Glycan engineering of proteins with whole living yeast cells expressing rat liver α2,3-sialytransferase in the porous cell wall

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Abstract The *N*-glycans of recombinant proteins produced via the secretory pathway of cultured mammalian cells are often undersialylated, and insect cells lack sialytransferases. Undersialylated glycoproteins are rapidly cleared from the circulation, compromising the effect of pharmaceuticals. We show that incubation with living *Saccharomyces cerevisiae* cells expressing the catalytic ectodomain of rat liver α2,3-sialyltransferase (ST3N$_e$) in the porous cell wall resulted in sialylation of glycoproteins. The K_m values of the yeast enzyme for several substrates were similar to those of recombinant ST3N$_e$ from insect cells and of authentic ST3N. The yeast strain provides an inexpensive self-perpetuating source of ST3N activity for glycan engineering of recombinant proteins.
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Key words: Sialyltransferase; Yeast; Glycan engineering; Recombinant pharmaceutical; Secretion

1. Introduction

Many mammalian pharmaceutical proteins manufactured in cultured cells by gene technology are secretory proteins with sialylated *N*-glycans. However, the recombinant proteins are often undersialylated, even when produced in mammalian cells, or sialylation is not authentic [1,2]. For instance cultured CHO and BHK-21 cells lack a functional α2,6-sialyltransferase, whereas in human and mouse cells glycoproteins are sialylated by both α2,3- and α2,6-linked residues [3,4]. Moreover, the host cell may lack sialyltransferases, like yeast and insect cells, or shed sialidase activity which desialylates the secreted protein product [5]. Lack of sialylation increases dramatically the plasma clearance rate of the proteins, compromising severely the pharmaceutical effect [6–10]. Clearance is accomplished by the hepatic asialoglycoprotein receptor, which recognizes terminal galactose residues of unsialylated *N*-glycans, resulting in elimination of the glycoprotein-receptor complex from the cell surface by endocytosis [11]. We show here that undersialylated and unsialylated native glycoproteins can be sialylated by incubation with living recombinant *Saccharomyces cerevisiae* cells harboring rat liver α2,3-sialyltransferase activity in the porous cell wall.

2. Materials and methods

2.1. Strains and media

S. cerevisiae strains H23 (Matα *his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 hsp150::URA3*) and H626 (Matα *his3-11,15 leu2-3,112 ade2-1 can1-100 hsp150::URA3 TRP1::HSP150Δ-ST3N$_e$*) and *Escherichia coli* DH5α were grown as described [12]. To fuse ST3N$_e$ to the

Hsp150 signal peptide, the DNA encoding amino acids 29–374 of ST3N was isolated from plasmid pKTH4638 (designated pKTH4636 in [12]), and inserted in plasmid pKTH4659 (Bluescript) between the sequences encoding amino acids 1–18 of Hsp150 (signal peptide) and the *ADH1* terminator, creating pKTH4674. The *HSP150SP-ST3N$_e$* fragment was ligated to the pFL35 shuttle vector resulting in plasmid pKTH4675, which was linearized with *Eco*RV at the *TRP1* locus and transformed to strain H23 to create strain H771. The *HSP150Δ-ST3N$_e$* and *ST3N$_e$* genes were driven by the *HSP150* promoter [13].

2.2. Sialyltransferase assays

Duplicate samples of 5×10^7 yeast cells were incubated in 70 μl of 50 mM imidazole buffer, pH 7.0 (glycoproteins), or of 50 mM Tris-maleate buffer, pH 6.7 (oligosaccharides), either with 0.2 nmol (10^5 cpm) of CMP[^{14}C]Neu5Ac (294 mCi/mmol, Amersham International, Buckinghamshire, UK) or with saturating concentrations of CMP-Neu5Ac (Sigma, St. Louis, MO, USA), and varying amounts of acceptor substrates, in a shaker at 24°C. The cells were pelleted and the supernatants subjected to precipitation with 20% TCA for 30 min on ice. For determination of intracellular plus extracellular ST3N activity, the cells were lysed with glass beads [12]. Recombinant ST3N$_e$ (0.34 mU) produced in Sf9 cells (Calbiochem-Novabiochem, La Jolla, CA, USA) was incubated at 37°C in 20 μl of 50 mM MOPS buffer, pH 7.5, containing 1% BSA, and 0.06 nmol of CMP[^{14}C]Neu5Ac (28 500 cpm) or saturating concentrations of CMP-Neu5Ac, and different amounts of acceptors before TCA precipitation. Oligosaccharides were applied on columns of Dowex AG 1 (acetate form, Bio-Rad, Hercules, CA, USA) and Dowex 50 (H$^+$ form, Fluka, Switzerland), which were eluted with 4 ml of water and then with 20 ml of 0.5 M acetic acid [14]. The test proteins were from Sigma (St. Louis, MO, USA), except transferrin (Finnish Red Cross, Helsinki, Finland), and prothrombin (ICN, Aurora, OH, USA). Desialylation was in 0.025 M H$_2$SO$_4$ for 1 h at 80°C [15].

2.3. Mass spectrometry and circular dichroism (CD) spectroscopy

Desialylated proteins were desalted on reversed-phase HPLC. The *N*-glycans were liberated by *N*-glycosidase F digestion and purified using Bond Elut C-18-columns [16]. Buffer salts were removed by drop dialysis against water on VSWP 02500 membranes (Millipore, Bedford, MA, USA) [17]. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was performed on a Biflex instrument (Bruker-Franzen Analytik, Bremen, Germany) equipped with a nitrogen laser operating at 337 nm. Oligosaccharides were analyzed with THAP matrix in the linear positive ion mode, which causes little fragmentation of sialic acids [16]. CD spectroscopy was performed using a Jasco J-720 spectropolarimeter. The spectra were recorded in the far UV region (190–259 nm). Each spectrum was the mean of five scans obtained with a time constant of 1 s and a speed of 20 nm/min.

2.4. Kinetic studies

The K_m determinations were according to Lineweaver-Burk. To determine the activity of Hsp150Δ-ST3N$_e$, H626 cells (5×10^7) were incubated with 0.4 mM lacto-*N*-tetraose and 10 mM CMP-Neu5Ac. The supernatant was subjected to ion exchange chromatography over the Dowex columns and the eluates were concentrated and chromatographed on a Superdex[7]Peptide PC 3.2/30 column (Pharmacia, Sweden) [16]. The oligosaccharides were quantitated against external lacto-*N*-tetraose (Sigma) and NeuNAc (Sigma).

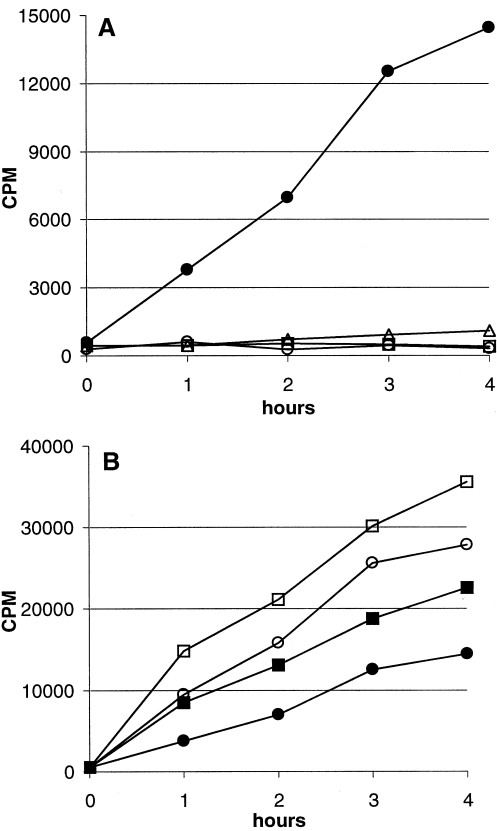*Corresponding author. Fax: (358) (9) 70859570.
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Fig. 1. Transfer of [14C]Neu5Ac to asialofetuin and asialomucin by whole yeast cells. A: H626 cells and CMP-[14C]Neu5Ac were incubated with asialofetuin (closed circles), fetuin (open circles), or asialomucin (triangles), and parental cells (H23) were incubated with asialofetuin (open squares), all in the presence of 10 mM NaN$_3$. B: Asialofetuin was incubated as above with NaN$_3$ (closed circles), or with 40 mM DTT (closed squares), or with 4% glucose (open circles), or with 4% glucose and 40 mM DTT (open squares). TCA-precipitated radioactivity is plotted against incubation time.

3. Results

3.1. Transfer of [14C]Neu5Ac to N-glycans of asialoglycoproteins

Sialyltransferases are type II transmembrane glycoproteins of the Golgi membrane [18]. We have constructed a *S. cerevisiae* strain, which expresses the ectodomain of rat liver α2,3-sialyltransferase (ST3N$_e$) as a fusion protein Hsp150Δ-ST3N$_e$ [12]. The Hsp150Δ polypeptide promotes proper folding of several foreign proteins in the yeast endoplasmic reticulum (ER) [19,20]. Hsp150Δ-ST3N$_e$ was transported to the yeast cell wall to which it was tightly bound. Incubation of the yeast cells with *N*-acetyllactosamine and CMP-Neu5Ac resulted in
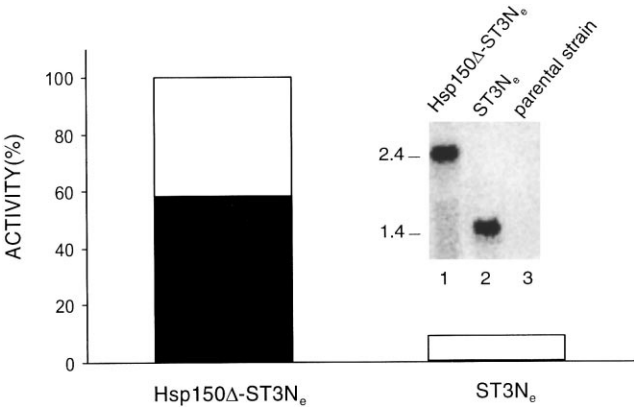

Fig. 2. Relative ST3N activities in yeast cells expressing Hsp150Δ-ST3N$_e$ (H626) and ST3N$_e$ (H771) using asialofetuin and CMP-[14C]Neu5Ac as substrates. Black area: activity in cell wall. White area: intracellular activity. Insert: Northern blot of strains H626 (lane 1), H771 (lane 2) and H23 (lane 3) probed with ST3N$_e$ cDNA. Size markers (kb) are on the left.

α2,3-sialylation of the disaccharide, as shown by NMR spectroscopy [12].

Here we studied whether asialoglycoproteins could be sialylated by the recombinant yeast cells. Bovine plasma fetuin (MW 48 kDa) carries two triantennary and one biantennary *N*-glycan, and three *O*-glycans, all completely sialylated [21,22]. Fetuin was desialylated with mild acid hydrolysis and incubated with CMP-[14C]Neu5Ac and yeast cells (strain H626) expressing Hsp150Δ-ST3N$_e$ in the cell wall. Sodium azide was included to block intracellular protein transport. Samples were removed at different times, the cells pelleted and the supernatants subjected to precipitation with trichloroacetic acid (TCA). Scintillation counting showed that the precipitated radioactivity increased with time (Fig. 1A, closed circles). It was confirmed by mass spectrometry to be fetuin with covalently linked [14C]Neu5Ac residues (see below). Very little radioactivity remained cell-associated, showing that the sialylated protein and CMP-[14C]Neu5Ac did not adhere to the cells. When desialylation was omitted (Fig. 1A, open squares), or when asialofetuin was incubated with yeast cells (H23) lacking the *HSP150Δ-ST3N$_e$* gene (open circles), no radioactivity was bound to fetuin. Hsp150Δ-ST3N$_e$ was specific for *N*-glycans, since no [14C]Neu5Ac was transferred to asialomucin, which carries only *O*-glycans (Fig. 1A, triangles). Thus, asialofetuin could be sialylated by the cell wall-borne Hsp150Δ-ST3N$_e$.

Sialylation of asialofetuin was enhanced when the incubation with H626 cells was carried out in the presence of dithiothreitol (DTT) (Fig. 1B, closed squares). DTT increases the porosity of the cell wall by reducing disulfide bonds [23]. When Hsp150Δ-ST3N$_e$ was allowed to be synthesized and

Table 1
Transfer of [14C]Neu5Ac to prothrombin and transferrin

Time (h)	Protein-bound [14C]Neu5Ac (cpm)			
	prothrombin	asialoprothrombin	transferrin	asialotransferrin
2	292	3856	428	994
4	657	9016	341	1885

The assay was performed as in Fig. 2A.

transported to the cell wall during the assay by omitting sodium azide and adding glucose to the reaction mixture, ^{14}C-sialylation of asialofetuin doubled (Fig. 1B, open circles). When both DTT and glucose were present, ^{14}C-sialylation was further increased (Fig. 2B, open squares). Also desialylated prothrombin (MW 73.6 kDa) and transferrin (MW 79.6 kDa) were ^{14}C-sialylated (Table 1). The differences in the efficiency of ^{14}C-sialylation reflect at least partly differences in the number of acceptor sites [21,22]. Fusion of ST3N$_e$ to the Hsp150Δ fragment was essential for activity and secretion competence. When ST3N$_e$ was linked directly to the Hsp150 signal peptide (strain H771), only little intracellular activity was found (Fig. 2), although the *HSP150Δ-ST3N$_e$* and *ST3N$_e$* genes were expressed at similar levels, as shown by Northern analysis (insert).

3.2. Efficiency of sialylation

Next the degree of protein sialylation was quantitated. Asialofetuin and saturating concentrations of unlabeled CMP-Neu5Ac were incubated with H626 cells. Thereafter the *N*-glycans of fetuin were released by *N*-glycosidase F digestion and analyzed by MALDI-TOF mass spectrometry. In 4 h 31.7% of the terminal galactose residues were sialylated (Fig. 3A,B). When a parallel cell suspension was pelleted after the 4 h incubation, and the supernatant incubated for another 4 h with fresh yeast cells and CMP-Neu5Ac, 55.3% of the galactose residues were sialylated (Fig. 3C). Thus, both asialoglycoproteins and partially sialylated proteins could be sialylated. After 16 h, 61.3% of the galactose residues of fetuin, and 41.5% of those of asialotransferrin were sialylated (not shown). Fetuin, prothrombin and transferrin were subjected to CD spectroscopy before and after desialylation. The spectra of the sialylated and desialylated proteins were superimposable in all three cases (data not shown), suggesting that proteins with native conformations could reach the cell wall-borne ST3N activity.

3.3. Kinetic properties of Hsp150Δ-ST3N$_e$

Finally we compared the kinetic properties of yeast cell wall Hsp150Δ-ST3N$_e$ and recombinant ST3N$_e$ produced in insect cells. The K_m values for asialofetuin, lacto-*N*-tetraose and *N*-acetyllactosamine were similar (Table 2A). The relative ratios V_{max}/K_m demonstrated that both enzyme preparations preferred lacto-*N*-tetraose (type 1: Galβ1-3GlcNAc) over *N*-ace-
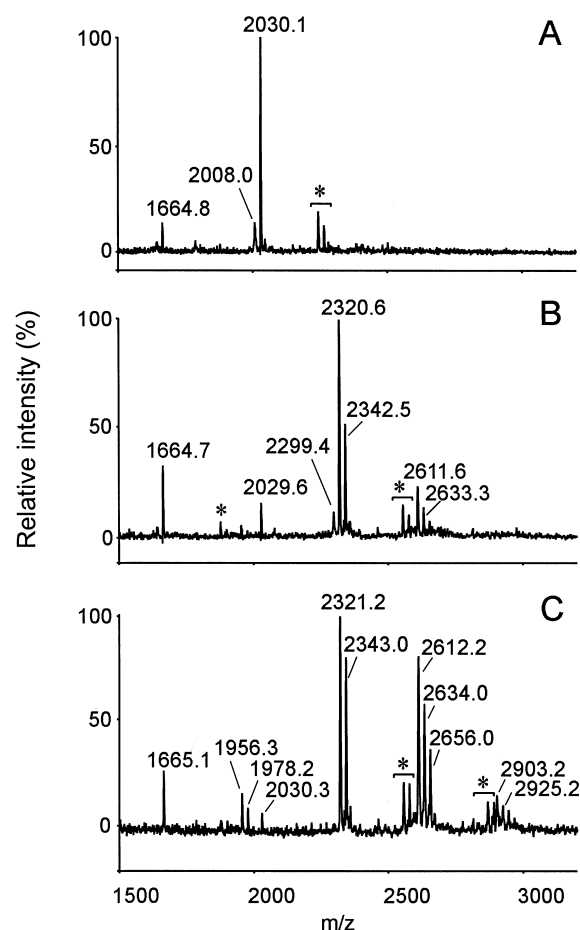

Fig. 3. MALDI-TOF mass spectra of the *N*-glycans. Oligosaccharides liberated from asialofetuin (A), or from asialofetuin incubated for 4 h (B) or 8 h (C) with H626 cells. In A, signals were assigned to non-sialylated triantennary glycans (*m/z* 2008, [M+H]$^+$; *m/z* 2030, [M+Na]$^+$) and non-sialylated biantennary glycans (*m/z* 1665, [M+Na]$^+$). In B and C, monosialylated biantennary (*m/z* 1956, [M+Na]$^+$; *m/z* 1978, [M-H+2Na]$^+$); monosialylated triantennary (*m/z* 2321, [M+Na]$^+$; *m/z* 2343, [M-H+2Na]$^+$), disialylated triantennary (*m/z* 2612, [M+Na]$^+$; *m/z* 2634, [M-H+2Na]$^+$; *m/z* 2656, [M-2H+3Na]$^+$) and trisialylated triantennary glycans (*m/z* 2903, [M+Na]$^+$; *m/z* 2925, [M-H+2Na]$^+$) were detected. The asterisk designates matrix adducts typical for this mode of analysis [16].

Table 2
Kinetic parameters of various ST3N preparations

	K_m (μM)		
	asialofetuin	lacto-*N*-tetraose	*N*-acetyllactosamine
A			
Hsp150Δ-ST3N$_e$	42.7 (5.8)	51.0 (100)	717.7 (5.9)
rST3N$_e$	34.4 (10.9)	43.0 (100)	1014 (11.3)
	CMP-Neu5Ac	Reference	
B			
Hsp150Δ-ST3N$_e$	55	this work	
rST3N$_e$	74.1	[24]	
ST3N	57.3	[25]	

CMP-Neu5Ac (A) or lacto-*N*-tetraose (B) was available in saturating concentrations. In B, varying concentrations of CMP-Neu5Ac up to 10 mM were mixed with a constant amount of CMP-[^{14}C]Neu5Ac. Enzyme preparations: Hsp150-ST3N$_e$ in the wall of yeast cells; recombinant ST3N$_e$ from insect cells (rST3N$_e$); authentic ST3N from rat liver. Figures in parentheses are the relative V_{max}/K_m values for the indicated substrates (highest value is 100).

tyllactosamine and asialofetuin (type 2: Galβ1-4GlcNAc) (Table 2A). The K_m value of Hsp150Δ-ST3N$_e$ for CMP-Neu5Ac was similar to those reported for recombinant ST3N$_e$ from insect cells and authentic ST3N (Table 2B). A 1 l overnight culture containing 18 g (dry weight) of yeast cells contained 117 mU of ST3N activity (lacto-*N*-tetraose as acceptor).

4. Discussion

We show here that unsialylated and undersialylated *N*-glycosylated proteins with apparently native conformations could be sialylated by incubating them with whole living recombinant yeast cells harboring the rat liver α2,3-sialyltransferase ectodomain (ST3N$_e$) fusion protein (Hsp150Δ-ST3N$_e$) in the cell wall. Proper folding of ST3N$_e$ and its intracellular transport from the ER to the cell surface required the yeast-derived polypeptide Hsp150Δ. ST3N$_e$ as such acquired an enzymatically inactive secretion-incompetent conformation, whereas the fusion protein was readily secreted and its kinetic properties were similar to those of isolated rat liver ST3N and recombinant ST3N$_e$ produced in insect cells. The Hsp150Δ polypeptide may have a chaperone-like activity, or attract chaperones which assist folding of heterologous fusion partners in the yeast ER [19,20]. Hsp150Δ-ST3N$_e$ was tightly bound to the cell wall covering the plasma membrane of yeast cells. Binding appears to be due to the ST3N$_e$ portion, because several other Hsp150Δ fusion proteins, the Hsp150Δ fragment alone and full length authentic Hsp150 are secreted to the culture medium [19,20,26]. Our recombinant yeast strain provides an inexpensive and self-perpetuating source of ST3N activity to complement sialylation of recombinant proteins, as well as for sialylation of oligosaccharides. Due to immobilization to the cell wall, the recombinant sialyltransferase needs not to be purified for use, and separation of the product from the transferase occurs simply by pelleting the cells.

The protein glycosylation machinery of insect cells is less complex than that of mammalian cells, and this has been a serious limitation for large-scale production of therapeutic proteins in cultured insect cells [27]. The most frequent types of *N*-glycans on proteins from cultured insect cells are of the high mannose types with 3–9 mannose residues [4]. The most developed *N*-glycan synthesized by *Spodoptera frugiperda* (Sf9) cells appears to be $Man_3GlcNAc_2$ with at least one *N*-acetylglucosamine residue linked to the mannose residues and fucose to the chitobiose [28,29]. Interestingly, a subclone of *Estigmena acrea* cells (Ea4) was able to galactosylate the *N*-glycans of human interferon γ, though incompletely [28]. Thus, Ea4 cells appear to possess most enzymes required for mammalian-type complex *N*-glycosylation, except sialyltransferases. If efficient terminal *N*-acetylglucosaminylation and galactosylation is achieved by clonal selection and/or expressing the appropriate mammalian glycosyltransferases, in vitro sialylation of insect cell-derived recombinant proteins for therapeutic use by our recombinant yeast cells should be a feasible approach.

References

[1] Curling, E.M.A., Hayter, P.M., Baines, A.J., Bull, A.T., Gull, K., Strange, P.G. and Jenkins, N. (1990) Biochem. J. 272, 333–337.
[2] James, D.C., Freedman, R.B., Hoare, M., Ogonah, O.W., Rooney, B.C., Larionov, O.A., Dobrovolsky, V.N., Lagutin, O.V. and Jenkins, N. (1995) BioTechnology 13, 592–596.
[3] Lee, E.U., Roth, J. and Paulson, J.C. (1989) J. Biol. Chem. 264, 13848–13855.
[4] Jenkins, N., Parekh, R.B. and James, D.C. (1996) Nature Biotechnol. 14, 975–981.
[5] Gramer, M.J., Goochee, C.F., Chock, V.Y., Brousseau, D.T. and Sliwkowski, M.B. (1995) BioTechnology 13, 692–698.
[6] Fukuda, M.N., Sasaki, H., Lopez, L. and Fukuda, M. (1989) Blood 73, 84–89.
[7] Goochee, C.F., Gramer, M.J., Andersen, D.C., Bahr, J.B. and Rasmussen, J.R. (1991) BioTechnology 9, 1348–1355.
[8] Maiorella, B.L., Winkelhake, J., Young, H., Moyer, B., Bauer, R., Hora, M., Andya, J., Thomson, J., Patel, T. and Parekh, R. (1993) BioTechnology 11, 387–392.
[9] Sareneva, T., Cantell, K., Pyhälä, L., Pirhonen, J. and Julkunen, I. (1993) J. Interferon Res. 13, 267–269.
[10] Szkudlinski, M.W., Thotakura, N.R., Bucci, I., Joshi, L.R., Tsai, A., East-Palmer, J., Shiloach, J. and Weintraub, B.D. (1993) Endocrinology 133, 1490–1500.
[11] Lodish, H.F. (1991) Trends Biochem. Sci. 16, 374–377.
[12] Mattila, P., Joutsjoki, V., Kaitera, E., Majuri, M.-L., Niittymäki, J., Saris, N., Maaheimo, H., Renkonen, O., Renkonen, R. and Makarow, M. (1996) Glycobiology 6, 851–859.
[13] Russo, P., Kalkkinen, N., Sareneva, H., Paakkola, J. and Makarow, M. (1992) Proc. Natl. Acad. Sci. USA 89, 3671–3675.
[14] Renkonen, O., Niemelä, R., Leppänen, A., Maaheimo, H., Seppo, A., Penttilä, L. and Vikman, A. (1991) Glycoconjugate J. 8, 368–375.
[15] Spiro, R.G. (1960) J. Biol. Chem. 235, 2860–2869.
[16] Nyman, T.A., Kalkkinen, N., Tölö, H. and Helin, J. (1998) Eur. J. Biochem. 253, 485–493.
[17] Börnsen, K.O., Mohr, M.D. and Widmer, H.M. (1995) Rapid Commun. Mass Spectrom. 9, 1031–1034.
[18] Field, M.C. and Wainwright, L.J. (1995) Glycobiology 5, 463–472.
[19] Simonen, M., Jämsä, E. and Makarow, M. (1994) J. Biol. Chem. 269, 13887–13892.
[20] Simonen, M., Vihinen, H., Jämsä, E., Arumäe, U., Kalkkinen, N. and Makarow, M. (1996) Yeast 12, 457–466.
[21] Spiro, R.G. (1973) Adv. Protein Chem. 27, 349–467.
[22] Rice, K.G., Rao, N.B.N. and Lee, Y.C. (1990) Anal. Biochem. 184, 249–258.
[23] de Nobel, J.G., Dijkers, C., Hooijberg, E. and Klis, F.M. (1989) J. Gen. Microbiol. 135, 2077–2084.
[24] Williams, M.A., Kitagawa, H., Datta, A.K., Paulson, J.C. and Jamieson, J.C. (1995) Glycoconjugate J. 12, 755–761.
[25] Gross, H.J., Rose, U., Krause, J.M., Paulson, J.C., Schmid, K., Feeney, R.E. and Brossmer, R. (1989) Biochemistry 28, 7386–7392.
[26] Jämsä, E., Holkeri, H., Vihinen, H., Wikström, M., Simonen, M., Walse, B., Kalkkinen, N., Paakkola, J. and Makarow, M. (1995) Yeast 11, 1381–1391.
[27] Jarvis, D.L. and Finn, E.E. (1996) Nature Biotechnol. 14, 1288–1292.
[28] Ogonah, O.W., Freedman, R.B., Jenkins, N., Patel, K. and Rooney, B.C. (1996) BioTechnology 14, 197–202.
[29] Wagner, R., Geyer, H. and Klenk, H.D. (1996) J. Virol. 70, 4103–4109.