ELSEVIER

Carbohydrate Research 337 (2002) 2181–2186

CARBOHYDRATE RESEARCH

www.elsevier.com/locate/carres

β-(1→4)-Galactosyltransferase activity in native and engineered insect cells measured with time-resolved europium fluorescence

Badarulhisam Abdul-Rahman,[a] Erik Ailor,[b] Donald Jarvis,[c] Michael Betenbaugh,[b] Yuan Chuan Lee[a,*]

[a]*Department of Biology, Johns Hopkins University, Baltimore, MD 21218, USA*
[b]*Department of Chemical Engineering, Johns Hopkins University, Baltimore, MD 21218, USA*
[c]*Department of Molecular Biology, University of Wyoming, Laramie, WY 82071, USA*

Received 19 March 2002; accepted 30 July 2002

Dedicated to Professor Derek Horton on the occasion of his 70th birthday

Abstract

To evaluate the ability of insect cells to produce complex-type *N*-glycans, β-(1→4)-galactosyltransferase (β4GalT) activity in several insect cell lines was analyzed. For this purpose, we developed a simple and highly sensitive assay for β-(1→4)-galactosyltransferase (β4GalT) activity, which is based on time-resolved fluorometry of europium. Bovine serum albumin (BSA) modified with GlcNAc ($GlcNAc_{44}$–BSA) was used as the acceptor. $GlcNAc_{44}$–BSA was coated on a 96-well microplate, and after incubation with the enzyme sample in the presence of UDP-Gal, Eu-labeled RCA_{120} (*Ricinus communis* aggutin I), was added. RCA_{120} binds to the Galβ(1→4)GlcNAc structure in the product, and the bound Eu-RCA_{120} was measured by the fluorescence of europium. When bovine β4Gal-T-I was used as a standard reference enzyme, a linear relationship between enzyme activity and fluorescent signal was obtained over the range of 0–1000 μUnits (IU). Using this system, we were able to measure a low but significant level of β4GalT activity in *Trichoplusia ni* cells ('High Five'). In contrast, no endogenous β4GalT activity was detected in a *Spodoptera frugiperda* (Sf-9) cell line. However, Sf-9 cells stably transfected with the bovine β4GalT-I gene and 'High Five' cells infected with a baculovirus containing the same gene produced activity levels that were comparable to or greater than those found in Chinese hamster ovary cells. We also showed that the β4GalT activity level observed in the baculovirus-infected *T. ni* cells under the control of immediate early promoter was highly dependent on the post-infection time, suggesting that galactosylation level may also be variable during the infection period.

Keywords: Galactosyltransferase; Insect cells; Time-resolved fluorescence; Europium

1. Introduction

There are numerous different *N*-glycan oligosaccharide structures found in nature. Most, if not all, *N*-glycans are synthesized from a common precursor with common initial processing steps, but they are eventually modified by various processing enzymes—glycosyltransferases and glycosidases—to form a vast array of different oligosaccharides. In mammalian systems, highly branched and complex oligosaccharide structures can be synthesized, and the latter are predominant structures expressed on circulating proteins and on cell-surface proteins in humans. Although insect cells are capable of performing *N*-glycosylation,[1,2] their *N*-glycans are generally either high-mannose or low-mannose (truncated) types, and complex or sialylated types are only rarely encountered.[1] In many instances, the low-mannose oligosaccharides are also α-(1→3)- and/or α-(1→6)-fucosylated at the innermost GlcNAc (see Ref. 3 for a review). There are a few reports of galactosylated *N*-glycans in insect cells.[4–6] About 20% of the IgG expressed in *Trichoplusia ni* cells was galactosylated,[6] as determined by a 2D HPLC mapping technique.[7] Interferon γ expressed in an *Estigmena acrea*

* Corresponding author. Tel.: 001-410-516-7041; fax: 001-410-516-5213
E-mail address: yclee@jhu.edu (Y.C. Lee).

0008-6215/02/$ - see front matter
PII: S0008-6215(02)00260-4

cell line was found to have galactosylated oligosaccharides as determined by mass spectrometry of the product.[5] Compositional analysis of glycoproteins from *T. ni* and *Danaus plexippus* eggs showed low but detectable amounts of galactose in native glycoproteins.[8] Recent sugar nucleotide analyses of *T. ni* and Sf-9 cells[9] suggest that these cells have as much UDP-Gal as in Chinese hamster ovary (CHO) cells, which avidly produce galactose-containing oligosaccharides. These results support a notion that both acceptor substrate (GlcNAc-terminal) and the donor for β4GalT (UDP-Gal), are available in sufficient quantities for galactosylation in insect cells, and yet the galactosylated glycoproteins are not as abundantly formed as in animal cells. Therefore, it became imperative to learn about the β4GalT activity in the insect cells. There has been no direct evidence for β4GalT activity in other insect cell lines except for a trace activity demonstrated in Ea4 cells.[10]

A number of methods are available for measurement of glycosyltransferase activities. Most commonly, sugar nucleotides radiolabeled in the sugar residue are used as donor, and the amount of radiolabeled sugar transferred to the acceptor is determined. Although this method is quite sensitive, it has considerable drawbacks because storage, usage, and disposal of radioisotopes must be carefully controlled. Some of these methods require an ion-exchange resin to remove the sugar nucleotide from the product.[11] Although there exists a highly sensitive (subfemtomol range) capillary electrophoresis method based on separation of fluorescein-conjugated glycosides by capillary electrophoresis and laser-induced detection,[12] it is not suitable for simultaneous determination of multiple samples. In another approach, lectins, capable of recognizing specific sugar epitopes, are utilized to measure various glycosyltransferase activities. For instance, when a biotin-labeled lectin specific for the product is added to the reaction mixture, the amount of the product formed can be determined by the amount of lectin bound to the product. The biotin on the lectin allows it to be captured by streptavidin or avidin conjugated with horseradish peroxidase or alkaline phosphatase to facilitate quantification.[13] Lectins modified with a fluorescent probe also had been used for a similar determination.[14]

A newer alternative to these techniques is to use lanthanide elements as fluorescent probes. The long Stoke's shift (over 200 nm) and exceptionally long fluorescence decay time (0.1–1 ms) are the major advantages of lanthanide fluorescence.[15] The latter allows time-resolved fluorescence (TR-F) measurement in a plastic microplate, since the lanthanide fluorescence can be measured after the fluorescence of plastic material of microplate has dissipated. By a special technique called 'dissociation enhanced lanthanide fluorescence immunoassay' (DELFIA)[16] which dissociates Eu from the chelating group in the probe and places it in a special micellar environment for maximum fluorescence intensity, sensitivity equal to or better than radioisotopic assays can be attained.[17,18] Eu is the most highly fluorescent among the lanthanide elements and is most frequently used in various biomedical assays.[18,19] Unlike some radioisotopes, Eu-labeled reagents are stable and sustain a long shelf life.

In this report, we present a newly developed and highly sensitive nonradioisotopic assay for β4GalT activity. The method uses microplate-coated GlcNAc-BSA as the acceptor and Eu-labeled RCA_{120} lectin as a probe for the newly formed Galβ(1→4)GlcNAc epitope. Activities of β4GalT in various insect cell lines were evaluated easily by this method.

2. Materials and methods

Materials.—Bovine serum albumin (BSA) was from Pentex (Bayer, Kankkee, IL). $GlcNAc_{44}$–BSA[20] was produced in the Y.C.L. laboratory. RCA_{120}, UDP-GlcNAc, UDP-Gal and bovine β4GalT-I were obtained from Sigma Chemical Co. (St. Louis, MO). Diethylenetriaminepentaacetic acid (DTPA) dianhydride was from Aldrich Chemical Co. (Milwaukee, WI). β-Galactosidase inhibitor (a D-galactose-type 1-iminosugar), a very potent β-galactosidase inhibitor (showing IC_{50} for *Aspergillus niger* β-galactosidase as 12 nM)[21] was a gift from Dr. Y. Ichikawa, Johns Hopkins University School of Medicine. FluoroNunc™ Module Maxisorp™ Surface 96 well Plates were from Nalge Nunc International (via Fisher Scientific, Pittsburgh, PA). The enhancing solution for maximizing Eu fluorescence was prepared as described.[16]

Cell culture and microsome preparation.—Chinese hamster ovary (CHO) cells (ATCC) were cultured at 37 °C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, 100 μM minimal essential amino acids and 4 mM L-glutamine. Cells were grown to confluency in T-75 flasks, washed twice with phosphate-buffered saline (PBS), and lysed in 50 mM Tris–HCl, pH 7.2, containing 2 mM EDTA, 1 mM dithiothreitol and 1% Triton X-100.

T. ni cells ('High Five', BTI-TN5B1-4) were obtained from Invitrogen (Carlsbad, CA). Sf-9 cells were obtained from ATCC (Manassas, VA). Sf-9 cells stably transformed with bovine β4GalT-I (SF-9β4GalT) were produced in the laboratory of D. J. (University of Wyoming, Laramie, WY). All cell lines were grown and maintained in a HyClone insect cell serum-free media (HyQR SFX-Insect) in T-75 flask or 20–30 mL culture in a 250-mL shaker flask, and the cells subcultured

every 3–4 days. The culture medium was supplemented with 5 mM glutamine and 100 μg/mL penicillin–streptomycin. For a typical enzyme analysis, cells at 3×10^6 cells/mL were harvested by centrifugation for 10 min at $3000 \times g$ (Sorvall RC6000 Benchtop centrifuge) and rinsed twice with 10 mL of PBS. Cells were suspended in a lysis buffer (50 mM Tris-HCl, pH 7.5, containing 1% Triton X-100, 10 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, PMSF) on ice for 45 min. Intact cells were removed by centrifugation for 5 min under the conditions described above.

For microsome preparation, the cell pellet was suspended in 5 mM imidazole-HCl buffer, pH 7.3, containing 0.25 M sucrose and 1 mM PMSF. The cells were hand-homogenized with a Potter–Elvehjem homogenizer for 5–10 min, monitoring the progress with a microscope. Intact cells and large particles were removed by centrifugation as above. The pellet was discarded and the supernatant was subjected to ultracentrifugation at $100{,}000 \times g$ for 1 h (Beckmann Ultracentrifugation, L7-65). The pellet was defined as the microsome fraction and was used for enzyme assays. Protein concentrations were determined by micro-BCA (bicinchoninic acid) assay following the manufacturer's instruction (Pierce, Rockford, IL). Bovine serum albumin was used as standard, and its absorbance measured with a Shimadzu UV-160U spectrophotometer at 562 nm.

Baculovirus and protein expression.—Two recombinant baculoviruses carrying the bovine β4GalT-I cDNA under the control of polyhedrin promoter[22] or under immediate early promoter (ie1)[23] have been described. Recombinant baculovirus carrying human transferrin cDNA was prepared and developed as described.[24] All recombinant baculoviruses were propagated in Sf-9 cells, and 0.45 μm of the filtered culture medium was used as a viral stock.

For protein expression, all viruses were seeded into a 50-mL culture of 'High Five' cells at a density of 1×10^6 cells/mL and a multiplicity of infection (MOI) of 5 at 27 °C on a rotary shaker. Cells were harvested by centrifugation at $3000 \times g$ (Sorvall SS32 rotor) at 72 h post infection and homogenized as described above for the enzyme assays.

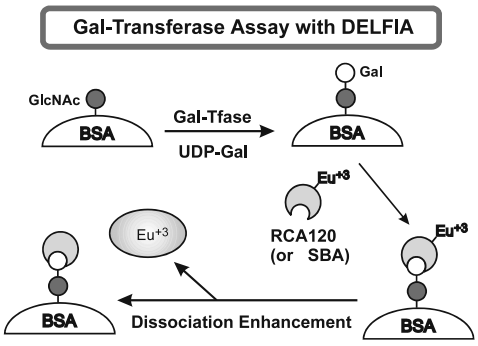

Fig. 1. Diagram for the galactosyltransferase assay using DELFIA technology.

Preparation of europium-labeled RCA_{120}.—All measurements of Eu fluorescence were conducted with a multilabel counter (Victor, Model 1420 by Wallac, Gaithersburg, MD). Ten mg of lyophilized RCA_{120} was dissolved in 200 μL of 10% (w/v) sodium bicarbonate solution, and 3.3 μmol of solid DTPA-dianhydride (equivalent to 40 mol DTPA-dianhydride per mol of RCA_{120}) was added. The reactants were gently mixed on a rotary shaker for 2 h. The solution was carefully adjusted to pH 4.0 with dilute acetic acid to decompose any excess bicarbonate, and after a few minutes, adjusted to pH 7.0 with NaOH. After 30 min, 3.3 μmol $EuCl_3$ (40 mol per mol protein) in 0.1 M acetic acid was added, and the mixture was gently stirred for 1 h. The mixture was separated by gel-permeation chromatography using a Sephadex G-10 column (1 × 90 cm) equilibrated in 10 mM ammonium bicarbonate, collecting 0.5-mL fractions. Fractions containing Eu-labeled RCA_{120} were pooled and lyophilized. About 2 mol Eu was incorporated per mol of RCA_{120} as determined by time-resolved fluorometry for total Eu and BCA assay for total protein using BSA as a standard.

Assay of β-(1→4)-galactosyltransferase activity.—$GlcNAc_{44}$–BSA, 100 nM in PBS, was placed into wells of a 96-well microplate and kept at 4 °C overnight. The excess unbound $GlcNAc_{44}$–BSA was removed by washing (3 times) with PBST (phosphate-buffered saline with Triton). The plate was blocked with BSA 1% (w/v) in PBST for 2 h at room temperature, and again washed three times with PBST to remove unbound BSA. The reaction mixture (100 μL per well) contained 125 μM UDP-Gal, 40 mM $MnCl_2$, 50 μM galactosidase inhibitor (see below), 1% Triton X-100, and up to 100 μg total protein from cell lysate or microsomes in 50 mM Na-cacodylate buffer, pH 6.8. β4GalT-1 from bovine milk was used as the standard. The transferase reaction was allowed to proceed at 37 °C for 1 h, and the reaction was stopped by washing three times with PBST. Fifty μL of 100 nM Eu^{3+}-RCA was then added to each well, and the mixture was kept at 4 °C for 1 h. The unbound Eu^{3+}-RCA was removed by washing (four times) with PBST, and the enhancement solution (200 μL per well) was added. After gentle shaking on a rotary shaker for 30 min at room temperature, the fluorescence was measured. All reaction mixtures contained 50 μM 'galactose-type iminosugar' to circumvent potential action of β-galactosidase.

3. Results

β-(1→4)-Galactosyltransferase assay.—The principle of the assay is diagramed in Fig. 1. After the

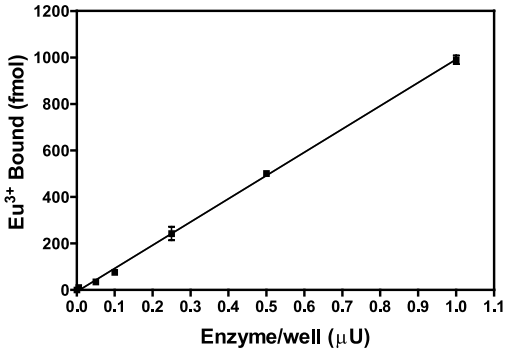

Fig. 2. Linearity of bovine β-(1→4)-galactosyltransferase activity assayed under the conditions proposed.

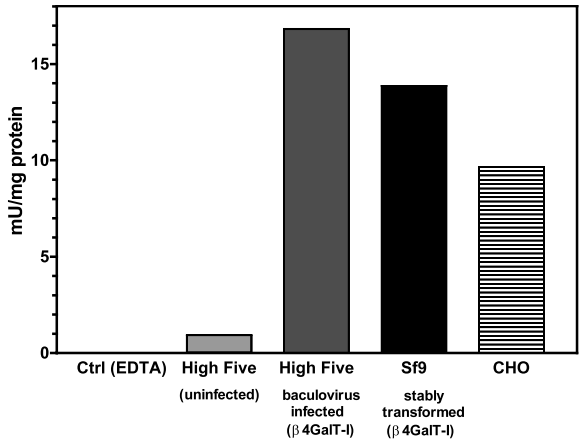

Fig. 3. Galactosyltransferase activities in normal, transiently transfected, or stable transformed insect cell lines and CHO cells.

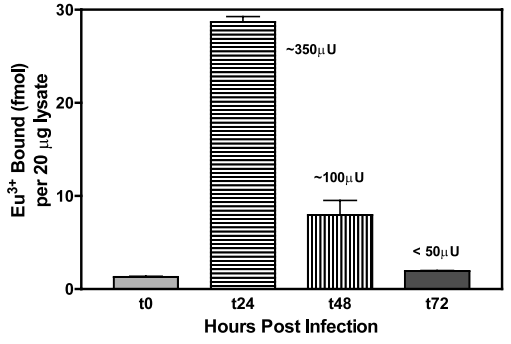

Fig. 4. Time course of the galactosyltransferase activity in 'High Five' cells post-infection.

enzyme has transferred Gal from UDP-Gal to the GlcNAc on BSA, Eu-labeled RCA_{120} will bind to the newly formed Galβ(1→4)GlcNAc structure. In the next step, Eu is dissociated from the lectin by the action of the enhancement solution and allowed to form a highly fluorescent complex, which was measured in a time-resolved manner. When bovine milk β4GalT-I was used as the enzyme standard, a linear relationship between the amount of Eu^{3+} and the amount of enzyme was obtained over the range of 0–1.0 μU (IU) (Fig. 2), which is comparable to 0–0.9 μU of a radioactivity method[11] or nonlinear range of 100–1000 pmol/h by an ELISA method.[25]

β4Gal-T level in insect cells.—Two widely used insect cell lines, Sf-9 (from *Spodoptera frugiperda*) and 'High Five' (from *T. ni*), were grown in serum-free culture medium and then lysed in order to evaluate their endogenous galactosylation potential in lysates or microsomal fractions. No β4GalT activity could be detected above the detection limit (0.01 μU) in Sf-9 cells in either cell lysate and its membrane fractions even after 24-h incubation. The low level of β4GalT may explain the previous report on the absence of galactose-terminated *N*-glycans in IFN γ expressed by Sf-9.[5] In contrast, a low level of β4GalT activity (ca. 1 mU/mg protein) was detected in 'High Five' cell lysates and the microsomal fractions. This result can be used to rationalize the low level of galactosylation in the 'High Five'-generated glycoproteins.[6,24] The amount of the galactosylation product was proportional to the incubation time and amount of the protein used in the incubation. Inclusion of EDTA in the mixture or boiling the enzyme sample (negative controls) reduced this activity to the background levels.The β4GalT activities in the lysates of genetically engineered insect cells (50 μg protein each) was also analyzed and compared to those in the widely used mammalian CHO cell line. As shown in Fig. 3, Sf-9 cells stably transformed with bovine β4GalT-1 contained ca.14 mU β4GalT/mg protein compared with 10 mU/mg in CHO cells. 'High Five' cells infected with the bovine β4GalT-1 gene via baculovirus under the control of the polyhedrin (pol) promoter[24] showed even higher β4GalT-1 levels (ca. 17 mU/mg protein). Since the level of the recombinant β4GalT-I in either stably transformed or baculovirus-vector infected insect cells exceeds that of CHO cells, this activity should be sufficient for galactosylation, if the enzyme is correctly localized and the proper substrates are available.

We further tested the effectiveness of the assay system in monitoring β4GalT-I expression level over time in 'High Five' cells infected with a baculovirus carrying the bovine β4GalT-I cDNA, under control of the immediate-early (ie1) baculovirus promoter. As shown in Fig. 4, the β4GalT-I activity peaked after 24 h of infection and quickly diminished after 48 h to almost the background level at 72 h post-infection. This expression profile is characteristic of the immediate-early promoter, which is most active immediately following the baculovirus infection. Consequently, the galactosylation activity can vary widely in infected insect cells and depends significantly on the particular promoter used in the baculovirus construct. Understanding the activity level with infection time will be useful in determining the optimal product harvesting time from the standpoint of galactosylation.

4. Discussion

The level of β4GalT activity in all systems described above has been evaluated using the newly developed assay based on Eu fluorescence. Samples of crude cell lysate containing 1% Triton X-100 were analyzed successfully in all assays. The proposed method of measuring the β4GalT activity represents a viable alternative to the assays that rely on radioisotopes. Moreover, the use of 96-well microplates allows a large number of samples to be processed simultaneously. Direct attachment of Eu to the lectin significantly shortens the processing time, compared with the some ELISA type assays where several steps of binding/washing are involved before the actual activity is measured. The method is highly accurate. For example, the correlation coefficient for the standard curve shown in Fig. 2 is 0.9976 (SD = 1%).

The results of our β-galactosyltransferase analyses (Fig. 3) indicate that β4GalT may be one of the limiting factors in generating complex type *N*-glycans in insect cells. Endogenous β4GalT activity was not detected in SF-9, while a low, but measurable level (1.0 ± 0.1 mU/mg protein) could be detected in 'High Five'. Even in these cells, the activity is much lower than the level found in CHO cells (5–10 mU/mg), and may be insufficient for proper galactosylation, especially during the period of high expression of recombinant proteins that occurs in the late phase of the baculovirus infection. It was reported that endogenous β-(1→4)-GalNAc-transferase activity in cell lines derived from *T. ni*, *S. frugiperda*, and *Mamestra brassicae* dropped to almost 1/100 of the original after baculovirus infection.[26] A similar reduction may occur for other transferases, and thus further reduce the cell's capacity for galactosylation.

Cell engineering to extend the glycosylation capabilities of insect cells and other expression systems has been applied widely in recent years. In the baculovirus system, *N*-glycan processing can be modified by the expression of necessary enzymes during the infection period. Since the levels of β4GalT activity are apparently insufficient in all insect cell lines, supplementing β4GalT activity would be expected to yield glycosylation patterns closer to human glycoproteins (i.e., 'humanization' of glycoproteins).

There are two strategies to enhance galactosylation during processing in insect cell lines. One is to use a stably transformed insect cell line expressing mammalian β4GalT, and transfect the cells with a baculovirus carrying the glycoprotein gene of interest. An alternative is to co-infect the cells with two baculoviruses, one carrying the gene of interest and another carrying bovine β4GalT, under the control of either the immediate early promoter (ie1)[23] or the very late polyhedrin promoter (pol).[22] As shown in Figs. 3 and 4, the activity levels of the β4GalT in the transfected 'High Five' and transformed Sf-9 cells, as compared with the CHO cells, may be sufficient for the effective galactosylation of the recombinant glycoprotein, provided the enzyme is properly localized and sufficient acceptor substrate is available. Future studies in our laboratory will examine the extent of galactosylation that can be achieved in these engineered cell lines

In conclusion, a simple and sensitive assay based on time-resolved fluorescence was developed to measure and monitor the recombinant bovine β4GalT-I expression in insect cells. With a working range comparable to radioisotopic methods, the microplate format presented here will greatly facilitate throughput of galactosyl transferase assays.

Acknowledgements

We thank the Government of Malaysia for the fellowship to support BAR, and the support of NSFResearch Grants BES9814100 and BES9905171. We are also grateful for Dr. Y. Ichikawa for providing an inhibitor for galactosidase, and Dr. Reiko Lee for reading the manuscript.

References

1. März, L.; Altmann, F.; Staudacher, E.; Kubelka, V. Glycoproteins. In *Protein Glycosylation in Insects*; Montreuil, J.; Schachter, H.; Vliegenthart, J. F. G., Eds.; Elsevier Science: Amsterdam, 1995; Vol. 3, pp 543–563.
2. Altmann, F. *Trends Glycosci. Glycotechnol.* **1996**, *8*, 101–114.
3. Altmann, F.; Staudacher, E.; Wilson, I. B.; Marz, L. *Glycoconjugate J.* **1999**, *16*, 109–123.
4. (a) Davidson, D. J.; Fraser, M. J.; Castellino, F. J. *Biochemistry* **1990**, *29*, 5584–5590;
   (b) Davidson, D. J.; Castellino, F. J. *Biochemistry* **1991**, *30*, 6689–6696;
   (c) Davidson, D. J.; Castellino, F. J. *Biochemistry* **1991**, *30*, 6165–6174.
5. Ogonah, O. W.; Freedman, R. B.; Jenkins, N.; Patel, K.; Rooney, B. C. *Bio/technology* **1996**, *14*, 197–202.
6. Hsu, T. A.; Takahashi, N.; Tsukamoto, Y.; Kato, K.; Shimada, I.; Masuda, K.; Whiteley, E. M.; Fan, J. Q.; Lee, Y. C.; Betenbaugh, M. J. *J. Biol. Chem.* **1997**, *272*, 9062–9070.
7. Tomiya, N.; Awaya, J.; Kurono, M.; Endo, S.; Arata, Y.; Takahashi, N. *Anal. Biochem.* **1988**, *171*, 73–90.
8. Park, Y. I.; Wood, H. A.; Lee, Y. C. *Glycoconjugate J.* **1999**, *16*, 629–638.
9. Tomiya, N.; Ailor, E.; Lawrence, S. M.; Betenbaugh, M. J.; Lee, Y. C. *Anal. Biochem.* **2001**, *293*, 129–137.
10. van Die, I.; van Tetering, A.; Bakker, H.; van den Eijnden, D. H.; Joziasse, D. H. *Glycobiology* **1996**, *6*, 157–164.
11. Holpert, M.; Cooper, T. G. *Anal. Biochem.* **1990**, *188*, 168–175.

12. Snow, D. M.; Shaper, J. H.; Shaper, N. L.; Hart, G. W. *Anal. Biochem.* **1999**, *271*, 36–42.
13. Oubihi, M.; Kitajima, K.; Kobayashi, K.; Adachi, T.; Aoki, N.; Matsuda, T. *Anal. Biochem.* **1998**, *257*, 169–175.
14. Stults, N. L.; Stocks, N. F.; Rivera, H.; Gray, J.; McCann, R. O.; O'Kane, D.; Cummings, R. D.; Cormier, M. J.; Smith, D. F. *Biochemistry* **1992**, *31*, 1433–1442.
15. Sinha, A. P. B. *Spectrosc. Inorg. Chem.* **1971**, *2*, 255–288.
16. Hemmila, I.; Dakubu, S.; Mukkala, V. M.; Siitari, H.; Lovgren, T. *Anal. Biochem.* **1984**, *137*, 335–343.
17. Yuan, J.; Wang, G.; Kimura, H.; Matsumoto, K. *Anal. Biochem.* **1997**, *254*, 283–287.
18. (a) Soini, E.; Lövgren, T. *CRC Crit. Rev. Anal. Chem.* **1987**, *18*, 105–153;
(b) Kropf, J.; Quitte, E.; Gressner, A. M. *Anal. Biochem.* **1991**, *197*, 258–265.
19. Yuan, J.; Matsumoto, K.; Kimura, H. *Anal. Chem.* **1998**, *70*, 596–601.
20. Stowell, C. P.; Lee, Y. C. *Methods Enzymol.* **1982**, *83*, 278–288.
21. Ichikawa, Y.; Igarashi, Y. *Tetrahedron Lett.* **1995**, *36*, 4585–4586.
22. Hollister, J. R.; Shaper, J. H.; Jarvis, D. L. *Glycobiology* **1998**, *8*, 473–480.
23. Jarvis, D. L.; Finn, E. E. *Nature Biotechnol.* **1996**, *14*, 1288–1292.
24. Ailor, E.; Takahashi, N.; Tsukamoto, Y.; Masuda, K.; Rahman, B. A.; Jarvis, D. L.; Lee, Y. C.; Betenbaugh, M. J. *Glycobiology* **2000**, *10*, 837–847.
25. Keusch, J.; Lydyard, P. M.; Isenberg, D. A.; Delves, P. J. *Glycobiology* **1995**, *5*, 365–700.
26. van Die, I.; van Tetering, A.; Bakker, H.; van den Eijnden, D. H.; Joziasse, D. H. *Glycobiology* **1996**, *6*, 157–164.