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Geun-Cheol Gil
University of Nebraska-Lincoln

William H. Velander
University of Nebraska-Lincoln, wvelander2@unl.edu

Kevin E. Van Cott
University of Nebraska-Lincoln, kvancott2@unl.edu

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N-glycosylation microheterogeneity and site occupancy of an Asn-X-Cys sequon in plasma-derived and recombinant protein C

Geun-Cheol Gil,[1] William H. Velander,[1,2] and Kevin E. Van Cott [1]

1 Department of Chemical and Biomolecular Engineering, University of Nebraska–Lincoln, Lincoln, NE, USA
2 Progenetics LLC, VA, USA

Corresponding author — K. E. Van Cott, Department of Chemical and Biomolecular Engineering, University of Nebraska–Lincoln, Lincoln, NE 68588, USA; fax 402 472-6989, email kvancott2@unl.edu

Abstract

Human protein C (hPC) is glycosylated at three Asn-X-Ser/Thr and one atypical Asn-X-Cys sequons. We have characterized the micro- and macro-heterogeneity of plasma-derived hPC and compared the glycosylation features with recombinant protein C (tg-PC) produced in a transgenic pig bioreactor from two animals having approximately tenfold different expression levels. The N-glycans of hPC are complex di- and tri-sialylated structures, and we measured 78% site occupancy at Asn-329 (the Asn-X-Cys sequon). The N-glycans of tg-PC are complex sialylated structures, but less branched and partially sialylated. The porcine mammary epithelial cells glycosylate the Asn-X-Cys sequon with a similar efficiency as human hepatocytes even at these high expression levels, and site occupancy at this sequon was not affected by expression level. A distinct bias for particular structures was present at each of the four glycosylation sites for both hPC and tg-PC. Interestingly, glycans with GalNAc in the antennae were predominant at the Asn-329 site. The N-glycan structures found for tg-PC are very similar to those reported for a recombinant Factor IX produced in transgenic pig milk, and similar to the endogenous milk protein lactoferrin, which may indicate that N-glycan processing in the porcine mammary epithelial cells is more uniform than in other tissues.

Keywords: Asn-X-Cys, glycosylation, mass spectrometry, N-glycan, protein C

Abbreviations: 2-AA, 2-aminobenzoic acid; **GluC,** glutamyl endopeptidase; **hPC,** human protein C (plasma-derived); **LysC,** lysyl endopeptidase; **MRM,** multiple reaction monitoring; **Neu5Ac,** Nacetylneuraminic acid; **Neu5Gc,** N-glycolylneuraminic acid; **NP,** normal phase; **tg-PC,** transgenic pig-derived human protein C

1 Introduction

Human protein C (hPC) is a vitamin K-dependent plasma glycoprotein having anti-coagulant activity, and circulates primarily as a disulfide-linked heterodimer composed of a light chain (25 kDa) and heavy chain (41 kDa) [1, 2]. Prior to secretion, hPC undergoes extensive PTM, including γ-carboxylation of nine Glu residues, β-hydroxylation of Asn-71, proteolytic maturation (propeptide and internal dipeptide removal), and N-glycosylation at up to four Asn residues [3, 4]. The potential N-glycosylation sites are in the epidermal growth factor (EGF) domain of the light chain at Asn-97 and in the heavy chain at Asn-248, 313, and 329.

The N-glycosylation site at Asn-329 contains the unusual sequon Asn-X-Cys (X is any amino acid except Pro) that is also found in bovine protein C [5]. The hPC has been reported to be 70–80% glycosylated at this sequon by SDS-PAGE analysis, and bovine protein C is reported to be fully glycosylated [5–8]. The N-glycosylation of the atypical Asn-X-Cys sequon has been characterized in the literature for only six other proteins: human alpha1T-glycoprotein, human von Willebrand factor, human CD69, human alpha-lactalbu-

min, murine and human fetal antigen 1, and recombinant human epidermal growth factor receptor [9–15]. In these proteins, the reported site occupancy at this sequon ranged from sparse (1% in alpha-lactalbumin) to full (von Willebrand factor, and human fetal antigen 1) [15]. Where glycan structures are reported for these sequons, they are complex bi- and tri-antennary with and without both sialylation and fucosylation. The N-glycans of hPC are important to function, and Grinnell *et al*. [7] showed by site-directed mutagenesis that the N-glycosylation of hPC affects protein secretion, protein folding and subsequent glycosylation at other sequons, proteolytic processing, and anticoagulant activity. Although N-glycosylation is an important factor for hPC biological functionality, there are no published reports of the N-glycan structures found in plasma-derived hPC, or of the glycosylation micro-heterogeneity at the four sites, especially with respect to the unusual sequon at Asn-329.

Several host systems have been employed to produce recombinant hPC. Recombinant activated PC (Drotrecogin alfa™ or Xigris) is produced in human kidney 293 cell culture by Eli Lilly for the treatment of sepsis [16–18], and the N-glycan structures of hPC produced in these cells have been published [19]. Transgenic animal bioreactors are an alternative mammalian expression system that can be used to produce recombinant glycoproteins [20]. Biologically active recombinant hPC has been produced in the transgenic pig mammary gland (tg-PC) at different expression levels [21, 22]. However, the N-glycan structures and the micro- and macro-heterogeneity, especially with respect to Asn-329, have not been reported.

In this work, we characterize the N-glycan structures and the micro- and macro-heterogeneity of hPC and tg-PC with a special emphasis on the atypical Asn_{329}-X-Cys sequon. We discuss the characteristics of the N-glycosylation of hPC and tg-PC with the intent to contribute towards an improved understanding of the differences and similarities between N-glycan processing in human hepatocytes *versus* transgenic animal mammary epithelial cells. Additionally, we investigate if there is evidence for rate limitations in the N-glycan attachment and processing in the porcine mammary gland by comparing two transgenic lineages having up to tenfold different expression levels. We also compare N-glycosylation of tg-PC with another recombinant vitamin K-dependent glycoprotein that has been produced in the transgenic pig mammary gland, Factor IX (tg-FIX) [23], and discuss the features of N-glycan processing of recombinant proteins that are evident in the transgenic pig bioreactor.

2 Materials and methods

2.1 Materials

Plasma-derived human protein C (hPC) was purchased from Enzyme Research Laboratories (South Bend, IN). PNGase F was purchased from New England Biolabs (Beverly, MA). Glutamyl endopeptidase (GluC) and triethylamine were purchased from EMD Chemicals (Gibbstown, NJ), and Lysyl endopeptidase (LysC) was purchased from Waco (Japan). Isotopically labeled standard peptides were custom synthesized by AnaSpec (San Jose, CA). Tetrahydrofuran (0.025% BHT inhibited) was purchased from J. T. Baker (Phillipsburg, NJ), and DTT, iodoacetamide, and all other chemicals were obtained from Sigma (St. Louis, MO).

2.2 Purification of transgenic hPC from pig milk

DEAE Sepharose FF (Amersham Biosciences, Piscataway, NJ) was packed in a Poros HP glass column (10 mm ID × 92 mm L, PerSeptive Biosystems, Framingham, MA). A BioCAD Vision chromatography system (Applied Biosystems, Foster City, CA) was used with 280 nm detection. Daily milk samples from days 5–40 were pooled together and mixed 1:1 v/v with 200 mM EDTA pH 7.4 and then centrifuged for 30 min at 12,000 rpm at 4°C. The solidified milk fat was removed and then diluted 1:5 v/v with loading buffer (20 mM Tris, 50 mM NaCl, 0.1% Tween 20, pH 7.4), and loaded onto the column at 0.5 cm/min. The column was washed with loading buffer at 3 cm/min. Tg-PC was eluted with 20 mM Tris, 250 mM NaCl, pH 7.4 at 3 cm/min. The column was washed with 20 mM Tris, 4 M NaCl, pH 7.4 and then cleaned with 1 M NaOH at 3 cm/min. The purified tg-PC was further purified by an immunoaffinity column. 12A8 monoclonal anti-hPC (a gift from the American Red Cross, Rockville, MD) was coupled onto HiTrap NHS-activated Sepharose (16 mm ID × 25 mm L, Amersham Biosciences, Piscataway, NJ) as described in the product manual. Briefly, the column was washed with ice-cold 1 mM HCl. The antibody was dissolved in coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) and loaded onto the column. The column was sealed and incubated for 12 h at 4°C, then washed with washing buffer A (0.5 Methanolamine, 0.5 M NaCl, pH 8.3), followed by washing buffer B (0.1 M acetate, 0.5 M NaCl, pH 4). Uncoupled sites on the column were blocked with buffer A for 4 h at 4°C. Then the column was washed with washing buffer A and B, and equilibrated with 20 mM Tris, 250 mM NaCl, pH 7.4. The product from the DEAE column was loaded onto the affinity column at 1 cm/min. After washing with 20 mM Tris, 250 mM NaCl, pH 7.4 at 1 cm/min, tg-PC was eluted with 0.1 M Na_2CO_3, 0.15 M NaCl, pH 10. The affinity column was cleaned with 2 M NaSCN, followed by 4 M NaCl.

2.3 Sialic acid analysis using RP-HPLC

Sialic acids were analyzed by RP-HPLC using the method described in Anumula *et al*. (1995) [24]. Protein C samples (5–10 μg in 50 μL) were mixed with 50 μL of 0.5 M sodium bisulfate ($NaHSO_4$) in 1.6 mL screw cap microcentrifuge tubes, and then hydrolyzed at 80°C for 20 min. The hydrolyzed samples were derivatized by adding 100 μL of o-phenyllenediamine-2HCl, OPD (20 mg/mL in 0.25 M

$NaHSO_4$). The tubes were heated at 80°C for 40 min. Solvent A consisted of 0.15% v/v 1-butylamine, 0.5% v/v phosphoric acid, and 1% v/v tetrahydrofuran in water. Solvent B was 50% of solvent A in ACN. After cooling the samples down to room temperature, 0.8 mL of solvent A was added and vortexed vigorously. Proteins and any other particles were precipitated by centrifugation and the supernatant (100 μL) was analyzed by RP-HPLC (Supelcosil LC318 HPLC column; 4.6 mm id × 250 mm L, 5 μm particles, Supelco, St. Louis, MO). The column was equilibrated with 5% B for 10 min. The OPD-derivatized sialic acids were eluted with 13% B for 20 min and the column was washed for 10 min with 100% solvent B. A Waters 2695 Separations module and 2475 fluorescence detector (Milford, MA) were used to detect OPD-derivatized sialic acids. The detector settings were 230 nm excitation, 425 nm emission, and 40 nm bandwidth. The sialic acid standard mixture (Neu5Ac and Neu5Gc) were also analyzed each time with the analysis of glycoproteins. Calibration curves were determined with 6 different levels of each sialic acid standard (100, 200, 400, 600, 800, and 1000 pmol). Linearity of the calibration curves was accepted if the correlation coefficient (R^2) was greater than 0.99. Empower software (Waters) was used to integrate peak areas and construct the calibration curves. Protein C concentrations were determined by measuring OD_{280nm} (extinction coefficient of 1.45 mL/mg-cm) [1]. The sialic acid to protein ratio (mol/mol) was calculated by obtained moles of sialic acid divided by protein moles injected.

2.4 PNGase F digestion and purification of N-glycans from protein C

Protein C samples were incubated with PNGase F (substrate: enzyme = 250:1 mass ratio) in 50 mM sodium phosphate at 37°C for 24 h. Released N-glycans were separated from protein using C18 Extract Clean columns (100 mg, 1.5 mL Alltech, Deerfield, IL). The cartridges were equilibrated with 6 mL of 5% acetic acid in water. Then, the sample was applied to the column. The flow through, along with the wash of 1 mL of 5% acetic acid, contained the N-glycans.

The N-glycan fraction was further desalted by using Carbograph Extract Clean columns (150 mg, 4 mL, Alltech). The solvent system was as follows: A, 0.1% TFA w/v in 50% ACN/50% water, B, 0.1% TFA w/v in 5% ACN/95% water. The cartridge was washed with 30% acetic acid in water first and then HPLC grade water, and primed with 3 mL of solvent A followed by 6 mL of solvent B. The glycan solution was applied to the column, and then washed with water and solvent B. Glycans were eluted with 2 × 0.5 mL of solvent A, and dried by speed-vac.

For MS analysis, the glycan samples were further purified by Stylus Protip 5–50 μL HILIC (Hydrophilic Interaction Chromatography) needle (The Nest Group, Southboro, MA). The dried glycan was dissolved in 50 μL of 90% ACN and then aspirated in and out of the tip to allow maximum binding to the media. The adsorbed sample was washed with 90% ACN. Glycans were eluted with 50 μL of water.

2.5 Derivatization of N-glycan with 2-aminobenzoic acid

N-glycan profiles were determined based on the HPLC method of Anumula and Dhume (1998) [25], where oligosaccharides can be separated based on size, charge, linkage, and overall structure. Dried N-glycans released by PNGase F digestion were reconstituted with 100 μL water prior to derivatization. The derivatization reagent was prepared fresh by dissolving 30 mg of 2-aminobenzoic acid (2-AA) and 20 mg of sodium cyanoborohydride in 1 mL of 4% sodium acetate trihydrate w/v and 2% boric acid w/v in methanol. Purified N-glycans (20 μL) were mixed with 100 μL of the derivatization reagent in 1.5 mL screw cap centrifuge tubes, and then reacted at 80°C for 40 min. After cooling, the sample was diluted with 1 mL 95% v/v ACN/water. Excess reagent was removed by Waters Oasis HLB cartridge (1 mL, Milford, MA). The cartridge was rinsed with 2 mL of 95% v/v ACN/water. The sample was applied to the cartridge, followed by washing with 2 mL of 95% v/v) ACN/water. The AA-derivatized N-glycans were eluted by 1 mL of 20% v/v ACN/water. The eluted N-glycans were stored at –80°C for normal phase (NP)-HPLC profiling analysis.

2.6 NP-HPLC profiling of AA-derivatized N-glycan

Profiling of AA-derivatized N-glycan was performed on an amine-bonded polymeric column (Polymer-NH_2, 5μm, 4.6 mm id × 250 mm L, Astec, Whippany, NJ) with a CPF10 prefilter (Vydac, Hesperia, CA). Solvent A was 2% acetic acid and 1% tetrahydrofuran (inhibited) in ACN, and Solvent B was 5% acetic acid, 3% triethylamine and 1% tetrahydrofuran (inhibited) in water. The derivatized sample (100 μL) was injected by autosampler. The gradient program started at 30% B for 2 min, followed by an increase to 95% B over 80 min. After separation, the column was isocratic at 95% B for 15 min and then re-equilibrated with initial conditions for 15 min prior to next injection. Column temperature was 50°C and the flow rate was constant at 1 mL/min. The HPLC system consisted of Waters 2695 Separations module and 2475 fluorescent detector (Milford). The detector settings were 360 nm excitation, 425 nm emission wavelength, and 20 nm bandwidth.

2.7 ESI-IT MS/MS analysis of N-glycans

MS analysis was performed on a 4000 Q-Trap hybrid triple quadruple/IT system (Applied Biosystems, Foster City, CA) with a MicroIon Spray II ion source. Approximately 30~50 pmol/μL of N-glycans was prepared in 50% methanol with 10 mM ammonium nitrate for negative ion mode. The sample solution was infused using a nanoflow Picotip emitter (Uncoated SilicaTip, 360 μm od/15 μm id, New

Objectives, Woburn, MA) at 0.5 μL/min. Source and inlet conditions were as follows: curtain gas = 20, collision gas = high, ion spray voltage = –2400 V, Gas 1 = 5, and interface heater temperature = 150°C. The declustering potential (DP) was set at –90 V. For MS/MS mode, the collision energy (CE) was dependent on the analyte. The scan rate was set to 1000 amu/s for enhanced MS (EMS) (IT mode) and enhanced product ion (EPI) scans, and 250 amu/s for enhanced resolution (ER) scan. For EMS, the linear IT (LIT) fill time was 100 ms with Q0 trapping activated. In EPI, Q1 was set to low resolution, with Q0 trapping activated, and an LIT fill time of 400 ms.

Information-dependent acquisition (IDA) was also used for data collection from underivatized N-glycan samples. Survey scans (MS) were performed in EMS, followed by ER of the four most intense peaks. Then, MS/MS spectra were taken on these four peaks. IDA criteria were set as follows: select 1-4 most intense peaks, include charge states 1-4 plus unknowns; ER was used to confirm charge state, and former target ions were excluded after one occurrence for 60 s. Tentative structures identified from MS spectra were based on matches with glycans from the GlycoMod database.

2.8 Glycopeptide preparation

Glycoprotein was reduced and alkylated prior to proteolytic digestion. Briefly, glycoprotein in 50 mM ammonium bicarbonate was mixed with DTT to make 5 mM final concentration and incubated at 60°C for 1 h. After cooling on ice, iodoacetamide was added to a final concentration of 10 mM and then the sample was incubated at room temperature in the dark for 30 min. The reduced and alkylated glycoprotein was incubated with PNGase F in 97 atom % $H_2{}^{18}O$ or regular water at 37°C overnight. The deglycosylated peptide was digested with GluC (1:20 = enzyme:substrate w/w) at 25°C for 12 h and then with LysC (1:20 = Enzyme:substrate w/w) at 30°C for 12 h to generate VSFL(D)$_{97}$CSLDNGGCTHYCLEE and IPVVPH(D)$_{329}$E. EVFVHP(D)$_{248}$YSK and R(D)$_{313}$RTFVLNFIK were obtained by digestion with LysC (1:20 = enzyme:substrate mass ratio) at 30°C for 12 h. The PNGase F digestion step was omitted to obtain glycosylated peptides. The reactions were quenched with 1% v/v formic acid to yield a final 0.1% v/v solution.

2.9 LC-ESI-IT MS/MS analysis of glycopeptides

The peptides were loaded onto an Acclaim PepMap100 column (C18, 3 μm, 75 μm id × 150 mm, Dionex) using an UltiMate 3000 LC system (Dionex, Sunnyvale, CA) at 300 nL/ min. Solvent A was 0.1% v/v formic acid in water and Solvent B was 0.1% v/v formic acid in ACN. Solvent B was held at 5% for 5 min, increased to 25% over 80 min and then increased to 95% over 5 min, and held at 95% for 10 min. The column was re-equilibrated for 10 min prior to next run. The eluted samples were continuously infused into a 4000 Q-Trap hybrid triple quadruple/IT mass spectrometer.

For quantitation of the Asn-329 site occupancy, isotopically labeled internal peptide standards were designed as described in Hulsmeier *et al.* [26]. We observed that the ionization efficiency of IPVVPHNE was 30–40% higher than IPVVPHDE. Therefore, as Hulsmeier *et al.* [26] discussed, isotopically labeled internal standards for both forms of a glycopeptide are best for accurate absolute quantification of site occupancy. Isotopically labeled amino acids are in bold and underlined, and the N-glycan sites in the peptide sequences are in parentheses in the text and figures. Two Pro of the peptide I**P**VV**P**H(N)E (+ 12.03 Da) and one Pro of IPVV**P**H(D)E (+ 6.01) were isotopically labeled to improve the distinction between the internal standards and the corresponding sample peptides. The deglycosylated sample peptides bearing Asp were also labeled with ^{18}O by digesting the glycoprotein with PNGase F in an $H_2{}^{18}O$ buffer as described earlier. Therefore, a 2.99-Da mass difference was obtained between the sample peptides containing a converted Asp *vs.* an unoccupied Asn. The multiple reaction monitoring (MRM) transition on the 4000 QTrap was used for the quantitation with Q1 and Q3 set at low resolution. The transition parameters and CE are given in Table 1 and were selected based on maximal signal intensities as judged from the MS/MS spectra of the peptides bearing Asp-329 (see Figure 5). The dwell time for each MRM transition was set to 100 ms. An equal amount (3 pmol each) of isotopically labeled standard peptide pairs was spiked into the samples prior to analysis. The peaks corresponding to Q1/Q3 MRM transition were integrated using Analyst quantitation software (Applied Biosystems). The sample peptide amount was calculated by dividing the sample peak area by the corresponding standard peak area, and

Table 1. MRM transition settings for the standard and sample peptides used for analysis of Asn-329 occupancy of hPC and tg-PC. Isotopically labeled Pro-P (+6.01 Da) and Asp-D (+2.00 Da) are bold and underlined, and the parentheses indicate the N-glycan sites.

Type	Sequence	Q1 $[M+2H]^{2+}$ [*m/z*]	Q3$[M+H]^{1}$ [*m/z*]	Q3 ion	CE [V]
Sample	IPVVPH(N)E	452.74	496.20	y4	35
Standard	I**P**VV**P**H(N)E	458.74	502.20	y4	40
Sample	IPVVPH(**D**)E	454.24	499.20	y4	35
Standard	IPVV**P**H(D)E	456.23	503.20	y4	40

multiplying with the standard peptide amount added. The N-glycan site occupancy at Asn-329 is the percentage of the amount of sample peptide IPVVPH(**D**)E among the summed amount of the two peptides IPVVPH(N)E and IPVVPH(**D**)E.

For the analysis of glycosylated peptides, precursor ion scans were performed for the product ion *m/z* 204.1. Source and inlet conditions were as follows: curtain gas = 20, collision gas = high, ion spray voltage = 4200 V, Gas 1 = 25, DP = 65 V, CE= 45 V, and interface heater temperature = 150°C. The scan rate was set to 250 amu/s for ER and 1000 amu/s for EPI. The resolution for Q1 and Q3 was set to low and dynamic fill time was used. The MRM analysis was also performed for relative quantitation of glycopeptides at each N-glycan site. Transition parameters were the *m/z* of glycopeptide precursor ions for Q1, 204.1 (HexNAc) for Q3, 40 for CE, and 100 ms for dwell time. The peaks corresponding to Q1/Q3 MRM transition were integrated using Analyst quantitation software. The source and gas conditions were the same as above.

3 Results and discussion

N-glycan structures and the micro-heterogeneity at each N-glycan site of a commercially available plasma-derived hPC and the recombinant version from transgenic animals were investigated. MRM analysis was performed to quantify site occupancy at Asn-329 and compared with previously published studies that used SDS-PAGE [6–8]. These studies were the basis for N-glycosylation analysis for two transgenic animals, denoted as 110-1 and 110-3, which were founder animals that contained a genomic hPC transgene construct with a 2.6-kb mouse whey acid protein (mWAP) promoter. The average tg-PC expression levels over the course of lactation appeared to be copy-number dependent for these animals: 100–300 mg/L for 110-1, and 1000–1600 mg/L for 110-3 [27], representing a variation of expression approaching tenfold. We sought to determine if differences in N-glycan attachment and processing (especially at Asn-329) were present in the transgenic animal bioreactor in this range of expression levels.

3.1 Sialic acid content of hPC and tg-PC

The first level of analysis was to quantify the type and amount of sialylation in the samples. Sialic acids were analyzed by RP-HPLC using the method described in Anumula *et al*. [24]. As summarized in Table 2, only N-acetylneuraminic acid (Neu5Ac) was detected in both hPC and tg-PC. N-glycolylneuraminic acid (Neu5Gc) was below the lower limit of the quantitation (0.05 mol/mol protein C). Total Neu5Ac content of hPC was 50–80% higher than tg-PC, which may mean that the N-glycans of hPC are more highly branched and/or have more complete sialylation than tg-PC. The amount of sialylation in the tg-PC samples does not appear to be rate limiting within this expression level range. We also found no Neu5Gc incorporation in the N-glycans of tg-FIX expressed in the milk of transgenic pigs [23]. These results indicate that the porcine mammary epithelial cells may glycosylate with only Neu5Ac termination, although the pig processes N-glycans containing both Neu5Ac and Neu5Gc in other tissues such as submandibular gland, lymph node, small intestine, spleen, and thymus [28].

3.2 NP-HPLC profiling of 2-AA-derivatized N-glycans

A "fingerprint" of the N-glycan structures was obtained by releasing the glycans with PNGase F, derivatizing with the fluorescent label 2-AA, and then separating with NP-HPLC as described in Anumula and Dhume (1998) [25], in which AA-derivatized oligosaccharides can be separated based on size, charge, linkage, and overall structure. The well-characterized glycoprotein, bovine fetuin, is used as a standard in assignment of N-glycan separation based on the number of sialic acid moieties. The separated N-glycans of bovine fetuin are grouped as 0S for neutral, 1S for mono-, 2S for di-, 3S for tri-, and 4S for tetra-sialylated oligosaccharides (Figure 1A). Figures 1B–D show that N-glycans of hPC are di- or tri-sialylated structures, while tg-PC contains primarily mono- and di-sialylated oligosaccharides. Sialylated N-glycans of both hPC and tg-PC are more than 98% of total the N-glycan population, based on relative peak area integration from the chromatograms (Table 2).

Table 2. Sialic acid content and percentages of sialylated glycan of hPC and tg-PC [a]

	Expression level [mg/L]	Neu5Ac [mol/mol protein][b]	Neu5Gc [mol/mol protein]	% of disialylated glycan [c]	% of total sialylated glycan [c]
hPC	–	7.4 ± 0.3	ND	87	99
tg-PC [pig 110-3]	1000~1600	4.9 ± 0.2	ND	12	99
tg-PC [pig 110-1]	100~300	4.1 ± 0.4	ND	7	98

a) Concentration of PC samples was obtained by measuring OD_{280nm} using an extinction coefficient of 1.45 mL/mg-cm [1]. ND = not detectable.

b) Molar ratio of Neu5Ac to Protein C was calculated from RP-HPLC analysis of sialic acid (run in triplicate).

c) Percentages were obtained from NP-HPLC profiles.

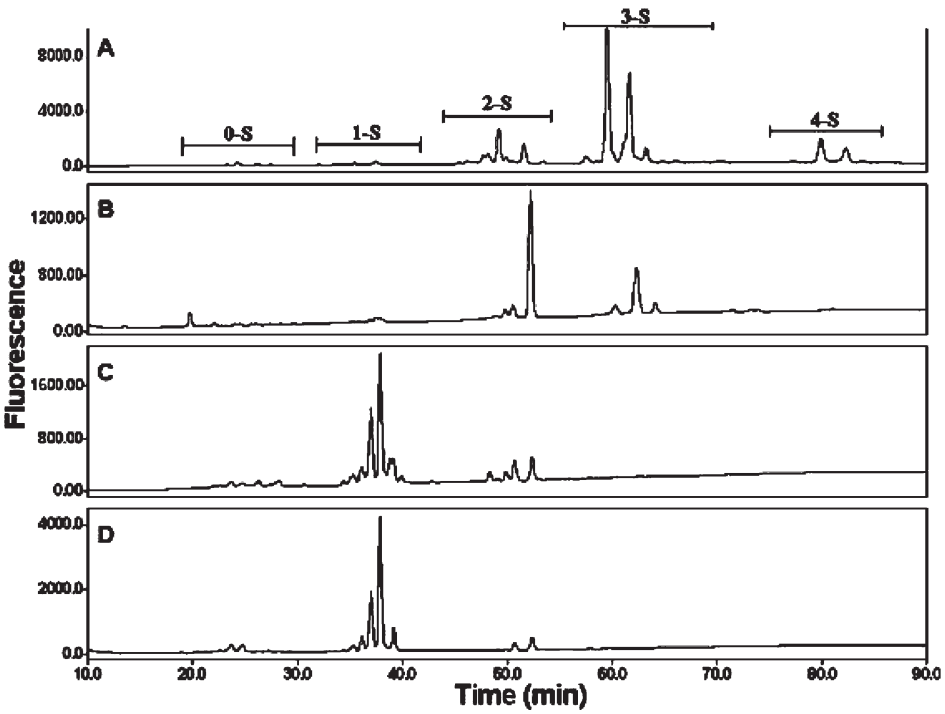

Figure 1. HPLC N-glycan profiles of (A) bovine fetuin standard, (B) hPC, (C) tg-PC [110–3], and (D) tg-PC [110–1]. The separated N-glycans of bovine fetuin are grouped as 0S for neutral, 1S for mono-, 2S for di-, 3S for tri-, and 4S for tetra-sialylated oligosaccharides as shown in panel (A).

3.3 N-glycan structures of hPC and tg-PC

Structural analysis of the sialylated N-glycans was performed by ESI-MS/MS IT analysis in negative mode (Figure 2). The tentative structures of the ions from N-glycan MS profiles of hPC and tg-PC were proposed by searching GlycoMod (http://ca.expasy.org/tools/glycomod) and our own databases of N-glycan structures, and then assigned after inspecting the MS/MS spectra (Figure 3). HPC glycans are denoted as H1, H2, H3, *etc.*, and tg-PC glycans are denoted as T1, T2, T3, *etc.* and are summarized in Figure 4.

The ESI-MS profile of hPC is given in Figure 2A. The ions at *m/z* 1110 $[M\text{-}2H]^{2-}$, *m/z* 1183 $[M\text{-}2H]^{2-}$, and *m/z* 1204 $[M\text{-}2H]^{2-}$ found on hPC were assigned as di-sialylated biantennary structures without and with core fucosylation, respectively. Peaks consistent with tri-sialylated triantennary structures were also found at *m/z* 1438 $[M\text{-}2H]^{2-}$ and 1511 $[M\text{-}2H]^{2-}$ (MS/MS data not shown).

Figures 2B and C show MS spectra of tg-PC N-glycans from pig 110-3 and 110-1, respectively, with structure identifications that were determined from MS/MS analysis. Representative MS/MS spectra are given in Figure 3. The doubly charged ions in the MS spectra are a mix of deprotonated species and nitrate adducts, because ammonium nitrate was used as an ion pairing reagent to improve sensitivity. The *m/z* 1971 $[M\text{-}H]^-$ peak is identified as a monosialylated biantennary with Gal and GalNAc residues on each antennae (Figure 3A). The ion *m/z* 424 indicates Hex + HexNAc + 59 as described in Harvey 2005 [29] and can be $^{1,3}A_{4\alpha}Y_6$ or $^{1,3}A_5Y_5$. The fragment ion $B_{3\alpha}$ (*m/z* 655) indicates Neu5Ac + Hex + HexNAc sequence, and $B_{3\beta}$ (*m/z* 696) indicates Neu5Ac + HexNAc + HexNAc, reflecting there are possible isomers according to the location of Neu5Ac termination. The same structure with a fucosylation is obtained from the *m/z* 2117 $[M\text{-}H]^-$ precursor ion (Figure 3B). The core fucosylation can be deduced from the 146-Da difference between the fragment ion $^{0,2}A_7$ from Figure 3A (*m/z* 1870) and Figure 3B (*m/z* 2016). Figure 3C shows the MS/MS spectrum of a monosialylated biantennary glycan with two fucosylations. The fragment ions, $^{2,4}A_7$ (*m/z* 1915) and $^{0,2}A_7$ (*m/z* 2121) were 146 Da higher than the corresponding ions, $^{2,4}A_7$ (*m/z* 1769) and $^{0,2}A_7$ (*m/z* 1975) found in the MS/MS spectrum of a monosialylated biantennary glycan with a core fucosylation (data not shown), indicating that the N-glycan structure in Figure 3C contains a core fucosylation and a fucosylation on the antennae. The position of this fucosylation could not be confirmed from our data. MS/MS spectra of the other molecular ions were interpreted to assign the N-glycan structures and were the same as we found in our previous report of tg-FIX [23].

As summarized in Figure 4, the N-glycans of both hPC and tg-PC are bi- or tri-antennary complex structures. Evidence for high-mannose and hybrid structures was not found, and we were also unable to find evidence for N-glycan structures terminated with Neu5Gc or α-Gal for both hPC and tg-PC. The N-glycans of hPC are less heterogeneous than those of tg-PC, but more highly branched and more fully sialylated, which is consistent with sialic acid content analysis and NPHPLC profiles.

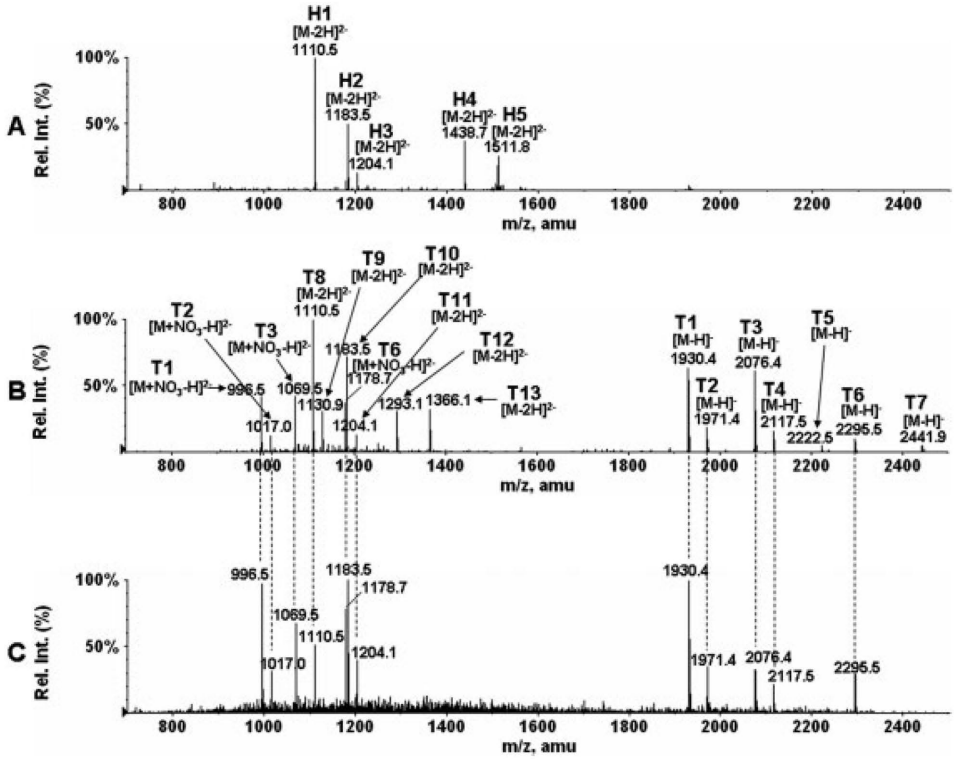

Figure 2. MS spectra of N-glycans released from (**A**) hPC, (**B**) tg-PC [110-3], and (**C**) tg-PC [110-1]. HPC glycans are denoted as H1, H2, H3, *etc*., and tg-PC glycans are denoted as T1, T2, T3, *etc*. The N-glycan structures of H1 ~ 5 and T1 ~ 13 are summarized in Figure 4.

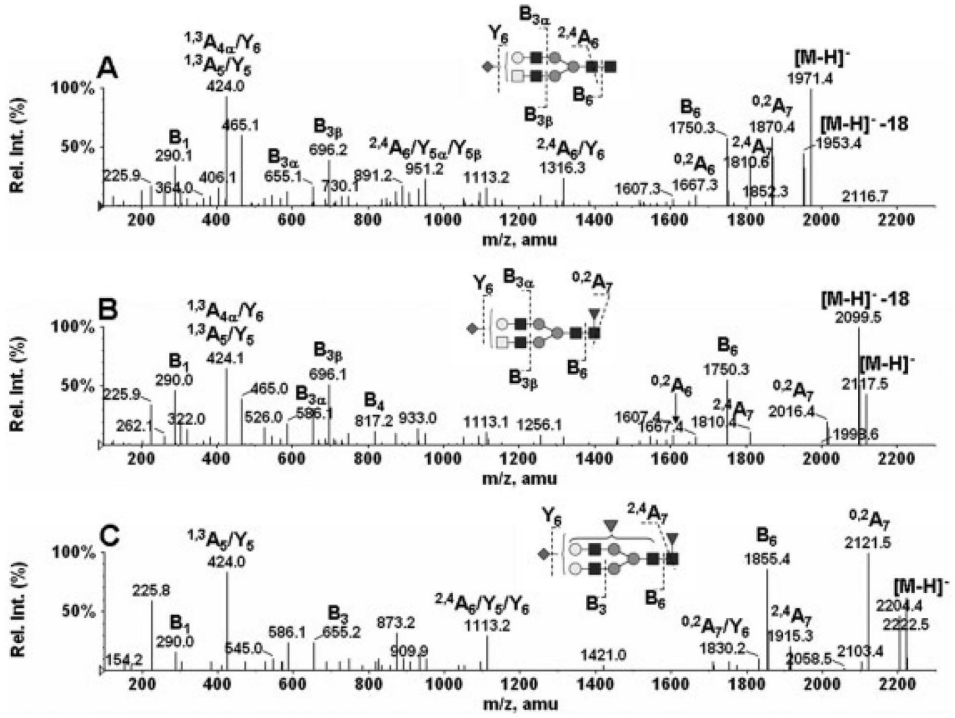

Figure 3. MS/MS spectra of tg-PC 110-3 N-glycan precursor ions (A) *m/z* [M-H]$^-$ 1971, (B) *m/z* [M-H]$^-$ 2117, and (C) *m/z* [M-H]$^-$ 2222. Key to symbols: ▼ = fucose; = ● mannose; = ○ galactose; ■ = GlcNAc; □ = GalNAc; ♦ = Neu5Ac.

3.4 Macroheterogeneity: N-glycan site occupancy in hPC and tg-PC

Protein C contains the atypical Asn-X-Cys sequon. Analysis by SDS-PAGE and Western blot techniques estimated a 70–80% occupancy of Asn-329 in hPC [6–8]. One objective of this study was to analyze site occupancy at Asn-329 using current technology (MS/MS quantitation) and determine how efficiently this sequon is glycosylated in both hPC and tg-PC. The proteins were deglycosylated with PNGase F in an $H_2{}^{18}O$ buffer prior to proteolysis, which converts a glycosylated Asn to Asp (2.99-Da increase), and leaves

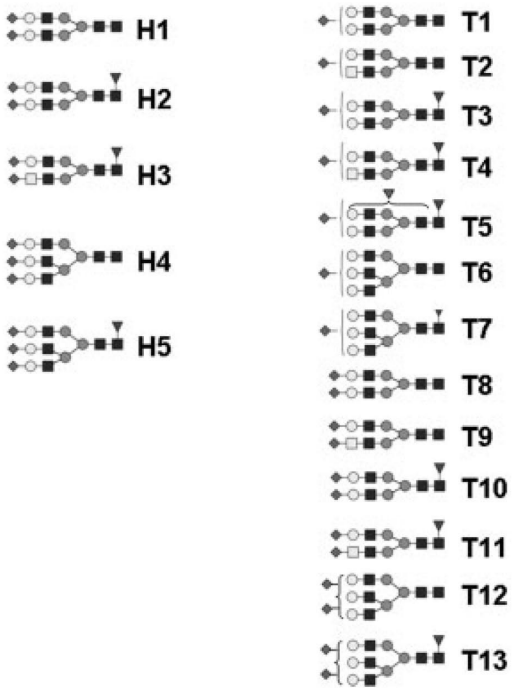

Figure 4. The identified N-glycan structures of hPC (H1 ~ 5) and tg-PC (T1 ~ 13). Keys to symbols are the same as Figure 3.

unglycosylated Asn unchanged. We were able to detect only the peptides containing ^{18}O-labeled Asp residues in the peptides containing Asn-97, 248, and 313 for both hPC and tg-PC (data not shown). These data indicate that these N-glycan sites of hPC and tg-PC are fully occupied. However, the peptides containing Asn-329 from both hPC and tg-PC had peptides bearing ^{18}O-labeled Asp or Asn, indicating the N-glycan site is partially occupied, and consistent with previous reports [6–8].

For quantitation of the Asn-329 site occupancy, MRM analysis was performed with the addition of isotopically labeled peptide standards as described in the experimental section. The parameters for MRM transition were selected empirically based on maximal signal intensities as judged from the MS/MS spectra of the peptides bearing Asn-329 or Asp-329 (Figure 5). The most intense fragment ion, y_4 was selected for Q3, and the corresponding Q1 and Q3 *m/z*, and CE for the sample and standard peptides are listed in Table 1.

Figure 6 shows the extracted ion chromatogram (XIC) of the MRM transitions of unglycosylated and deglycosylated sample peptides containing Asn-329 of hPC and tg-PC, along with the internal standards. The peptide containing the unoccupied Asn elutes earlier than the peptide having Asp. The average XIC peak areas of the peptides were used to calculate the site occupancy at Asn-329. As

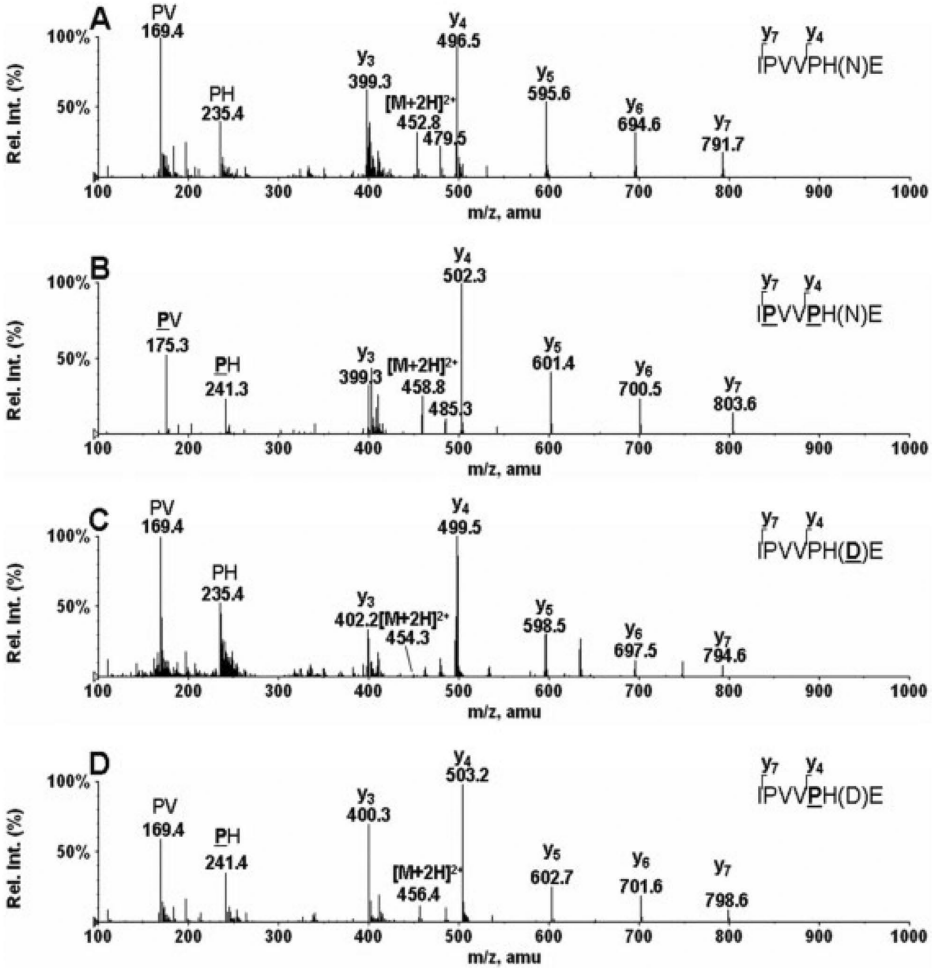

Figure 5. MS/MS spectra of (**A**) the sample peptide IPVVPH(N)E, (**B**) the isotopically labeled standard peptide I**P**VV**P**H(N)E, (**C**) the sample peptide IPVVPH(**D**)E, which is labeled with ^{18}O during PNGase F digestion, and (**D**) the isotopically labeled standard peptide IPVV**P**H(D)E. Isotopically labeled Pro-P (+6.01 Da) and Asp-D (+2.00 Da) are bold and underlined, and the parentheses indicate the N-glycosylation sites.

Table 3. N-glycan site occupancy of hPC and tg-PC

N-glycan site	Peptide sequence	Protease used	Occupancy (%) hPC	tg-PC [110-3]	tg-PC [110-1]
Asn-97	VSFL(N)[a]CSLDNGGCTHYCLEE	GluC+LysC	Full	Full	Full
Asn-248	EVFVHP(N)YSK	LysC	Full	Full	Full
Asn-313	R(N)RTFVLNFIK	LysC	Full	Full	Full
Asn-329	IPVVPH(N)E	GluC+LysC	78.4 ±0.5[b]	77.0 ±1.3[b]	74.3 ±0.9[b]

a) The parentheses indicate the N-glycan sites.
b) The data are from the average of triplicate digests and duplicate injections.

summarized in Table 3, the site occupancy at Asn-329 for hPC is 78%, which is within the range of the previous reports showing 70–80% occupancy [6–8]. There was no significant difference of the site occupancy between hPC (78%) and tg-PC (74–77%), indicating that the Asn-X-Cys sequon is recognized by the oligosaccharyl transferase complex of the porcine mammary epithelial cells with the same efficiency as that observed in human hepatocytes. We did not observe a decrease in N-glycan site occupancy of the high expressor 110-3 (77.0 ± 1.3%) *vs.* the low expressor 110-1 (74.3 ± 0.9%), indicating that attachment of the dolichol-oligosaccharide precursor in the ER at this Asn-X-Cys sequon is not a rate limiting step in this range of expression levels. Giuffrida *et al*. [12] reported that only 1% of the human alpha-lactalbumin Asn-X-Cys sequon is occupied. From these results, it appears that site occupancy of the Asn-X-Cys sequon in milk proteins is protein specific (as it appears to be for proteins synthesized in other tissues), and the capacity for filling these sites in the mammary epithelial cells (at least in the pig) can be very high.

3.5 Micro-heterogeneity of hPC and tg-PC

Another objective of this work was to evaluate the micro-heterogeneity of both hPC and tg-PC, and to determine if there is any bias as to what glycan structures are found at a particular Asn residue, particularly Asn-329. Glycopeptides containing each N-glycan site were analyzed by LC-MS/MS. The glycosylated peptides were detected using a precursor ion scan IDA method (*m/z* 204.1, HexNAc), and identity was confirmed by analysis of MS/MS spectra. Representative MS/MS spectra of glycosylated peptides from hPC and tg-PC are shown in Figure 7. The presence of N-glycan was confirmed by oxonium ions observed at *m/z* 204 (HexNAc), 292 (Neu5Ac), 366 (Hex + HexNAc), 454 (Neu5Ac + Hex), and 657 (Neu5Ac + Hex + HexNAc) in the spectra of glycopeptides. In this manner, the acquired spectra were searched for all possible glycopeptides bearing the identified N-glycan structures for the four N-glycosylation sites.

The relative amounts of the glycopeptides were compared for each N-glycan site using MRM analysis of all iden-

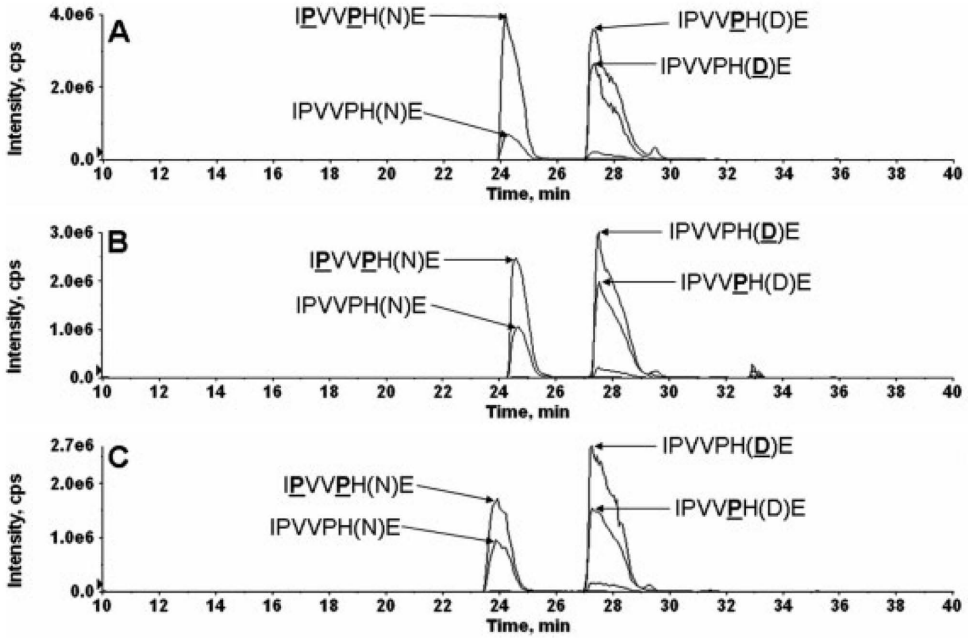

Figure 6. Extracted ion chromatograms (XIC) of the MRM transitions of the Asn-329 glycopeptides from (**A**) hPC, (**B**) tg-PC 110-3, and (**C**) tg-PC 110-1. Isotopically labeled Pro-P (+6.01 Da) and Asp-D (+2.00 Da) are bold and underlined, and the parentheses indicate the N-glycan sites.

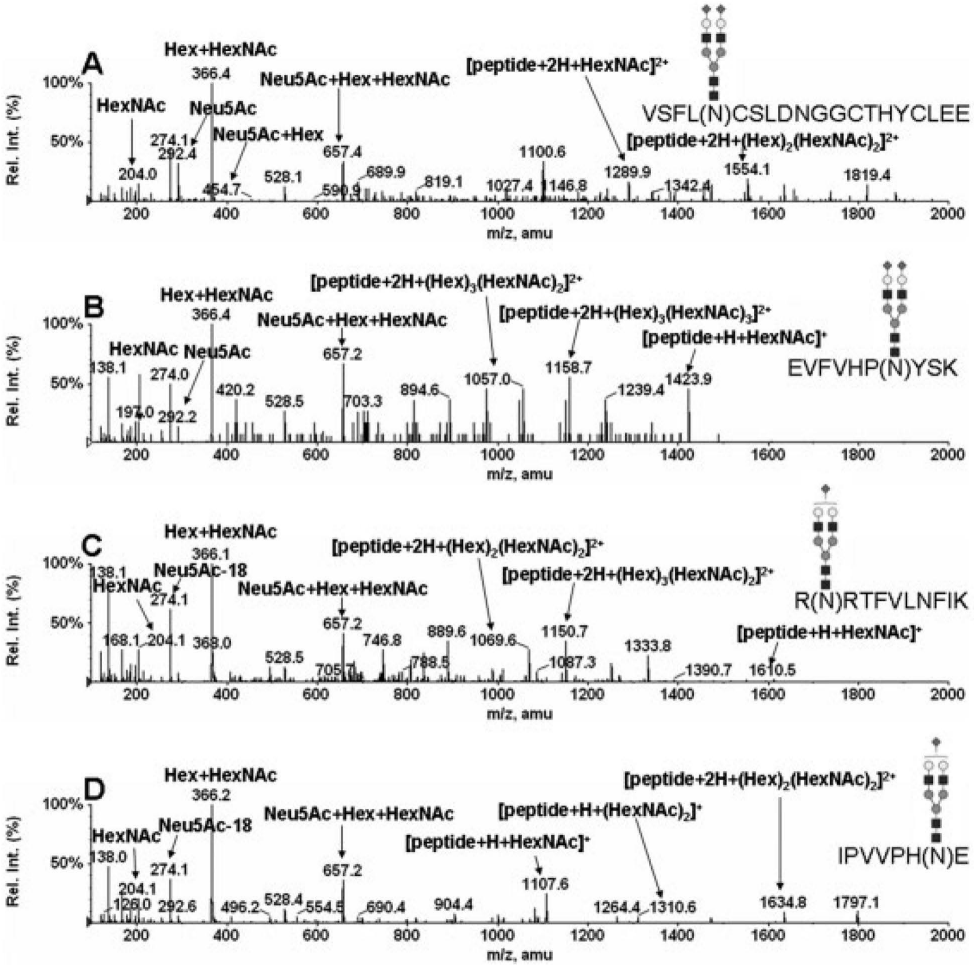

Figure 7. MS/MS spectra of hPC glycopeptides (**A**) VSFL(N)$_{97}$CSLDNGGCTHYCLEE and (**B**) EVFVHP(N)$_{248}$YSK bearing the H1 N-glycan structure, and tg-PC glycopeptides (**C**) R(N)$_{313}$RTFVLNFIK and (**D**) IPVVPH(N)$_{329}$E bearing the T1 N-glycan structure. H1 and T1 structures are shown in Figure 4.

tified glycopeptides. It is understood that the sialic acid content can affect the ionization efficiency of a glycopeptide, and so our results are not strictly quantitative, but represent a first-order estimate of the micro-heterogeneity at each site. It has been shown that glycan processing is a function of protein folding, and can be influenced by glycosylation at other sites [30], but the mechanisms responsible have not been determined. Our results for hPC showed that there was a distinct bias for particular N-glycan structures at each Asn residue (Figure 8A). Asn-97 contained a more even distribution of glycan structures than other sites, with the predominant being the fucosylated triantennary sialylated species H5. Asn-248 was glycosylated predominantly with the biantennary sialylated species H1 (not fucosylated), and contained lower amounts of fucosylated glycans H2, H3, and H5. Asn-313 was predominantly glycosylated with the tri-antennary sialylated species H4 (non-fucosylated), and had low levels of fucosylation at this site. Asn-329 was largely glycosylated with the fucosylated biantennary sialylated species H3, which contains GalNAc in one of the antennae.

The microheterogeneity of tg-PC from the two expression levels is summarized in Figures 8B and 8C. At Asn-97 and Asn-248, the biantennary monosialylated T1 and T3 (fucosylated) species were predominant, with no clear preference for fucosylation. At Asn-313, both animals had a significant amount of species T2, which has GalNAc in one antennae, in addition to the T1 and T3 species. At Asn-329, the majority of the MRM signal was due to glycans which has a GalNAc in one antenna (T4, T9, and T11), although there was also a presence (~15–25% of peak areas) from the T1 species. Although some animal-to-animal variation in glycosylation is to be expected and is observed, these features are consistent for both animals with high and low expression levels. Consistent with the NP-HPLC profile data, we do not see gross differences in glycan structures between the animals, and the distribution of glycan structures at each Asn residue is similar. Thus, we can conclude that a tenfold difference in expression level does not affect N-glycan processing at each of the four sites.

One interesting feature of the microheterogeneity analysis is that for both hPC and tg-PC, glycans with GalNAc in the antennae (H3 for hPC and T4, T9, T11 for tg-PC) were more abundant at Asn-329 than at Asn-97, 248, and 313. The reason for this is not known at this time. Sato *et al*. [15] reviewed the structures found at Asn-X-Cys sequons in other proteins, and there does not appear to be any specific inter-protein bias for glycan processing at Asn-X-Cys sequons. For the case of hPC and tg-PC, it may be that the GalNAc transferase has more access to the nascent glycan at Asn-329 than the other locations.

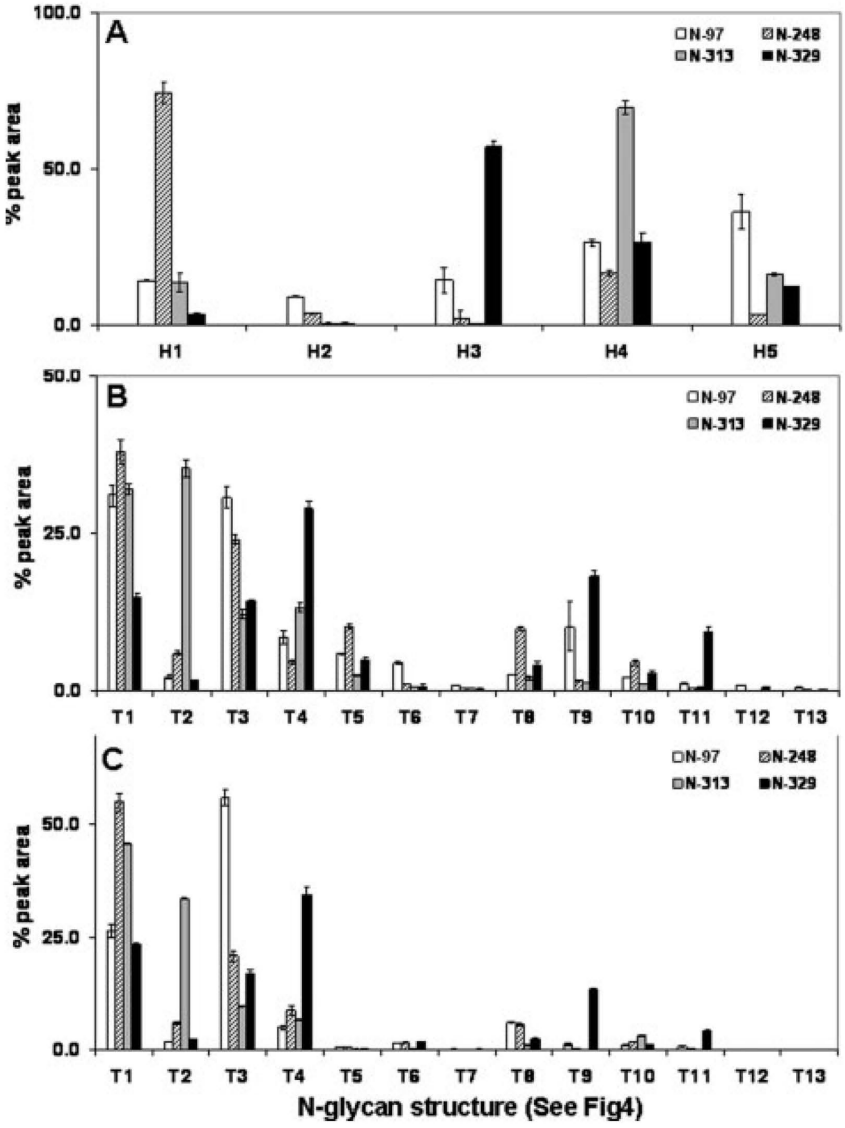

Figure 8. Percentage of glycopeptide peak area at each N-glycan site of (**A**) hPC, (**B**) tg-PC 110-3, and (**C**) tg-PC 110-1. Peak areas were obtained from MRM analysis of all detectable glycopeptides. The N-glycan structures of H1 ~ 5 and T1 ~13 on the *x*-axis are summarized in Figure 4.

4 Concluding remarks

The N-glycans of hPC are complex di- and tri-sialylated structures, and MRM analysis resulted in a measured 78% site occupancy at Asn-329, which agrees with previous reports that used SDS-PAGE methods. We analyzed the microheterogeneity of hPC N-glycosylation, and found that there was a distinct bias for particular structures being present at each of the four sites, and that glycans with GalNAc in the antennae were predominant at the Asn-329 site.

We compared hPC N-glycosylation with a recombinant protein C produced in the milk of transgenic animals having expression levels that ranged from about 100–1600 mg/L. The N-glycans of tg-PC were complex sialylated structures (with Neu5Ac only), but were less branched and partially sialylated. The porcine mammary epithelial cells glycosylate the Asn-X-Cys sequon with a similar efficiency as human hepatocytes, and the extent of site occupancy at this sequon was not affected by a ten-fold difference in expression level. Thus, the mammary epithelial cells have a very high capacity for glycosylation of Asn-X-Cys sequons. We have also shown that this range of expression level does not significantly affect the tg-PC N-glycan structures at each site: N-glycan processing in the Golgi is not rate limiting. Similar to hPC, we found that glycans with GalNAc in the antennae were predominant at Asn-329. The reason for this is not understood at this time, but it appears to be specific for protein C, and not a general feature of glycans at the Asn-X-Cys sequon.

We found that the tg-PC N-glycan structures were very similar to those found in the analysis of tg-FIX [23] and endogenous lactoferrin [31], and thus we have three cases of porcine mammary epithelial cells processing glycoproteins at gram/liter/hour expression levels with similar N-glycan structures. This is in contrast to N-glycosylation of hPC and human Factor IX (hFIX) by hepatocytes. HFIX is homologous to hPC: both are vitamin K-dependent glycoproteins that are synthesized in hepatocytes, and they

are homologous with respect to amino acid sequence, domain structure, and function (both are serine proteases). HFIX has two N-glycosylation sites [32] and significantly more heterogeneity of N-glycan structures than we have found for hPC. Since both hPC and hFIX are synthesized and secreted by hepatocytes, where the N-glycan biosynthesis and processing pathways should be the same for both proteins, one could hypothesize that the folding of the glycoprotein substrate plays an important role on N-glycan processing in human hepatocyte Golgi. However, the data clearly show that this hypothesis cannot be extrapolated to tg-PC and tg-FIX; the N-glycans of tg-PC are very similar to those found for tg-FIX with respect to both the level of sialylation and the structures [23]. The reason for this is not known at this time. It could be that in the porcine mammary epithelial Golgi, recombinant proteins are not folded to the extent that they are in human hepatocytes, and thus the glycosidases and glycosyltransferases see more uniform substrates. The glycosidases and glycosyltransferases present in the porcine mammary epithelial cells could also be inherently less sensitive to the nascent protein structure. In addition, fewer processing enzymes may be present in the mammary epithelial cells during lactation. Whatever the cause, these results indicate that the N-glycosylation machinery of porcine mammary epithelial cell is consistent with respect to the N-glycan processing of two different recombinant glycoproteins, and consistent with respect to a previously reported endogenous milk glycoprotein.

Acknowledgments

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Conflict of interest statement

W. H. Velander is a co-founder and stockholder in Progenetics LLC, a company that develops transgenic animal bioreactors for production of therapeutic proteins.

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