The lactose analog GalNAcβ1→4Glc is present in bovine colostrum

Enzymatic basis for its occurrence

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Abstract We have isolated from bovine colostrum the lactose analog GalNAcβ1→4Glc. The enzymatic basis for its occurrence was studied by assaying the activities of GlcNAcβ-R β4-*N*-acetylgalactosaminyltransferase (β4-GalNAcT) and GlcNAcβ-R β4-galactosyltransferase (β4-GalT) in primary milk and several lactating bovine mammary gland fractions. As the β4-GalNAcT, which appears to be tightly membrane bound, is induced by the milk protein α-lactalbumin (α-LA) to act on Glc, it is concluded that β4-GalNAcT is responsible for the synthesis of GalNAcβ1→4Glc in the gland. The comparatively low level (15–20 mg/l) at which this disaccharide is produced may be due to the relatively poor interaction of β4-GalNAcT with α-LA as well as to the fact that α-LA does not inhibit the action of the enzyme on *N*-acetylglucosaminides.

Key words: Bovine mammary gland; Bovine colostrum oligosaccharide; α-Lactalbumin; β4-Galactosyltransferase; β4-*N*-acetylgalactosaminyltransferase

1. Introduction

During lactation in the mammary gland of almost all mammals, β4-galactosyltransferase (β4-GalT) (EC 2.4.1.38) interacts with α-lactalbumin (α-LA) to form the lactose-synthase complex (EC 2.4.1.22) yielding Galβ1→4Glc (lactose) [1–4]. Lactose, concentrated in Golgi and secretory vesicles in epithelial cells of the lactating mammary gland, is osmotically active and pulls water into the vesicles resulting in volume increase [3,5,6]. The content of the swollen vesicles is then secreted in alveoli and ducts as milk which contains the disaccharide as the major carbohydrate source for the newborn [6]. β4-GalT-deficient mice are born healthy but show growth retardation and early death [7,8]. Because the lactose-synthase complex cannot be formed, deficient females produce no lactose in their milk and cannot rear their offspring probably because the milk is too viscous [7,8]. A similar inability is observed in mice in which the gene encoding α-LA has been disrupted [9].

Milk may contain, in addition to lactose, a variety of other free oligosaccharides (ranging from 3 to 14 saccharide units). Most of these oligosaccharides are based on lactose and are produced by the enzymatic attachment of additional residues (fucose, galactose, *N*-acetylneuraminic acid, *N*-acetylglucosamine) to this disaccharide. The structures of these oligosaccharides and their concentrations in milk are determined by the expression levels of the glycosyltransferases involved in their synthesis as well as the availability of the nucleotide-sugar donor substrates. Because these vary widely between different mammals, the oligosaccharide composition of milk is species dependent. Human milk contains a rather complex mixture of oligosaccharides [10,11]. Bovine milk, however, contains very few oligosaccharides other than lactose and only the presence of relatively minor amounts of sialylated [12] and some additional neutral oligosaccharides [13] has been reported.

Recently, we showed that bovine mammary gland contains a β4-*N*-acetylgalactosaminyltransferase (β4-GalNAcT) with properties that strikingly resemble those of the β4-GalT expressed in this tissue [14]. β4-GalNAcT can act on terminal GlcNAc residues on protein *N*-linked glycans, yielding GalNAcβ1→4GlcNAc (*N,N′*-diacetyllactosediamine, lacdiNAc) units. This enzyme therefore appears to function in the synthesis of lacdiNAc based complex type glycans that frequently occur on bovine milk glycoproteins (reviewed in [14,15]). Interestingly, however, β4-GalNAcT also appears to be sensitive to α-LA and is induced by this modifier protein in vitro to act on Glc, yielding the lactose analog GalNAcβ1→4Glc [14]. In this study, we investigated whether this interaction also takes place in vivo in the bovine mammary gland by specifically searching for the occurrence of GalNAcβ1→4Glc in bovine colostrum. We also determined the activities of β4-GalNAcT and β4-GalT in primary milk and several bovine mammary gland fractions in order to obtain enzymatic evidence for the synthesis of GalNAcβ1→4Glc in this tissue.

2. Materials and methods

2.1. Materials

Bovine colostrum was obtained from a local farm. Bovine mammary gland was from a local slaughterhouse. UDP-[^{3}H]GalNAc (8.7 Ci/mmol) and UDP-[^{3}H]Gal (10.6 Ci/mmol) were purchased from New England Nuclear (Boston, MA, USA). The sugar nucleotide donors were diluted with unlabelled UDP-GalNAc and UDP-Gal (Sigma, St. Louis, MO, USA), respectively, to give the desired specific radioactivity. *p*-Nitrophenyl 2-acetamido-2-deoxy-1-thio-β-D-glucopyranoside (GlcNAcβ-S-pNP) was purchased from Sigma (St. Louis, MO, USA). β-Galactosidase from *Escherichia coli* was obtained from Boehringer Mannheim (Mannheim, Germany). All other chemicals were obtained from commercial sources and were of the best quality available.

2.2. Fractionation of oligosaccharides from bovine colostrum

Bovine colostrum (750 ml) was defatted by centrifugation (30 min at 5000×*g*) and filtered through a column of glasswool to remove remaining traces of fat [10]. The colostrum was then dialyzed against 2 l water for 24 h, whereafter the dialysate containing the milk oligosaccharides was lyophilized. Subsequently, the lyophilized material was dissolved in 700 ml 50 mM sodium-cacodylate pH 6.5 and incubated for 48 h at 37°C with 600 U of β-galactosidase in order to digest lactose into monosaccharides. The latter enzyme was used to this end because it acts with a very high rate on lactose [16]. The

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reaction was terminated by inactivation of the enzyme by heating for 10 min at 90°C. The digested material was then passed over a calibrated column (1.6×200 cm) of Bio-Gel P-4 (200–400 mesh) equilibrated and eluted in 50 mM ammonium acetate pH 5.2 at a flow of 10–15 ml/h [17]. Fractions of 3.5 ml were collected and the hexose content was estimated by the phenol-sulfuric acid method [18].

2.3. Isolation and identification of a disaccharide other than lactose

Fractions in which disaccharides are known to elute were pooled and again passed over the column of Bio-Gel P-4. Fractions of 3.5 ml were collected and the hexose content was estimated as described in Section 2.2. In addition, the fractions containing disaccharide material were analyzed by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The HPAEC system used consisted of a Dionex Bio-LC gradient pump, a CarboPac PA-100 column (4×250 mm) and a PAD model-2 detector (Dionex). The pulse potentials used for detection were $E_1 = 0.05$ V (480 ms), $E_2 = 0.60$ V (120 ms) and $E_3 = -0.60$ V (60 ms). The column was run in 0.1 M NaOH for 10 min after which a gradient was applied of sodium acetate by increasing the concentration from 0 to 0.25 M by 2.5 mM per min at a flow rate of 1 ml per min. This system was calibrated with a panel of reference disaccharides amongst which was GalNAcβ1→4Glc obtained previously [19]. The signal of the peak in the chromatogram with the same retention time as this disaccharide was quantified for each fraction analyzed. From the fraction of the Bio-Gel column that gave the highest signal, the disaccharide of interest was isolated by HPAEC as described above except that a CarboPac PA-100 column (9×250 mm) was used which was run isocratically in 0.01 M NaOH. The isolated material was desalted on a column (1.0×42 cm) of Bio-Gel P-2 (200–400 mesh) run in water and the sample was prepared for analysis by 400 MHz ^{1}H-NMR spectroscopy by treating it three times with ^2H_2O (99.75 atom%; Merck, Darmstadt, Germany) at pH 7 and room temperature with intermediate freeze-drying. Finally, the sample was redissolved in 360 μl of ^2H_2O (99.95 atom%; Aldrich, Milwaukee, WI, USA). ^{1}H-NMR spectroscopy was performed as described previously [14].

2.4. Fractionation of bovine mammary gland

Primary milk was isolated from large pieces of freshly obtained bovine mammary gland by collecting the fluid dripping out. Ten g of the gland tissue was cut into small pieces of about 1 g, extensively washed with PBS to remove remaining milk and then homogenized in 50 ml 20 mM sodium-cacodylate pH 7.2 containing 10 mM $MnCl_2$ using a Polytron homogenizer (2×30 s at maximum speed). The homogenate (60 ml) was centrifuged for 10 min at 600×*g* to obtain a debris free homogenate, which was then centrifuged for 60 min at 100 000×*g* to yield a pellet consisting of total cellular membranes and a supernatant which represented the soluble cell fraction. Subsequently, the cellular membrane fraction was extracted by resuspending in 20 mM sodium-cacodylate pH 7.2 containing 10 mM $MnCl_2$ and 2.5% Triton X-100, using a Polytron homogenizer. Following stirring for 90 min, this mixture was centrifuged for 60 min at 100 000×*g*, resulting in an extract and an extracted membrane fraction. All manipulations were carried out at 0–4°C. The protein concentration was determined according to Peterson [20] using bovine serum albumin as a standard.

2.5. Assay of β4-GalNAcT activity

The standard incubation mixture contained 5 μmol sodium-cacodylate pH 7.0, 2 μmol $MnCl_2$, 0.4 μl Triton X-100, 0.2 μmol ATP, 25 nmol UDP-[^{3}H]GalNAc (1.5 Ci/mol), 50 nmol GlcNAc-S-pNP and enzyme in a volume of 50 μl. This system appeared to be optimal in pH and $MnCl_2$ concentration (data not shown), while the donor and acceptor substrate concentrations were about two times the K_m. Control incubations were carried out without acceptor substrate to correct for incorporation into endogenous acceptors. The mixtures were incubated for 60–150 min at 37°C. The reactions were stopped by addition of 950 μl 10 mM acetic acid. Incorporation of [^{3}H]GalNAc into the acceptor was estimated by use of Sep-Pak C-18 reverse-phase cartridges [21]. The mixtures were applied to the Sep-Pak columns, which were washed with 30 ml water. Radioactive products were eluted with 5 ml methanol. The eluate was evaporated to dryness, dissolved in 1 ml water and assayed for radioactivity. One unit of enzyme activity is defined as the amount of enzyme catalyzing the transfer of 1 μmol sugar per min to the acceptor substrate under the assay conditions.

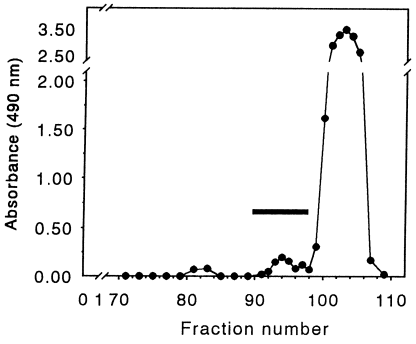

Fig. 1. Fractionation of oligosaccharides isolated from bovine colostrum. Total oligosaccharides, after digestion with β-galactosidase [16], were applied to a column (1.6×200 cm) of Bio-Gel (200–400 mesh), equilibrated and eluted with 50 mM ammonium acetate pH 5.2 at a flow of 10–15 ml/h [17]. The system was calibrated with a number of reference oligosaccharides. Fractions of 3.5 ml were collected and the hexose content (solid circles) was estimated by the phenol-sulfuric acid method [18]. Fractions indicated by the bar, containing the disaccharide material, were pooled for a second run on the Bio-Gel column.

2.6. Assay of β4-GalT activity

The standard incubation mixture contained 5 μmol sodium-cacodylate pH 7.2, 1 μmol $MnCl_2$, 0.4 μl Triton X-100, 0.2 μmol ATP, 25 nmol UDP-[^{3}H]Gal (1.0 Ci/mol), 50 nmol GlcNAc-S-pNP and enzyme in a volume of 50 μl. Controls, incubation conditions and determination of the incorporated radioactivity were done as described for β4-GalNAcT.

3. Results

3.1. Fractionation of colostrum oligosaccharides

The crude oligosaccharide mixture obtained from bovine colostrum was depleted in lactose by digestion with β-galactosidase prior to fractionation. Subsequent filtration of the mixture on a column of Bio-Gel yielded a major peak of monosaccharides (consisting of Gal and Glc, fractions 99–107, Fig. 1) and minor material that eluted as sialylated oligosaccharides (fractions 80–84, Fig. 1) [12] and as disaccharides (fractions 90–98, Fig. 1), respectively. Quantification of the monosaccharides indicated that the colostrum contained approximately 40 g/l lactose, which is a normal value [22]. The pooled disaccharides were subjected to a second run on Bio-

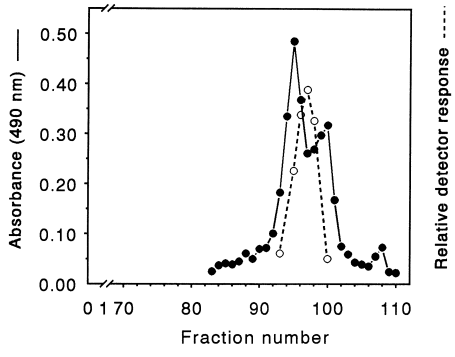

Fig. 2. Fractionation of β-galactosidase resistant disaccharides from bovine colostrum. The disaccharide material obtained as described in Fig. 1 was re-applied to the Bio-Gel column under identical conditions. In addition to the hexose content (solid circles), fractions were monitored for the occurrence of GalNAcβ1→4Glc (open circles) by HPAEC-PAD analysis as described under Section 2.

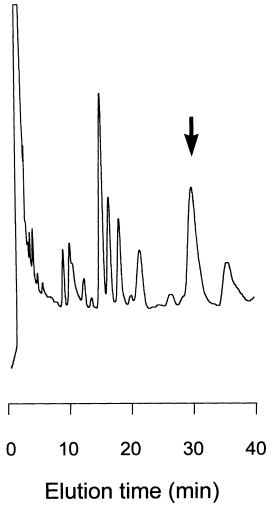

Fig. 3. Isolation of GalNAcβ1→4Glc from bovine colostrum by HPAEC-PAD. Fraction 97 from the Bio-Gel run shown in Fig. 2 was applied to the Dionex HPAEC-PAD system as described in Section 2. Material eluting with the same retention time as authentic GalNAcβ1→4Glc (at the arrow) was pooled and prepared for analysis by 400 MHz ^{1}H-NMR spectroscopy.

Gel. Specific detection in the eluate of material with a HPAEC retention time corresponding to that of GalNAc1→4Glc showed a disaccharide eluting in Bio-Gel fractions 93–100 (Fig. 2). Quantification showed that this material was present in colostrum at a concentration of 15–20 mg/l.

3.2. *Isolation of GalNAcβ1→4Glc and confirmation of its structure*

The oligosaccharide material present in the peak fraction 97 (Fig. 2) was subjected to preparative HPAEC. Of the peaks appearing in the resulting chromatogram (Fig. 3), the material eluting at approximately 29.5 min was pooled and analyzed by 400 MHz ^{1}H-NMR. The partial ^{1}H-NMR spectrum of this disaccharide is shown in Fig. 4. The spectrum and chemical shift values (not shown) are essentially identical to those of authentic GalNAcβ1→4Glc [19]. Hence, the structure of the disaccharide is GalNAcβ1→4Glc.

3.3. *β4-GalNAcT and β4-GalT activities in primary bovine milk and bovine mammary gland fractions*

In primary bovine milk, a high β4-GalT activity was found using GlcNAc-S-pNP as an acceptor substrate, while only a minor β4-GalNAcT activity was detected amounting less than 0.2% of the β4-GalT activity (Table 1). By contrast, in all of the bovine mammary gland fractions, a distinct β4-GalNAcT activity was found in addition to a major β4-GalT activity (Table 1). This is particularly true for the membranous mammary gland fraction, showing that in bovine mammary gland, β4-GalNAcT for the greater part is a membrane bound enzyme, while β4-GalT appears to occur in a membrane bound as well as a soluble form (cf. soluble cell fraction and total cellular membranes, Table 1). Furthermore, it is noteworthy that the β4-GalNAcT activity appears to be largely resistant to extraction with 2.5% Triton X-100, while β4-GalT can almost entirely be solubilized (Table 1).

4. Discussion

In the present study, we describe the isolation and identification of the lactose analog GalNAcβ1→4Glc from bovine colostrum. The presence of this disaccharide has been suggested before [13], but here, we provide the enzymatic basis for its occurrence in addition. We recently reported that bovine mammary gland contains a β4-GalNAcT, with an activity amounting 1–5% of that of the β4-GalT in the gland, that was concluded to function in the synthesis of lacdiNAc-based complex type oligosaccharide chains on bovine milk glycoproteins [14]. Since this β4-GalNAcT can be induced by α-LA to act on Glc [14], it seems that in the lactating mammary gland, in which α-LA is highly expressed, the enzyme supports the synthesis of GalNAcβ1→4Glc analogously to the synthesis of lactose by the β4-GalT/α-LA (lactose-synthase) complex. The

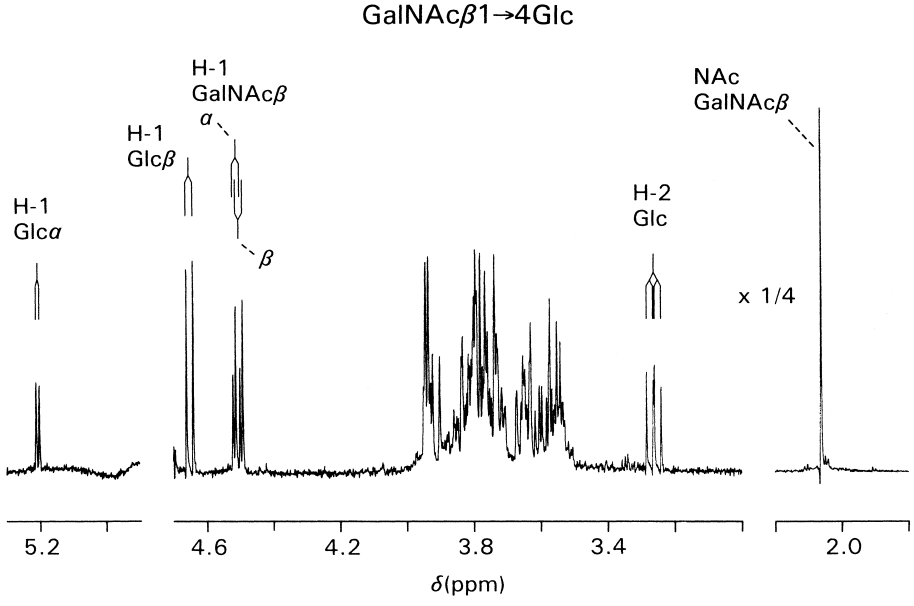

Fig. 4. Diagnostic areas of the 400 MHz ^{1}H-NMR spectrum of GalNAcβ1→4Glc isolated from bovine colostrum featuring the structural reporter groups.

Table 1
β4-GalT and β4-GalNAcT activities[a] in primary bovine milk and several bovine mammary gland fractions

Bovine mammary gland fraction	β4-GalT activity		β4-GalNAcT activity		Ratio of β4-GalNAcT/β4-GalT activity × 100
	mU/mg protein	% Recovery	mU/mg protein	% Recovery	
Primary bovine milk	1.65		0.003		0.19
Debris free homogenate	3.19	100	0.034	100	1.07
Soluble cell fraction	1.94	49	0.006	15	0.32
Total cellular membranes	5.50	35	0.120	72	2.18
Triton X-100 extract	10.10	28	0.035	9	0.34
Extracted membranes	0.28	2	0.109	59	39.00

Standard assays were performed using GlcNAc-S-pNP as an acceptor at a concentration of 1 mM as described under Section 2.
[a]Optimal assay conditions in terms of pH and [$MnCl_2$] were used, while the donor and acceptor concentrations were about two times the K_m.

concentration of GalNAcβ1→4Glc, however, amounts only 0.05% of that of lactose, which was unexpected in view of the distinct activity of β4-GalNAcT in the mammary gland ([14], this study). Apparently, the supply of the required donor nucleotide-sugar (UDP-GalNAc) is not a limiting factor in its synthesis in view of the relative abundance of lacdiNAc-based chains on bovine milk glycoproteins [14,15].

β4-GalT is known to be secreted in milk in a soluble, enzymatically active form resulting from proteolytic processing of the membrane bound enzyme [23] and in accord with this, we found considerable β4-GalT activity in the soluble cell fraction. It is believed that lactose is primarily formed within the lumen of the Golgi vesicles [3], but it is unknown to what extent the complexes with membrane bound β4-GalT and with soluble enzyme each contribute to lactose formation. By contrast, we found that bovine mammary gland β4-GalNAcT is tightly membrane bound, is hardly secreted in the milk and largely withstands extraction with the non-ionic detergent Triton X-100, suggesting that β4-GalNAcT is differently embedded in the membranes of the Golgi vesicles and is not subject to proteolytic release. This might result in a relatively low overall capability to combine with α-LA to a complex resulting in the production of only minor amounts of GalNAcβ1→4Glc. This is indeed consistent with kinetic data showing that the increase in activity with Glc brought about by α-LA is lower for β4-GalNAcT [14] than for β4-GalT [2,24]. In addition, it has to be noted that while α-LA inhibits the action of β4-GalT on GlcNAc and terminally linked GlcNAc on protein-linked glycans [2,19,24], this modifier protein stimulates the action of mammary gland β4-GalNAcT on both GlcNAc and Glc [14]. This means that in the presence of α-LA, both types of substrates will compete for the β4-GalNAcT and that consequently only a fraction of the β4-GalNAcT enzyme molecules will be engaged in the synthesis of GalNAcβ1→4Glc.

Another mechanism by which GalNAcβ1→4Glc was considered to be formed is by the promiscuous usage of UDP-GalNAc by β4-GalT [25], particularly because β4-GalT has been reported to be induced to utilize this nucleotide-sugar donor in the presence of α-LA [19,26]. Attempts to synthesize GalNAcβ1→4Glc by incubating bovine milk β4-GalT with UDP-GalNAc and Glc in the presence of α-LA, however, failed to yield detectable amounts of the product (data not shown). The possibility that GalNAcβ1→4Glc may be synthesized in vivo by the action of β4-GalT can therefore be discounted.

Finally, this study provides additional evidence that bovine mammary gland β4-GalT and β4-GalNAcT activities are due to different enzyme species which can physically be separated by extraction of membranes with non-ionic detergent.

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References

[1] Brodbeck, U., Denton, W.L., Tanahashi, N. and Ebner, K.E. (1967) J. Biol. Chem. 242, 1391–1397.
[2] Brew, K., Vanaman, T.C. and Hill, R.L. (1968) Proc. Natl. Acad. Sci. USA 59, 491–497.
[3] Hill, R.L. and Brew, K. (1975) Adv. Enzymol. Relat. Areas Mol. Biol. 43, 411–490.
[4] Bell, J.E., Beyer, T.A. and Hill, R.L. (1976) J. Biol. Chem. 251, 3003–3013.
[5] Linzell, J.L. and Peaker, M. (1971) Physiol. Rev. 51, 564–597.
[6] Sasaki, M., Eigel, W.N. and Keenan, T.W. (1978) Proc. Natl. Acad. Sci. USA 75, 5020–5024.
[7] Asano, M., Furukawa, K., Kido, M., Matsumoto, S., Umesaki, Y., Kochibe, N. and Iwakura, Y. (1997) EMBO J. 16, 1850–1857.
[8] Lu, Q., Hasty, P. and Shur, B.D. (1997) Dev. Biol. 181, 257–267.
[9] Stinnakre, M.G., Vilotte, J.L., Soulier, S. and Mercier, J.C. (1994) Proc. Natl. Acad. Sci. USA 91, 6544–6548.
[10] Kobata, A. (1972) Methods Enzymol. 28, 262–271.
[11] Kobata, A., Yamashita, K. and Tachibana, Y. (1978) Methods Enzymol. 50, 216–220.
[12] Veh, R.W., Michalski, J.C., Corfield, A.P., Sander Wewer, M., Gies, D. and Schauer, R. (1981) J. Chromatogr. 212, 313–322.
[13] Saito, T., Itoh, T. and Adachi, S. (1984) Biochim. Biophys. Acta 801, 147–150.
[14] Van den Nieuwenhof, I.M., Schiphorst, W.E.C.M., van Die, I. and Van den Eijnden, D.H. (1999) Glycobiology 9, 115–123.
[15] Van den Eijnden, D.H., Bakker, H., Neeleman, A.P., Van den Nieuwenhof, I.M. and van Die, I. (1997) Biochem. Soc. Trans. 25, 887–893.
[16] Huber, R.E., Kurz, G. and Wallenfels, K. (1976) Biochemistry 15, 1994–2001.
[17] Van den Eijnden, D.H., Koenderman, A.H.L. and Schiphorst, W.E.C.M. (1988) J. Biol. Chem. 263, 12461–12471.
[18] Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Anal. Chem. 28, 350–356.
[19] Neeleman, A.P. and Van den Eijnden, D.H. (1996) Proc. Natl. Acad. Sci. USA 93, 10111–10116.
[20] Peterson, G.L. (1977) Anal. Biochem. 83, 346–356.
[21] Palcic, M.M., Heerze, L.D., Pierce, M. and Hindsgaul, O. (1988) Glycoconjug. J. 5, 49–63.
[22] Narendran, R., Hacker, R.R., Batra, T.R. and Burnside, E.B. (1974) J. Dairy Sci. 57, 1334–1340.
[23] Smith, C.A. and Brew, K. (1977) J. Biol. Chem. 252, 7294–7299.
[24] Schanbacher, F.L. and Ebner, K.E. (1970) J. Biol. Chem. 245, 5057–5061.
[25] Palcic, M.M. and Hindsgaul, O. (1991) Glycobiology 1, 205–209.
[26] Do, K.Y., Do, S.I. and Cummings, R.D. (1995) J. Biol. Chem. 270, 18447–18451.