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Structure of the O-glycopeptides isolated from bovine milk component PP3

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The heat-stable acid-soluble phosphoglycoprotein component PP3 was isolated from the bovine milk proteose peptone fraction by concanavalin A affinity chromatography. Glycopeptides from the ConA-bound fraction corresponding to the component PP3 were obtained by Pronase digestion and were separated by gel filtration into high and low-molecular-mass glycopeptides. In a previous work, we have investigated the structure of the N-glycans from the high-molecular-mass glycopeptides [Girardet *et al.* (1995) *Eur J Biochem* 234: 939–46]. Here, we describe the structure of the O-glycans from the low-molecular-mass glycopeptides. By combining methylation analysis, mass spectrometry, 400 MHz ^{1}H-NMR spectroscopy and peptide sequence analysis, we show that the low-molecular-mass fraction contains several neutral glycopeptides. A mixture of the following three glycan structures linked to the Thr^{86} has been identified: GalNacα1-O-Thr, Gal(β1-3)GalNAcα1-O-Thr and Gal(β1-4)GlcNAc(β1-6)[Gal(β1-3)]GalNAcα1-O-Thr.

***Keywords*: bovine milk, lactophorin, proteose peptone, component PP3, O-glycan**

***Abbreviations*: ConA, concanavalin A; GlyCAM, glycosylation-dependent cell adhesion molecule; HPAE, high-pH anion-exchange, MFGM, milk fat globule membrane; PP, proteose peptone.**

Introduction

Proteose peptone, a bovine milk heat-stable and acid-soluble protein fraction is known to contain a complex mixture of protein hydrolysates, mainly from caseins and component PP3 also termed lactophorin or lactoglycophorin [1, 2]. This last component, isolated recently, is a phosphoglycoprotein with an apparent molecular mass of 28 kDa [3]. The peptide sequence of component PP3 was established and the presence of two O-glycosylation sites (Thr^{16} and Thr^{86}) and one N-glycosylation site (Asn^{77}) were identified [3]. Two of them (Asn^{77} and Thr^{86}) located in the C-terminal region of the protein are also found in the 18 kDa glycoprotein called PP3 f(54–135) associated to component PP3 and corresponding to the amino acid sequence 54 to 135 of the component PP3 released by plasmin hydrolysis in milk [3]. Component PP3 was identified as a bovine homologue of the murine glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1), a murine like endothelial cell surface ligand for the L-selectin leukocyte adhesion molecule [4, 5]. Moreover, bovine milk component PP3 possesses several common epitopes with the milk fat globule membrane glycoproteins [6, 7].

Previous studies [8] have determined the structure of the N-linked glycans of the high-molecular-mass glycopeptides from bovine milk component PP3. In the present data, by using high-pH anion-exchange (HPAE) chromatography, methylation, MALDI/TOF spectrometry and ^{1}H-NMR spectroscopy, we have determined the structure of the O-glycans from the low-molecular-mass glycopeptides. Three carbohydrate molecular variants linked to the main O-glycosylation site (Thr^{86}) of component PP3 have been characterized.

Materials and methods

Materials

ConA-Sepharose 4B was from Pharmacia LKB Biotechnology AB (Uppsala, Sweden), BioGel P-2 and Chelex 100 from Bio-Rad Laboratories (Richmond, CA, USA). Pronase EC

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3.4.24.4 from *Streptomyces griseus* was purchased from Merck (Darmstadt, Germany) and D_2O (99.95 atom % D) from the Commissariat à l'Energie Atomique (Saclay, France). All reagents were of the highest purity and all solvents were redistilled before use.

Preparation of component PP3

Proteose peptone extracts were prepared from bovine raw milk sample produced by a single animal with the following phenotype: α_{s1}-casein BB, β-casein A_1A_2, κ-casein AB, α-lactalbumin AA and β-lactoglobulin BB as previously reported [9]. The heat-stable acid-soluble glycoproteins were fractionated on a ConA-Sepharose 4B column (1.5 cm × 15 cm), eluted successively with the equilibrating buffer: 5 mM sodium acetate buffer, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 M NaCl, pH 6.8 (ConA-unbound fraction) and with the same buffer containing 0.5 M methyl-α-D-glucopyranoside (ConA-bound fraction) [10]. Each fraction was dialysed and lyophilized. Trace amounts of methyl-α-D-glucopyranoside contained in the ConA-bound fraction corresponding to the component PP3 were removed on a Sephadex G-25 column (3 cm × 53 cm) eluted with water.

Preparation of component PP3 glycopeptides

The ConA-bound fraction was subjected to exhaustive digestion by Pronase [11]. The reaction was stopped by decreasing the pH to 4 with 10% (v/v) acetic acid. Glycopeptides were precipitated by nine volumes of ethanol at 4 °C and the procedure of proteolysis was repeated twice. Residual proteins and Pronase were denatured and removed by the addition of 12% (w/v) trichloracetic acid. The glycopeptides were desalted and fractionated twice on a Bio-Gel P-2 column (1.5 cm × 53 cm) onto high and low-molecular-mass glycopeptides. Elution was carried out with water and the monosaccharide containing fractions were stained on silicagel plates with orcinol/H_2SO_4 reagent. Trace amounts of divalent cations or heavy metals were removed on a Chelex 100 column (1.5 cm × 10 cm).

High-pH anion-exchange chromatography

The low-molecular-mass glycopeptides were fractionated by HPAE chromatography on a Dionex liquid chromatography system (Dionex, Camberley, Surrey, UK) equipped with a column CarboPac PA-1 (250 mm × 4 mm, 5–10 μm). The column was equilibrated with 25 mM sodium acetate in 0.1 M NaOH (buffer A). For the first 10 min of separation, 100% buffer A was used. Then, a linear gradient was run for the next 100 min to reach the sodium acetate concentration of buffer B consisting of 250 mM sodium acetate in 0.1 M NaOH. The eluted glycopeptides were monitored on-line by pulse amperometric detector. The column flow rate was 1 ml min^{-1} [12].

Carbohydrate composition

The monosaccharide molar ratios of the glycopeptides were determined after methanolysis [13] and gas-liquid chromatography of the trimethylsilylated methyl glycosides on a capillary CPSIL 5 CB column (0.2 mm × 25 m) [14].

Methylation analysis

The glycopeptides were permethylated [15] and the partially methylated methyl glycosides obtained after methanolysis were peracetylated with pyridine : acetic anhydride (0.2 : 1, v/v) at 37 °C, overnight. The peracetylated permethylated methyl glycosides were then analysed by gas–liquid chromatography [16] and also identified by mass spectrometry using a mass spectrometer RIBERMAG R 10-10 (Riber, Rueil-Malmaison, France) coupled to the data system Sydar 121.

MALDI/TOF mass spectrometry

The experiments were carried on a Vision 2000 MALDI mass spectrometer (Finnigan MAT, Bremen, Germany), a reflection time-of-flight (TOF) instrument equipped with a nitrogen laser at 337 nm and operated in the positive or negative detection with 6 kV acceleration potential. Targets were loaded with the matrix solution (1 μl 2,5 dihydroxybenzoic acid, 10 mg ml^{-1} dissolved in water) and sample (generally 1–10 pmol of permethylated oligosaccharides dissolved in methanol) and the mixture was allowed to crystallize at room temperature. External calibration was performed using angiotensin I standard purchased from Sigma [MM (Molecular Mass): 1296.7]. Spectra obtained from single laser shots were accumulated until a satisfactory signal-to-noise ratio was obtained (usually 10–20 shots).

^{1}H-NMR spectroscopy

For 400 MHz ^{1}H-NMR spectroscopy analysis, the glycopeptides were repeatedly dissolved in D_2O at room temperature and at *pD 7* with intermediate freeze-drying [17]. The deuterium-exchanged glycopeptides were submitted to ^{1}H-NMR spectroscopy performed on a Bruker AM 400-WB spectrometer operating at 400 MHz in the fourier-transform mode with the reverse 5 mm BBI probe at a temperature of 300 °K. Chemical shifts were expressed in ppm downfield for internal sodium 4,4′-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone ($\delta = 2.225$ ppm in D_2O at 25 °C). The two-dimensional homonuclear correlated spectroscopy (COSY 90) experiment was performed using the Bruker standard pulse sequence.

Amino acid composition analysis

The glycopeptides were hydrolysed for 1 h in the PICO-TAG station (Millipore Corp., Bedford, MA, USA) at 150 °C under vacuum in the presence of 6 M HCl containing

1% phenol (v/v). Released amino acids were derivatized with phenylisothiocyanate in the amino acid analyser model 420A (Applied BioSystems, Forster City, CA, USA) and separated at 34 °C by reverse-phase HPLC on a Brownlee PTC C_{18} column (2.1 mm × 220 mm). Buffer A was 45 mM sodium acetate at pH 5.9 and buffer B was a mixture of 105 M sodium acetate at pH 4.6 and acetonitrile in a ratio 3 : 7. The column was first equilibrated with 4% buffer B and the successive gradient slopes were subsequently performed: 4–14% buffer B gradient for 5 min, 14–38% buffer B for 7 min, and a 38–67% buffer B gradient for 12 min. Detection was carried out at 254 nm and a flow rate of 300 µl min^{-1} was applied.

Peptide sequence analysis

For sequencing, the high-molecular-mass glycopeptides were previously dissolved in 20% (v/v) acetonitrile water containing trifluoroacetic acid 0.1% (v/v). Peptide sequence analysis was performed on an Applied BioSystems model 476A sequencer with on-line identification of the phenylthiohydantoin derivatives.

Results

Fractionation and characterization of the glycopeptides

Immobilized ConA affinity chromatography was performed to separate proteose peptone into ConA-unbound and ConA-bound fractions. The ConA-unbound fraction contained non-glycosylated peptides generated by *in situ* plasmin hydrolysis of the main milk proteins, especially β-casein [9] and was not further analysed. The ConA-bound fraction was resolved by SDS/PAGE electrophoresis into two major protein bands with an apparent M_r of 28 kDa and 18 kDa which were identified respectively to component PP3 and to fragment PP3 f(54–135) [18]. After Pronase digestion of the component PP3, two subfractions of purified glycopeptides were obtained by gel filtration: a major one (91%) which contained high-molecular-mass glycopeptides (MM > 2 kDa) and a minor one (9%) which contained low-molecular-mass glycopeptides (MM < 2 kDa). The high-molecular-mass glycopeptides were previously identified as N-glycosidically linked glycans of *N*-acetyllactosamine type with a GalNAc(β1-4)GlcNAc or NeuAc(α2-6)GalNAc(β1-4)GlcNAc group [8]. The heterogeneity and the structure of the low-molecular-mass glycopeptides whose monosaccharide composition is given in Table 1 was further analysed.

Structural characterization of the low-molecular-mass glycopeptides from component PP3

This fraction was examined by HPAE chromatography (Figure 1) and three peaks in the relative ratios of 16%; 34% and 50% were characterized. The acidic behaviour of the three glycopeptides may be explained by their amino acids composition. Upon analytical pellicular alkaline chromatography, the elution follows the increasing number of sugar residues. However, the amount of collected materials was not sufficient to study by ^{1}H-NMR spectroscopy each peak separated by HPAE chromatography. Therefore,

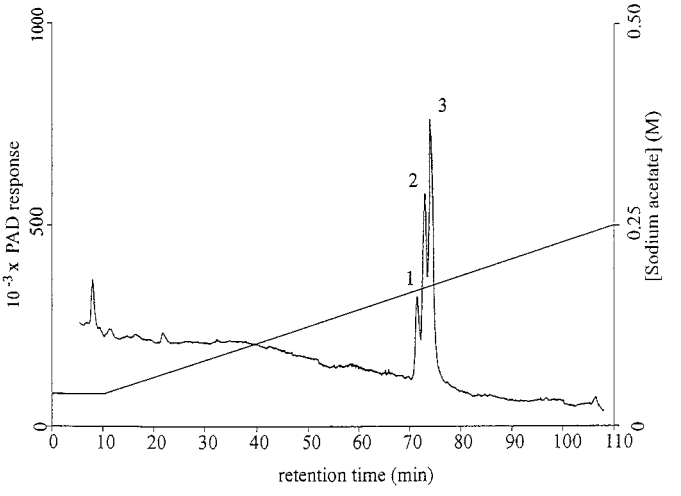


Figure 1. High-pH anion-exchange chromatography profile of the fraction containing low-molecular-mass glycopeptides isolated from component PP3.

Table 1. Monosaccharides molar ratios of glycopeptides isolated from milk component PP3 after gel filtration.

Fraction	*Sugar*					
	Fuc	*Gal*	*Man*	*GalNAc*	*GlcNAc*	*NeuAc*
Component PP3	0.9	2.2	3.0	2.5	3.9	0.8
High-molecular-mass glycopeptides	0.9	2.4	3.0	2.5	3.9	0.8
Low-molecular-mass glycopeptides	0.0	1.4	0.0	1.0	0.4	0.2

Monosaccharide molar ratios for component PP3 and high-molecular-mass glycopeptides were calculated on the basis of 3.0 Man residues and on the basis of 1.0 GalNAc residue for the high-molecular-mass glycopeptides.

the structure determination was performed on the mixture of glycopeptides. For a 1.0 residue of 2,3,4,6-Me_4Gal, permethylation analysis of the low-molecular-mass glycopeptides indicated the presence of a 0.2 residue of 3,4,6-Me_3GalNAcNMe at the terminal non-reducing position, a 0.3 residue of GalNAc and 0.4 residue of GlcNAc substituted in positions 3 and 4 as indicated by the presence of 4,6-Me_2GalNAcNMe and 3,6-Me_2GlcNAcNMe respectively. The presence of a 0.4 residue of 4-MeGalNAcNMe was attributed to an additional branching. Sialic acid has been found in the monosaccharide composition of global low-molecular-mass glycopeptide fractions but was not found by methylation analysis.

The mass spectrum of the permethylated oligosaccharides (Figure 2) contained three abundant ions at *m/z*: 314.2, *m/z*: 518.6 and *m/z*: 968.3 (Table 2). These ions were mainly found with $[M + Na]^+$, but ions $[M + K]^+$ *m/z*: 327.3, 534.6 and 984.3 were also present. Although the first component is near the matrix background, we may distinguish the molecular mass of a *N*-acetylhexosamine (HexNAc). The two other components are constituted by a disaccharide HexHexNAc and a tetrasaccharide $Hex_2HexNAc_2$. The MALDI/TOF mass spectrometry confirms the presence of three peaks evidenced upon HPAE chromatography by the elution profile from the lightest to the heaviest compound. We may also observe that the molecular masses do not account for the peptides which are then lost during the methylation procedure. In order to further elucidate the primary structure of the mixture of oligosaccharides, 400 MHz ^{1}H-NMR two dimensional homonuclear correlated spectroscopy was recorded on the glycopeptides, the COSY 90 spectrum is given in Figure 3 and the characteristic chemical shifts are summarized in Table 3. The presence of three compounds was evidenced by the spectra of GalNAc proton signals. Typical signals at $\delta = 5.062$; 5.039 and 4.980 ppm were assigned to H-1 and $\delta = 4.316$; 4.308

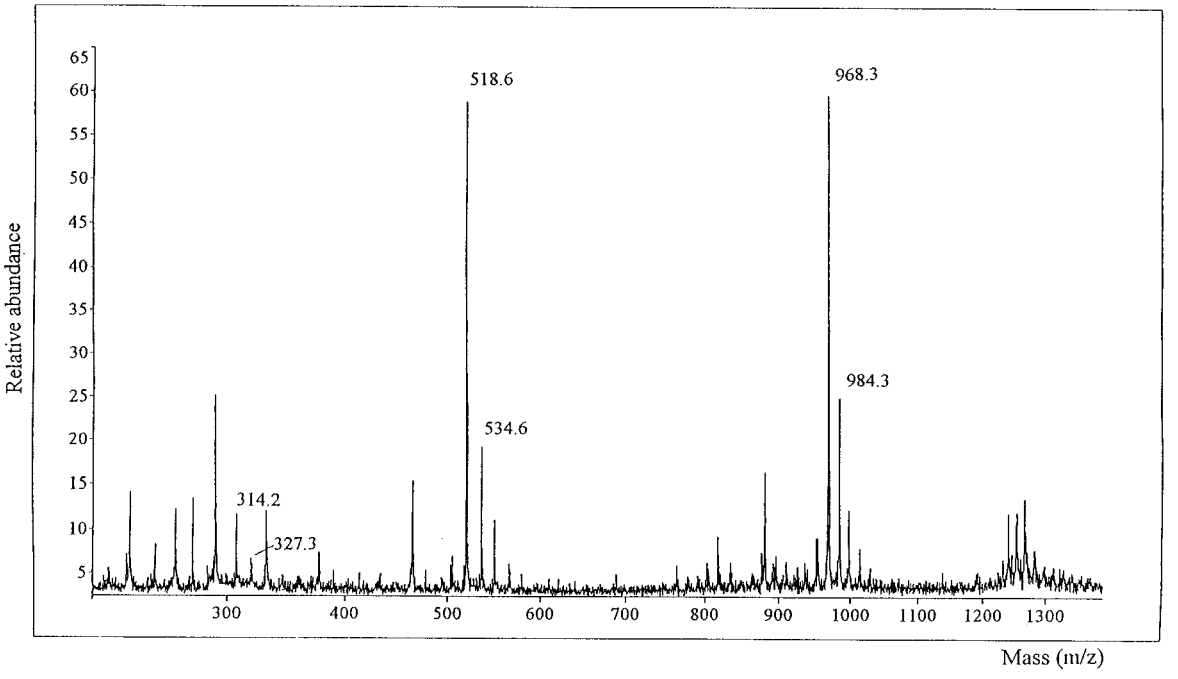


Figure 2. MALDI/TOF mass spectrometry of permethylated low-molecular-mass oligosaccharides from component PP3.

Table 2. Masses of molecular ions of permethylated low-molecular-mass oligosaccharides isolated from component PP3.

Low-molecular-mass oligosaccharides	*Molecular ion*			*Description*
	Theoretical	*Observed [M + Na]$^+$*	*Observed [M + K]$^+$*	
	291.13	314.2	327.3	HexNAc
	495.26	518.6	534.6	HexHexNAc
	944.49	968.3	984.3	$Hex_2HexNAc_2$

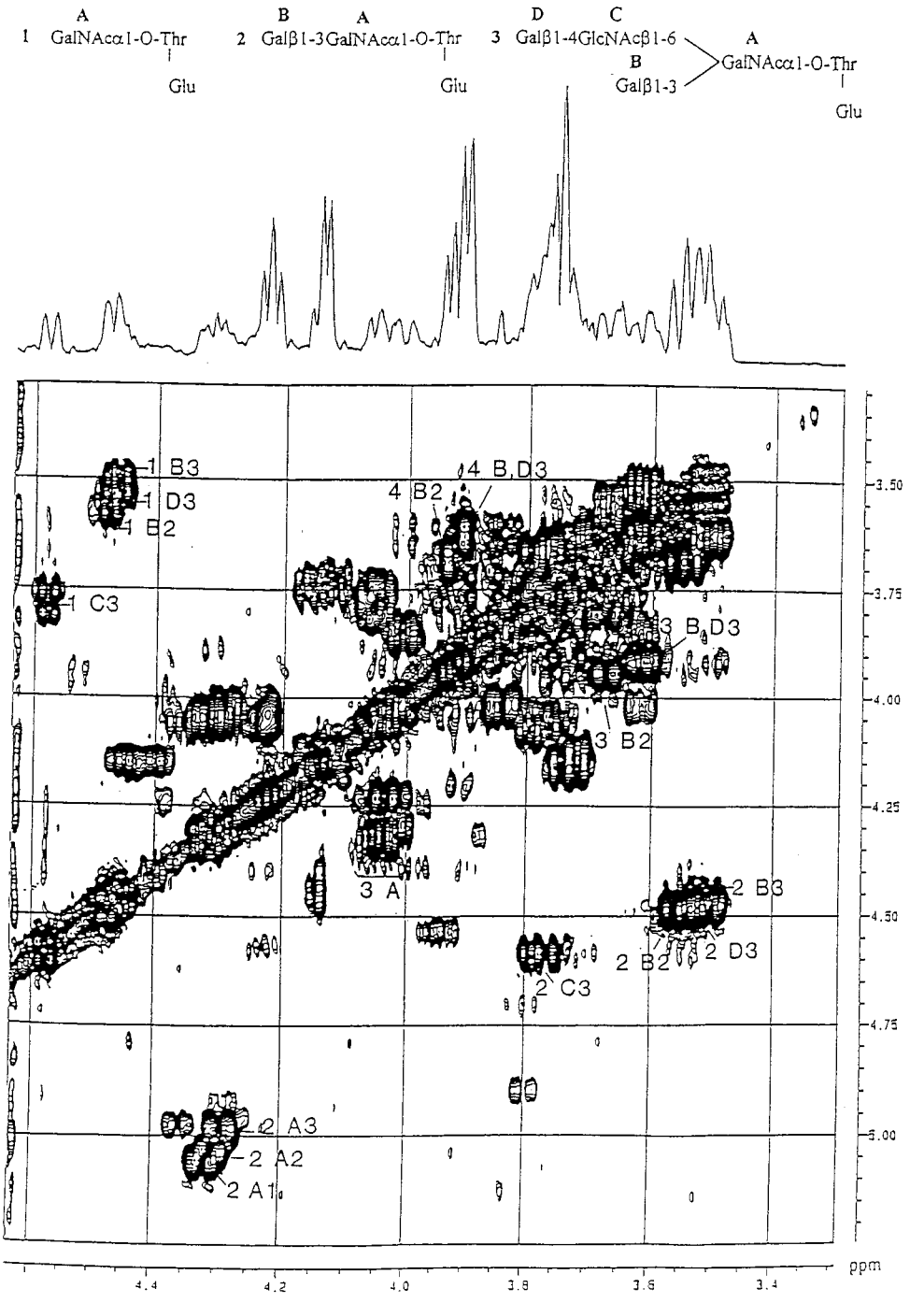
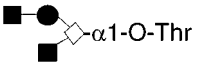


Figure 3. Relevant portion of the COSY spectrum for the low-molecular-mass glycopeptides isolated from component PP3. The first number refers to the proton, the following letter to the sugar residue and then the last number to the structure.

and 4.293 ppm signals were assigned to H-2 and corresponded respectively to the GalNAc itself, to a monosubstituted GalNAc and to disubstituted GalNAc. Due to the presence of the peptide chain, these values differed from those of the corresponding non-reduced [19] and reduced oligosaccharide-alditols [20], which therefore cannot be taken as references. The presence of two types of Gal^3 residues is reflected by the doublet for H-1 at $\delta = 4.474$ and 4.465 ppm and for H-2 at $\delta = 3.589$ and 3.549 ppm. Characteristic Gal^3 H-4 signal at $\delta = 3.910$ ppm is also observed. Chemical shifts observed for GlcNAc H-1 ($\delta = 4.586$ ppm) and GlcNAc H-2 ($\delta = 3.776$ ppm) and those of a $Gal^{4,6}$ residue (H-1,

Table 3. 400 MHz ^{1}H-NMR chemical shifts to structural reporter groups of constituent monosaccharides for the low-molecular-mass glycopeptides isolated from component PP3. Compounds are represented by schematic structures according to Vliegenthart *et al.* [17]: ◇, GalNAc; ●, GlcNAc; ■, Gal.

Reporter	*Residue group*	*Chemical shifts*		
		LM Fraction *1*	*2*	*3*
		◇-α1-O-Thr	■-◇-α1-O-Thr	
H-1	Gal[3]	–	4.474	4.465
	Gal[4,6]	–	–	4.468
	GalNAc	5.062	5.039	4.980
	GlcNAc	–	–	4.586
H-2	Gal[3]	–	3.589	3.549
	Gal[4,6]	–	–	3.570
	GalNAc	4.316	4.308	4.293
	GlcNAc	–	–	3.776
H-4	Gal[3]	–	3.910	3.910
	Gal[4,6]	–	–	3.926
NAc	GalNAc	2.015	2.015	2.021
	GlcNAc	–	–	2.056

$\delta = 4.468$ ppm; H-2, $\delta = 3.570$ ppm; H-4, $\delta = 3.926$ ppm) showed the terminal sequence Gal(β1-4)GlcNAcβ at C-6 position of GalNAcα1-O-Thr. No significant signal could be seen either in the NeuAc axial proton nor in the equatorial one. In the same way, no downfield shift was observed which could correspond to a sulfate substitution. The glycan structures of the three compounds were established by the combined data of mass spectrometry and ^{1}H-NMR (Figure 4). The first one was identified as GalNAcα1-O-Thr, the second one as Gal(β1-3)GalNAcα1-O-Thr and the last one contained the sequence Gal(β1-4)GlcNAc(β1-6)[Gal(β1-3)]GalNAcα1-O-Thr. These different structures have been characterized, in particular, in cow colostrum κ-casein [21] in ovarian cyst mucins [22] and in the human secretory immunoglobulin A hinge region [23] in the alditol form.

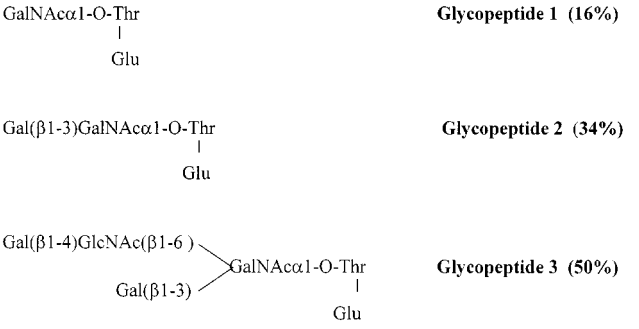


Figure 4. Structures proposed for the low-molecular-mass glycopeptides isolated from component PP3.

Peptide sequence analysis

Determination of the peptide sequence by Edman degradation and amino acid composition of the glycopeptides indicated that peptide moiety was very short and identified as Thr-Glu for the three glycopeptides. This dipeptide sequence corresponded certainly to the O-glycosylation site of component PP3 and PP3 f(54–135) located in the C-terminal part ^{86}Thr-Glu^{87} characterized in lactophorin [3].

Discussion

Three glycans were identified as GalNAcα1-O-Thr, Gal(β1-3)GalNAcα1-O-Thr and Gal(β1-4)GlcNAc(β1-6)[Gal(β1-3)]GalNAcα1-O-Thr by homonuclear bidimensional correlation spectroscopy (COSY 90). Yet, we have characterized the O-glycan structures of the O-glycosylation site corresponding to Thr^{86}. This Thr^{86} residue is fully glycosylated both in the whole component PP3 molecule and in the fragment PP3 f(54–135). This result is in agreement with that obtained by Sørensen and Petersen [3] who have shown that the amino sugars detected by the amino acid analyser at Thr^{86} was mainly galactosamine with a small amount of glucosamine. By using enzymatic digest

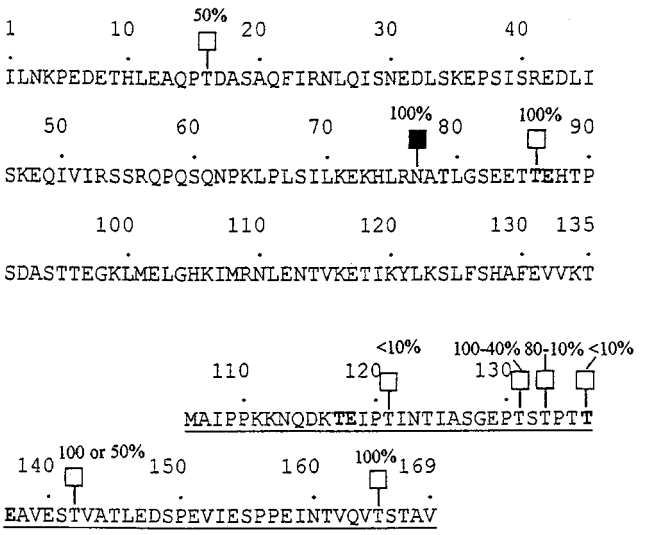


Figure 5. Comparison of the peptide sequence of component PP3 with the peptide sequence of the glycosylated C-terminal domain (106–169) of bovine κ-casein (underlined characters) [24, 25]. The N- (■) and O-glycosylation sites (□) together with their glycosylation rates are indicated, The sequences T-E of PP3 and of κ-casein are shown in bold.

(endoprotease V8 and plasmin), these authors isolated a small peptide containing a residue of Thr at position 16. This peptide was present in a glycosylated and an unglycosylated form in a ratio of 1 : 1. In our preparation, we have never found glycopeptides with the sequence Thr-Asp corresponding to residues 16 and 17 of component PP3. The sequence Thr-Glu is also found in κ-casein and corresponds to fragments 117–118 and 136–137 [24] (Figure 5). However, the first fragment is not glycosylated and the second one is only glycosylated by less than 10% and only in the κ-casein variant A. The variant B had no glycosylation site at position 136 [25]. Moreover, the relative ratios of the glycopeptides found in low-molecular-mass fraction: GalNAcα1-O-Thr and Gal(β1-3)GalNAcα1-O-Thr are minor in κ-casein: 0.8% and 6.3% respectively [24]. The glycopeptide 3: Gal(β1-4)GlcNAc(β1-6)[Gal(β1-3)]GalNAcα1-O-Thr was not encountered in κ-casein isolated from milk but only from colostrum [26].

Component PP3 is synthesized in the lactating mammary gland like the milk fat globule membrane (MFGM) glycoprotein pool and shares common epitopes with butyrophilin (the major MFGM glycoprotein) and MFGM CD 36 [27–29]. In fact, MFGM proteins might be *N*-acetylgalactosaminylated by the same glycosyltransferase as component PP3 and the immunological cross reactivity is probably due to the presence of the N-linked carbohydrate chains containing GalNAc residue [8]. The O-glycans present in component PP3 but absent in butyrophilin [27] do not seem to be involved in the immunological cross reactivity between component PP3 and MFGM glycoproteins.

Recently, the complete primary structures of the 27 kDa lactophorin (accession number D 26176) and bovine glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1) (accession number L 36854) were deduced by conceptual translation of linear single-strand mRNA. Moreover, the cDNA corresponding to a gene coding for the bovine glycoprotein was similar to that coding for the murine GlyCAM-1 glycoprotein and has been identified as the PP3 component [30]. Component PP3 primary structure [3] is identical to the mature 27 kDa lactophorin and to GlyCAM-1 [5]. GlyCAM-1 is one of the mucins produced in the mammary gland, the lymph nodes and the lung [31] and is originally identified as a sulfated glycoprotein which operates as an endothelial cell surface ligand for the leukocyte adhesion molecule L-selectin [4, 32]. However, the binding of L-selectin to GlyCAM-1 requires sialylated, sulfated and probably fucosylated oligosaccharides. In contrast to the lymph nodes and the lung, the epithelial cells for the mammary gland synthesize a non-sulfated GlyCAM-1, which is unable to bind to L-selectin. GlyCAM-1 possesses a heterogeneous array of O-linked carbohydrate chains [33] and recently 6′-sulfated Lewis and sialylated T-antigens have been identified as the major capping structures of this glycoprotein [34–36]. Only one potential site for N-glycosylation is present in the cloned sequence [36]. Although component PP3 cDNA reveals homology with the mouse and rat adhesion molecules GlyCAM-1 cDNA [5], the glycan structures of these compounds are totally different. Component PP3 possesses together *N*-acetylgalactosaminylated N-linked carbohydrate chains and O-glycans without either sialic acid, sulfate or fucose. Thus, component PP3 should not be able to bind the L-selectin.

Acknowledgements

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