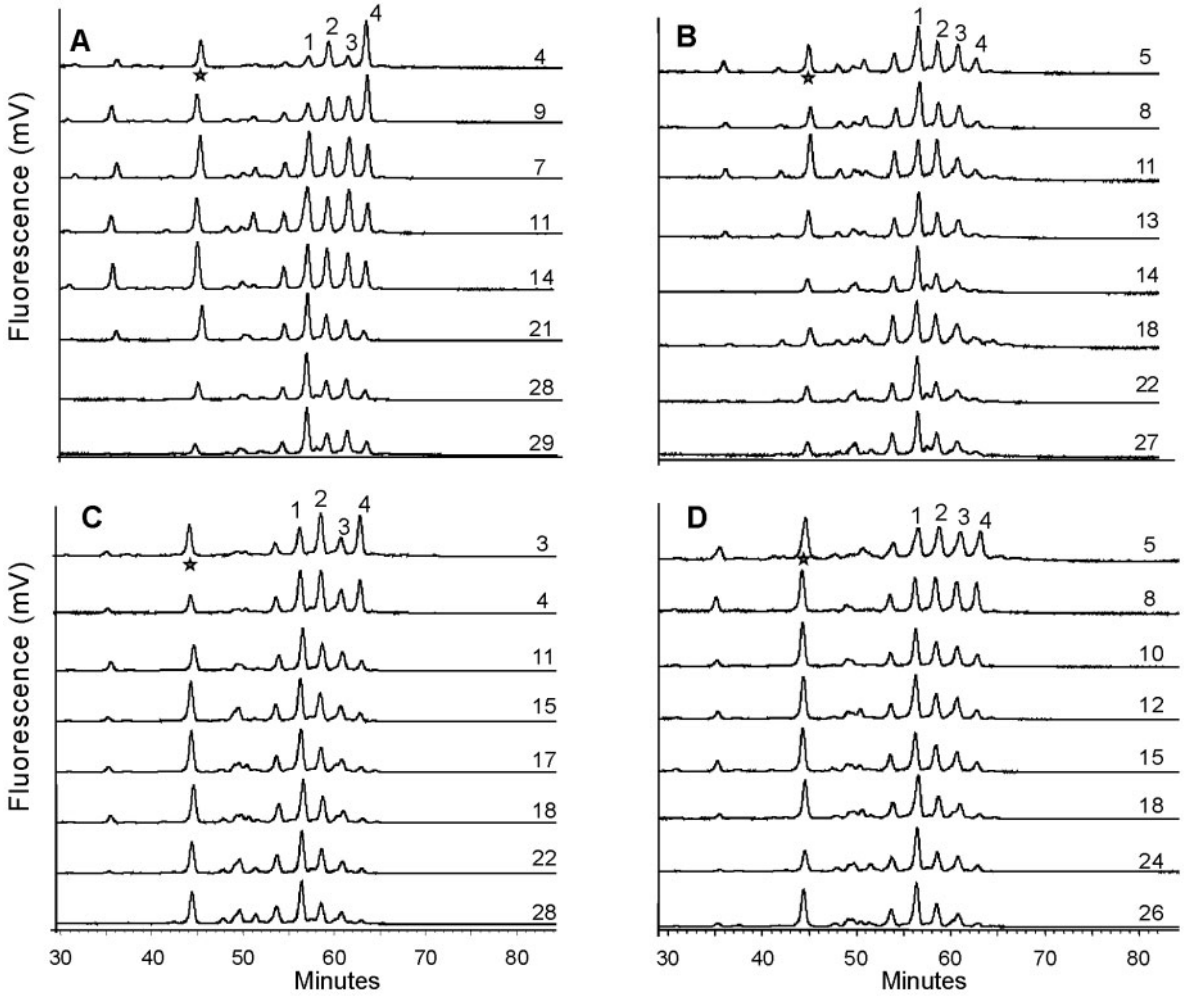


Fig. 4. N-glycan profiles on GlycoSepN of rhC1INH of high-expressor lines. (**A**) Rabbit C, 1st lactation. (**B**) Rabbit C, 2nd lactation. (**C**) Rabbit F, 3rd lactation. (**D**) Rabbit F, 5th lactation. The number on each chromatogram refers to the day of lactation. The major peaks 1 to 4 represent mono- and di-(α2-6)-sialylated partially core fucosylated complex-type N-glycans, as explained in Figure 1. The peak marked with an asterisk corresponds to $Man_5GlcNAc_2$.

high-expressor lines when compared with the samples of low-expressor lines. In low-expressor lines, the relative amount of $Man_5GlcNAc_2$ is very low or strongly reduced throughout lactation. These data suggest that expression levels influence the amount of oligomannose-type N-glycans on rhC1INH. To examine this relationship further, an additional series of seven animals were selected with rhC1INH expression levels ranging from 1 g/L to 16 g/L, and the proportion of $Man_5GlcNAc_2$ in the respective N-glycan pools was determined at the early (days 2–6) and the late (days 23–27) stages of lactation. The results, shown in Figure 5, suggest a relationship between rhC1INH expression levels and oligomannose-type N-glycan content. As opposed to the earlier stages of lactation, where no significant correlation could be found ($r^2 = 0.33$, $p = 0.23$), later stages of lactation showed a significant correlation between expression level and $Man_5GlcNAc_2$ content ($r^2 = 0.84$, $p = 0.01$). The lack of correlation at early stages could be explained by the higher individual variations during this period (see Figures 3 and 4).

Batch-to-batch consistency of rhC1INH glycosylation

Therapeutic applications require significantly higher amounts of recombinant glycoproteins than can be purified from milk collected during a single lactation day of a rabbit. Batches of rhC1INH were produced by purifying the glycoprotein from pooled milk originating from 20–25 high-expressing animals, each producing ~12 g rhC1INH per L of raw milk. The milk pools were composed in such a way that early and late lactation milk contributed equally to the total pool. Briefly, on a 10-L scale, the purification process was made up of centrifugation, filtration, and standard chromatography techniques, yielding ~50 g highly purified glycoprotein (van Veen *et al.*, unpublished data). Thus far, 20 batches have been produced, of which 7 were subjected to N-glycan profiling. As can be concluded from Figure 6, the GlycoSepN profiles of these batches were remarkably similar, indicating that even though N-glycosylation of rhC1INH is subject to individual heterogeneity and changes throughout lactation, N-glycosylation at the batch level is highly consistent.

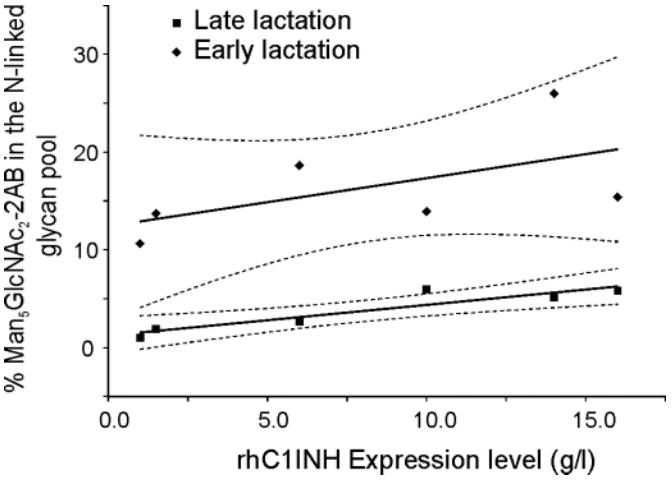


Fig. 5. Correlation between the expression level of rhC1INH and the relative $Man_5GlcNAc_2$ content in N-glycan pools derived from rhC1INH samples of seven animals with increasing expression level. Diamonds and squares illustrate the relationship during early stages (days 2–6; $r^2 = 0.33$, $p = 0.23$) and late stages (days 23–27; $r^2 = 0.84$, $p = 0.01$) of lactation, respectively. The dotted lines represent the 95% confidence interval of the linear fits.

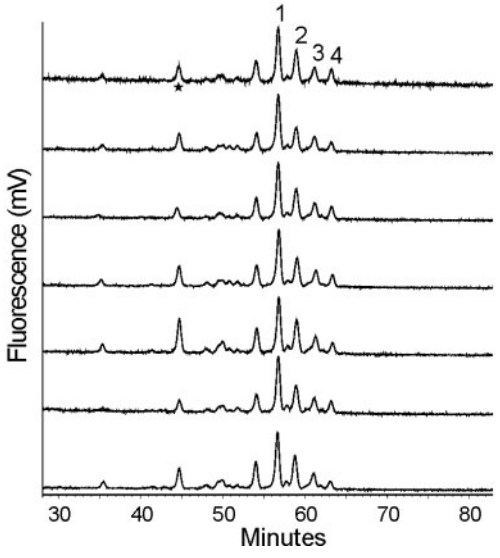


Fig. 6. Consistency of the GlycoSepN N-glycan profiles of seven batches of rhC1INH from milk pooled from ~20–25 transgenic rabbits belonging to a high-expressor line. The major peaks 1 to 4 represent mono- and di-(α2-6)-sialylated partially core fucosylated complex-type N-glycans, as explained in Figure 1. The peak marked with an asterisk corresponds to $Man_5GlcNAc_2$.

The lot-to-lot consistency of glycosylation was further addressed by monosaccharide analysis using high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). In agreement with our previous report (Koles *et al.*, 2004), the monosaccharides Fuc, Man, Gal, GalNAc, GlcNAc, and Neu5Ac were present in 20 rhC1INH batches in the molar ratio of (0.14 ± 0.02):(3.0):(2.5 ± 0.2):(1.7 ± 0.1):(2.4 ± 0.1):(1.2 ± 0.1). Thus monosaccharide analysis across different batches indicated a highly consistent N,O-glycosylation.

Discussion

Our experiments aimed at identifying possible changes in the N-glycosylation of rhC1INH expressed in the milk of transgenic rabbits. In particular, we focused on the N-glycosylation of rhC1INH in relation to the expression levels of the glycoprotein in the milk, possible differences in rhC1INH N-glycosylation between individual animals, and changes in N-glycosylation of rhC1INH that might occur during the progress of lactation. Additionally, the consistency of a number of rhC1INH batches, prepared from pooled milk of several animals, was investigated.

The various results show that sialylation levels and total rhC1INH-bound sialic acid content decreased with the progress of lactation independent of expression levels. These findings are in accordance with earlier data reported for a few native human and bovine milk glycoproteins. A decrease in protein-bound sialic acid after the first 2 weeks of lactation has been reported for pooled human milk glycoproteins (Carlson, 1985). A change in the glycosylation status of the human milk bile salt–stimulated lipase during lactation has been described, whereby sialic acid levels were at highest at the early stages of lactation (Landberg *et al.*, 2000). The two bovine milk glycoproteins MGP 57 and MGP 53 were more negatively charged immediately after parturition than at later stages, probably due to higher sialylation at the early stages (Aoki *et al.*, 1994). The observations suggest that a decrease in sialic acid content during lactation in mammals might be a general phenomenon. Because sialylation level plays an important role in the pharmacokinetic properties of many glycoproteins, understanding the biochemical details of this down-regulation might help maximize the degree of sialylation of recombinant proteins.

Two interesting observations were made in relation to the oligomannose-type N-glycans found on rhC1INH. First, the amount of oligomannose-type glycosylation strongly decreased during lactation, even though the expression levels remained constant. Because oligomannose (and neutral hybrid) type N-glycans are readily recognized by the mannose receptors present on epithelial cells and macrophages, resulting in enhanced clearance from the circulation, for therapeutic applications, where a long plasma residency is of importance, a low amount of such structures is desirable. In addition to a decrease in the oligomannose and sialic acid content, a significant decrease in core fucosylation was also seen during lactation. This could be an advantage when considering expression of recombinant monoclonal antibodies in transgenic animals, because recently it has been shown that the absence of core fucose on human IgG strongly increases its antibody-dependent cellular cytotoxicity (Shinkawa *et al.*, 2003). Second, a correlation between the oligomannose-type N-glycan content and expression levels of rhC1INH, particularly at late stages of lactation, was noticed. This suggests that at a high level of expression, the glycosylation machinery of the mammary epithelial cells may become limiting.

It is worth pointing out that on pooled rhC1INH the total amount of oligomannose-type glycan is on average 15% of total, and the majority of the N-glycans (55%) are of the complex type (Koles *et al*., 2004). These findings are remarkable for a heavily glycosylated glycoprotein expressed at such high levels (12 g/L), illustrating the huge glycosylation capacity of the rabbit mammary gland.

Finally, the differences that were observed at the individual level were virtually absent when analyzing large pools of purified rhC1INH. Indeed, both the N-glycan profiles and monosaccharide composition of different batches were remarkably consistent.

Further detailed studies in other transgenic species might help unravel the contribution of species-specific factors to the glycosylation profile of individual recombinant glycoproteins, like rhC1INH, and this in turn might aid the choice for the most suitable host animal. In this context the observed differences in sialylation between recombinant hC1INH produced in the mammary glands of mice and rabbits are quite striking indeed.

Materials and methods

Purification of rhC1INH from milk on research scale

Milk was obtained from transgenic rabbits engineered to express rhC1INH under a mammary gland–specific promotor (details to be published elsewhere; Heus *et al*., unpublished data). The whey fraction, prepared from 2 ml rabbit milk by centrifugation at 15,000 rpm for 1.5 h at 4°C, was diluted twofold in 20 mM sodium citrate buffer, pH 7.0. After filtration through a 0.22-μm filter, the fraction was applied to a Mono-S column (Pharmacia, Uppsala, Sweden; HR 5/5), equilibrated in 20 mM sodium citrate buffer, pH 7.0, and eluted with a linear concentration gradient of 0–250 mM NaCl in 17.5 min. Fractions containing rhC1INH were pooled and concentrated to 0.5 ml using 10-kDa cutoff centrifugal filters (Nalgene, Rochester, NY; ~3 h at 4,000 rpm). The purity of rhC1INH was verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and its concentration was determined by measuring the absorbance at 280 nm (extinction coefficient, 0.4). The overall yield of purification was more than 75%.

The hC1INH and rm-hC1INH (from pooled milk of a transgenic mouse line expressing the glycoprotein on a g/L scale) samples were gifts of Pharming Technologies BV.

Sialic acid quantification

rhC1INH (20 μg) in 20 μl 20 mM sodium phosphate buffer, pH 7.2, was incubated with 40 mU *Arthrobacter ureafaciens* sialidase (Roche Molecular Biochemicals, Mannheim, Germany) in 20 μl of the same buffer for 1 h at 37°C. Then 70 μl water and 50 μl 40 μM 3-deoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid (internal standard; Toronto Research Chemicals, Toronto, Canada) were added, and 20 μl of the mixture was analyzed by HPAEC-PAD on a Dionex DX-500 system, equipped with a CarboPac PA-1 column (4.0 × 250 mm) and a CarboPac PA-100 guard column (4.0 × 50 mm). Sialic acids were eluted isocratically with 125 mM sodium acetate in 150 mM NaOH for 15 min at a flow rate of 1.0 ml/min. Neu5Ac was quantified using a linear calibration curve of standard Neu5Ac (Sigma, St. Louis, MO).

Monosaccharide analysis

To 50 μg rhC1INH in 267 μl 20 mM sodium phosphate buffer, pH 7.2, was added 133 μl 36–38% HCl (final concentration, 4 M HCl). The mixture was incubated for 3 h at 80°C and then dried under vacuum in a SpeedVac; the residue was dissolved in 50 μl water. Prior to chromatography, 50 μl 2-deoxy-D-glucose (15.0 μg/ml) was added as internal standard. Each sample (20 μl) was analyzed by HPAEC-PAD (CarboPac PA-1), using a sodium acetate gradient from 0–75 mM in 150 mM NaOH (10 min; flow rate, 1.0 ml/min). For the quantification of the monosaccharides, calibration curves were used. All peak areas were corrected for the peak area of the internal standard.

Enzymatic release of N-glycans

Samples of 100 μg rhC1INH (50 μl) were denatured in 10% sodium dodecyl sulfate (w/v) for 5 min at 100°C and digested with 5 U/mg PNGase F (Roche Molecular Biochemicals, Indianapolis, IN) in 20 mM phosphate buffered saline, pH 7.2, containing 10 mM ethylenediamine tetra-acetic acid, 10 mM β-mercaptoethanol, and 6.4 mg/ml 3-[(3-cholamidodopropyl)dimethylammonio]-1-propane sulfonate (Fluka, Buchs, Switzerland) for 24 h at 37°C. Digests were filtered through 30-kDa cutoff centrifugal filters (Nalgene), and filtrates were treated with Calbiosorb beads (Calbiochem, San Diego, CA) to remove detergents. Released N-glycans were purified on graphitized carbon columns (Alltech, Breda, The Netherlands) and then fluorescently labeled using 0.35 M 2-AB (Sigma) and 1 M sodium cyanoborohydride in a mixture of dimethylsulfoxide:acetic acid (7:3) for 2 h at 65°C (Koles *et al*., 2004). The 2-AB-labeled glycans were purified via paper chromatography on acid-pretreated quartz fiber filter paper strips (Millipore, Dublin, Ireland), eluted with water, and analyzed by HPLC.

HPLC

N-Glycan profiles at 30°C were obtained on GlycoSepC (4.6 × 100 mm) and GlycoSepN (4.6 × 250 mm) columns (Glyko, Novato, CA), using a Waters 2690 HPLC system equipped with a fluorescence detector ($\lambda_{exc.max} = 373$ nm, $\lambda_{em.max} = 420$ nm). For weak anion-exchange chromatography on GlycoSepC, two solvent systems were used: solvent A, 50% 500 mM ammonium formate, pH 4.5, 30% HPLC-quality water, and 20% acetonitrile (Biosolve, Valkenswaard, The Netherlands); and solvent B, 80% HPLC-quality water and 20% acetonitrile. After running 100% B for 5 min, charged N-glycans were eluted with a linear gradient of 0–100% A over 35 min at a flow rate of 0.4 ml/min. For normal phase chromatography on GlycoSepN, solvent A was 50 mM ammonium formate, pH 4.4, and solvent B was 20% 50 mM ammonium formate, pH 4.4, in acetonitrile. The gradient consisted of a linear increase of A from 6.5% to 43.8% over 100 min at a flow rate of 0.8 ml/min, followed by 100% B for 10 min at a flow rate of 1 ml/min, and a reequilibration step to starting conditions for 25 min at a flow rate of 0.8 ml/min.

MALDI-TOF MS

For experimental details, see Koles *et al*. (2004).

Exoglycosidase digestions

The following enzymes and digestion conditions were used: β-galactosidase (*Streptococcus pneumoniae*; Calbiochem), 2–4 mU per 25 µl digest in 100 mM sodium acetate buffer, pH 6.0; β-*N*-acetylhexosaminidase (jack bean; Glyko), 0.25–0.5 U per 25 µl digest in 100 mM sodium phosphate/citrate buffer, pH 5.0; α-fucosidase (bovine kidney, Glyko), 5 mU per 25 µl digest in 100 mM sodium citrate buffer, pH 6.0; sialidase (*A. ureafaciens*, Glyko), 25 mU per 25 µl digest in 100 mM sodium acetate buffer, pH 5.0. Combined digestions (i.e., more than one exoglycosidase in the digestion mixture) were performed in 100 mM sodium acetate buffer, pH 5.5. Approximately 30–80 pmol of 2-AB-labeled glycans were used for the digestions, which were performed for 18 h at 37°C. Prior to HPLC analysis the samples were deproteinated through a 5-kDa cut-off centrifugal filter (Millipore, Bedford, MA), and the digested glycan mixtures, still containing the buffer salts, were analyzed by HPLC on GlycoSepN. A 2-AB-labeled dextran hydrolysate served as external calibration standard.

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Abbreviations

2-AB, 2-aminobenzamide; C1INH, C1 inhibitor; HPAEC, high-pH anion-exchange chromatography; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; PAD, pulsed amperometric detection; PNGase F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F).

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