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Study of the enzymatic transformation of fluorescently labeled oligosaccharides in human epidermoid cells using capillary electrophoresis with laser-induced fluorescence detection

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Abstract

Isomeric oligosaccharides of both βGal(1→3)βGlcNAc (type I) series and βGal(1→4)βGlcNAc (type II) series were studied by using capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection. A mixture of phenylboronic acid and sodium tetraborate was used in the CE running buffers to improve the electrophoretic separation of the oligosaccharides. Both series of the tetramethylrhodamine (TMR)-labeled substrates [βGal(1→3)βGlcNAc-O-TMR and βGal(1→4)βGlcNAc-O-TMR) and their potential enzymatic products were baseline resolved using CE. The high resolution provided by CE and the excellent detection limit ($8 \cdot 10^{-23}$ mol, or 50 molecules) by LIF allowed for the determination of minor enzyme products in the presence of excess unreacted substrate. The action of competing enzymes acting on the common type I sequence was monitored after the incubation of human epidermoid carcinoma cells (A431) with a fluorescent substrate (βGal(1→3)βGlcNAc-O-TMR). The CE–LIF analyses showed the formation of both synthetic and hydrolytic products, suggesting the actions of glycosyltransferases and glycosidases in the cells.

Keywords: Oligosaccharides; Tetramethylrhodamine; Enzymes

1. Introduction

Glycosyltransferases are important synthetic enzymes, which are responsible for transferring a single monosaccharide unit from nucleotide donor sugar molecules to other carbohydrates or aglycons (peptides or lipids) [1–4]. With few exceptions, a single enzyme makes one specific glycosidic linkage; this has been referred to as "the central dogma of glycobiology" [1,2]. Glycosyltransferase assays using defined acceptor substrates are essential for understanding both oligosaccharide biosynthesis and their biological roles.

Glycosyltransferases are often present at very low levels in mammalian systems. Several conventional assays, including enzyme-linked immunosorbent assay (ELISA), gel-permeation, ion-exchange, affinity chromatography and radiochemical methods, have been reported for these enzymes [5–10]. These methods require large amounts of sample and/or tedious sample manipulations. The objective of this study is to develop an ultra-sensitive assay using capillary electrophoresis (CE) separation with laser-induced fluorescence (LIF) detection, taking advantages of the excellent detection limit obtainable by LIF and the small sample volume (under 1 nl) required by CE.

The substrates and potential enzyme reaction

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products of glycosyltransferases and glycosidases are oligosaccharides and monosaccharides, which do not have chromophores or fluorophores for sensitive spectrometric detection. Several molecular absorption and fluorescence detection methods for carbohydrates have been reviewed [11–13]. These generally involve the derivatization of the oligosaccharides at their reducing end with a chromophore or fluorophore prior to spectrometric detection. Among these methods, fluorescent labeling followed by LIF detection provides the best detection limits [14–21]. We have achieved detection limits of 50 molecules of tetramethylrhodamine (TMR)-labeled oligosaccharides [21]. The oligosaccharides were aminated and then reacted with the N-hydroxysuccinimide ester of tetramethylrhodamine [18–20]. For this study, a disaccharide, (βGal(1→3)βGlcNAc, Type I) and the potential enzyme reaction products from glycosyltransferases acting on βGal(1→3)βGlcNAc have been labeled with TMR.

A previous study from our group [18] has demonstrated a successful assay for fucosyltransferase, which was isolated from human milk. The purified enzyme acted on the substrate, producing only one reaction product, which was readily separated by CE from the unreacted substrate. In the present study, we wish to investigate enzyme activities present in whole mammalian cells. The cells, however, may contain several competing enzymes and, consequently, several enzyme reaction products are expected. Therefore, it is essential to separate these potential products in order to identify the enzymes responsible for their formation. Oligosaccharides in general do not have readily ionizable functions. This characteristic makes it difficult to separate oligosaccharides using capillary zone electrophoresis. Borate has been commonly added to the electrophoretic separation buffer to assist CE separation because oligosaccharides can readily form negatively charged species by complexation with tetrahydroxyborate under alkaline conditions [11–13,22–25]. We found that the use of both borate and phenylboronic acid in the micellar electrokinetic capillary chromatography (MECC) format dramatically improves the separation. In this paper, we report baseline resolutions of isomeric oligosaccharides of both a βGal(1→3)βGlcNAc series and a βGal(1→4)βGlcNAc series. We also demonstrate an application of CE–LIF to monitoring the enzymatic biotransformation of TMR-labeled oligosaccharides in a human epidermoid cell line, A431.

2. Experimental

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were obtained from Gibco (Gibco BRL). Cell culture flasks (T 25 cm^2) were obtained from Falcon. Stock solutions including 0.1 *M* Na_2HPO_4 (Fisher), 0.1 *M* sodium tetraborate (Fisher), 0.1 *M* sodium dodecyl sulfate (SDS; BDH) and 0.1 *M* phenylboronic acid (Sigma), were prepared in deionized water (Barnstead NANO pure system) and filtered using 0.2 μm pore size disposable filters (Nalgene). The electrophoresis running buffers were prepared by mixing these stock solutions to the desired final concentrations. The pH of the buffers was adjusted using dilute NaOH or HCl solutions.

The TMR-labeled oligosaccharides used in this study are listed in Table 1. These fluorescent compounds were prepared by derivatizing synthetic 8-methoxycarbonyloctyl oligosaccharides as previously reported [18–20]. They were purified using column chromatography and thin-layer chromatography techniques, and characterized using both mass spectrometry and nuclear magnetic resonance spectroscopy [20]. The overall yields were in the 70% range. The solid form (red fluffy powder) of these compounds are stable at −20°C in the dark for over two years. In solution, they are stable for several months at room temperature in a dark room. These compounds have the general structure of sugar-$O(CH_2)_8CONHCH_2CH_2NHCO$-TMR and are abbreviated sugar-O-TMR.

2.2. Capillary electrophoresis with laser-induced fluorescence detection

The CE–LIF instrument used for the determination of the fluorescent substrate and enzyme products of oligosaccharides has been reported in detail elsewhere [21]. Briefly, the electrophoresis was driven by a CZE1000R high voltage power

Table 1
List of the saccharide derivatives of tetramethylrhodamine (TMR) that were used in this study

No.	Structure of TMR derivatives	Trivial name
1	$HO(CH_2)_8CONHCH_2CH_2NHCO$-TMR	HO-TMR(Linker arm)
2	βGlcNAc-O-TMR	GlcNAc
3	βGal(1→4)βGlcNAc-O-TMR	LacNAc (Type II)
4	αFuc(1→2)βGal(1→4)βGlcNAc-O-TMR	H-type II
5	βGal(1→4)[αFuc(1→3)]βGlcNAc-O-TMR	Le^x
6	αFuc(1→2)βGal(1→4)[αFuc(1→3)]βGlcNAc-O-TMR	Le^y
7	βGal(1→3)βGlcNAc-O-TMR	Type I
8	αFuc(1→2)βGal(1→3)βGlcNAc-O-TMR	H-type I
9	βGal(1→3)[αFuc(1→4)]βGlcNAc-O-TMR	Le^a
10	αFuc(1→2)βGal(1→3)[αFuc(1→4)]βGlcNAc-O-TMR	Le^b

supply (Spellman, Plainview, NY, USA). Separation was carried out in a 40–50 cm long, 10 μm inner diameter fused-silica capillary in an electric field of 400 V/cm. A 1.0-mW helium–neon laser (Melles Griot) beam, λ=543.5 nm, was focused into a post-column sheath flow cuvette. Fluorescence was collected at a right angle with a high numerical aperture (0.7 N.A.) microscope objective (60×), spectrally filtered with a band-pass filter (580DF40), imaged onto one end of a fiber collimator (SELFOC) and detected at the other end of the fiber collimator with a R1477 photomultiplier tube. Data was digitized by a NB-MIO-16X data acquisition board in a Macintosh computer.

Samples were electrokinetically injected onto the separation capillary typically by applying a 500–1000 V potential for 5 s. The electrophoretic separation was performed at room temperature. Electropherogram peak identity was obtained by comparing the migration time of the analyte in the sample with those of the standards. Further confirmation of the enzyme products was achieved by analysis of the co-injected standard compound and the reaction mixture. Co-migration of an analyte and the standard compound is evidence that the reaction mixture contained that standard compound.

2.3. Incubation of A431 cells with a fluorescent oligosaccharide

A TMR-labeled disaccharide derivative, βGal(1→3)βGlcNAc-O-TMR (Type I), was used as a substrate. The potential enzymatic transformations of this saccharide include both degradation and biosynthesis. The degradation of βGal(1→3)-βGlcNAc-O-TMR, involving galactosidase action, gives N-acetyl-β-D-glucosaminide-O-TMR (GlcNAc), and further degradation by hexosaminidase gives $HO(CH_2)_8CONHCH_2CH_2NHCO$-TMR (Linker arm: HO-TMR). Biosynthesis involving fucosyltransferases and galactosyltransferases can result in the formation of αFuc(1→2)βGal(1→3)βGlcNAc-O-TMR (H-type I), βGal(1→3)[αFuc(1→4)]βGlcNAc-O-TMR (Le^a) and αFuc(1→2)βGal(1→3)[αFuc-(1→4)]βGlcNAc-O-TMR (Le^b).

A human epidermoid carcinoma cell line, A431, was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in DMEM supplemented with 10% FCS at 37°C in a water-saturated 5% CO_2 atmosphere (pH 7.2–7.4). After the cells were grown to 60–70% confluency (72 h of incubation, a total of $3 \cdot 10^6$ cells per 25 cm^2 culture flask), they were washed with 6 ml of phosphate-buffered saline (PBS). Fresh culture medium supplemented with 25 μg/ml of the substrate βGal(1→3)βGlcNAc-O-TMR was added to the cell culture flask. The cell incubation was continued for 20 h at 37°C in a 5% CO_2 atmosphere. The culture medium containing βGal(1→3)βGlcNAc-O-TMR was removed and the cells were washed with a total volume of 250 ml of PBS. Then, 2 ml of a trypsin–EDTA solution containing 0.05% trypsin and 0.53 m*M* EDTA were added to the culture flask and incubated for 10 min at 37°C. The cells were detached from the bottom of the flask, transferred to a centrifuge tube and centrifuged at 1000 *g* for 10 min. The cell pellets were lysed with 500 μl of Milli-Q water and vortex-

mixed. The lysate was loaded onto a Sep-Pak Plus C_{18} cartridge (Millipore/Waters), then washed with 30 ml of Milli-Q water. Sugar-O-TMR compounds were eluted with 3.5 ml of methanol and diluted in the CE running buffer prior to CE–LIF analysis to determine enzymatic products and the unreacted substrate. Parallel controls were carried out by incubating the cells with the culture medium without the addition of substrate. No fluorescence was detected from CE–LIF analyses of the controls.

3. Results and discussion

Fig. 1 shows an electropherogram obtained from the CE separation of five oligosaccharide derivatives,

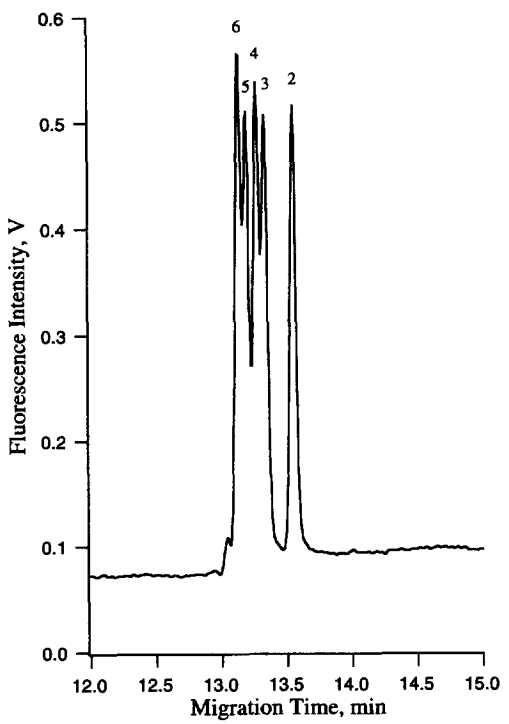


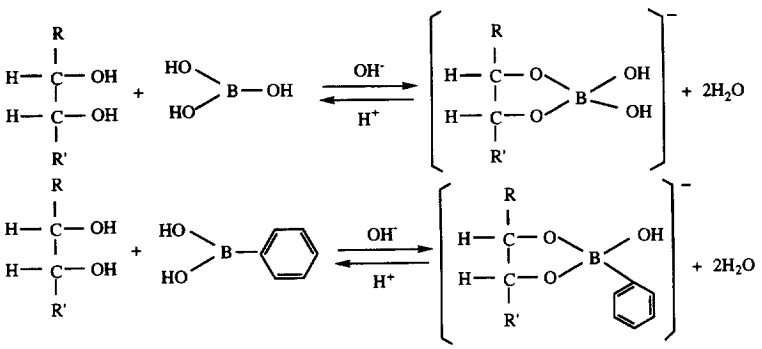
Fig. 1. Capillary electrophoresis separation and laser-induced fluorescence detection of five fluorescently labeled oligosaccharides. A mixture of 20 m*M* borate and 100 m*M* SDS was used as the running buffer. A voltage of 18 kV was applied to a 45-cm capillary (10 μm I.D., 144 μm O.D.) for the electrophoretic separation. Peak identities are shown in Table 1.

using 20 m*M* sodium borate and 100 m*M* SDS as a separation buffer. These saccharides are the potential enzyme products and substrate of glycosyltransferases and glycosidases acting on βGal(1→4)-βGlcNAc-O-TMR (LacNAc) (type II). A partial separation is obtained, as shown in Fig. 1, however, a baseline resolution is desirable in order to study enzymatic transformation of these compounds. Attempts to further improve the resolution by adjusting the pH of the buffer and the concentrations of sodium borate (10–60 m*M*) and SDS (10–100 m*M*) were not successful. Also, the addition of small amounts (10–30 m*M*) of cyclodextrin and hydroxypropylmethylcellulose to the above buffers did not improve the separation.

When a small amount (10 m*M*) of phenylboronic acid is added to the separation buffer, the resolution of the above oligosaccharides is dramatically improved. Fig. 2 shows electropherograms obtained from the CE–LIF analysis of the oligosaccharide mixture using buffers containing 10 m*M* each of borate, phenylboronic acid, phosphate and SDS. Baseline resolutions are achieved for all of these saccharides over a wide pH range (pH 7.5–10). The structural differences between some of these compounds (e.g. isomers) are very small, especially after the saccharides are labeled with a relatively large TMR fluorophore. A comparison of the electropherograms in Figs. 1 and 2 clearly shows the benefit of phenylboronic acid in separating similar oligosaccharides.

In alkaline solutions, borate acts as a Lewis acid to form anionic tetrahydroxyborate, $B(OH)_4^-$, which reacts with vicinal diols (preferably *cis*-) in a carbohydrate molecule forming a negatively charged complex [22–26]. Similarly, phenylboronic acid can also form complexes with diols in the carbohydrates, as shown below.

The stability of these complexes is dependent upon the structure of the carbohydrates. The electrophoretic mobility is in turn dependent on the formation and stability of these complexes. Thus, the use of borate in electrophoretic separation buffers has been very successful in resolving some carbohydrates. We include phenylboronic acid because the relatively larger size of the benzene group compared to the hydroxyl group introduces additional steric effects on the formation of borate complexes with



some carbohydrates. Therefore, the use of both sodium borate and phenylboronic acid in the separation buffer enhances electrophoretic mobility differences among the oligosaccharides, leading to the improved separation. Phenylborate esters of carbohydrates will also be more hydrophobic, affecting their partition into the SDS micelles.

A similar baseline resolution is obtained for the separation of another series of oligosaccharides, as shown in Fig. 3. These are from type I series; H-type I, Le^a and Le^b are potential enzymatic synthesis products of the type I disaccharide, βGal(1→3)-βGlcNAc, whereas the linker arm and GlcNAc are the enzymatic hydrolysis products. Baseline resolu-

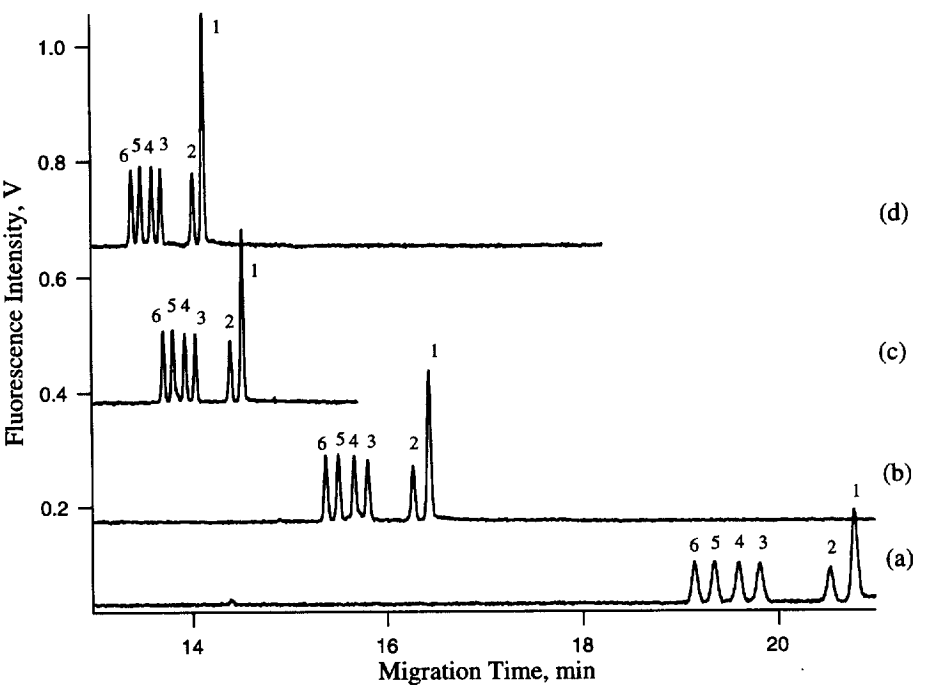


Fig. 2. Electropherograms obtained from the CE–LIF analyses of five fluorescent saccharide derivatives (type II sequence) and the linker arm. The separation was carried out on a 50-cm capillary (10 μm I.D., 144 μm O.D.) with a running voltage of 20 kV. The running buffers contained 10 m*M* each of phosphate, borate, phenylboronic acid and SDS. The pH of the buffers was (a) 7.5, (b) 8.3, (c) 9.1 and (d) 9.3. Approximately 10 pl of a standard solution containing 10^{-9} *M* of each compound was injected using an electrokinetic injection (1000 V for 5 s). Peak identities are shown in Table 1.

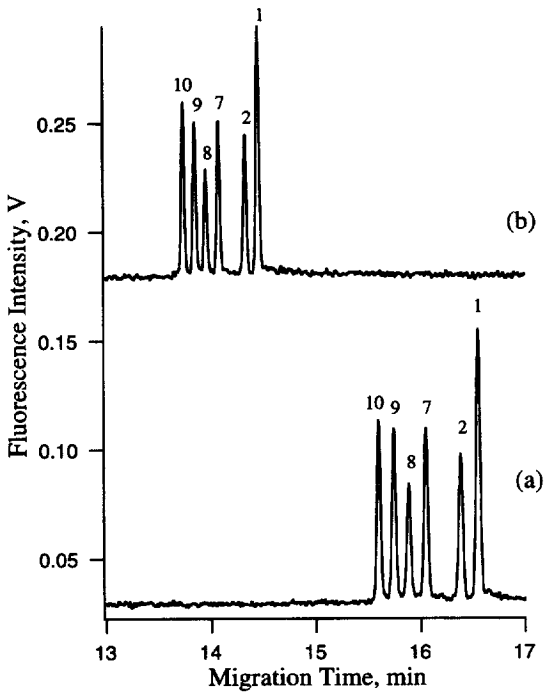


Fig. 3. Electropherograms obtained from the CE–LIF analyses of oligosaccharide derivatives from the βGal(1→3)βGlcNAc-O-TMR (type I) sequence. Peak identities are shown in Table 1. The same experimental conditions as shown in Fig. 2 were used, except that the pH of the buffers was (a) 8.3 and (b) 9.1.

tion of these compounds, including two pairs of isomeric oligosaccharides, H-type II and Le^x (Fig. 2) and H-type I and Le^a (Fig. 3), demonstrates the excellent separation efficiency, which is achieved by using an optimized buffer solution containing borate, phenylboronic acid, phosphate and SDS.

Direct spectrophotometric detection of oligosaccharides is difficult because this class of compounds has only very low UV absorption. Several approaches have been shown to improve the detection sensitivity, including borate complexation [11–13,22,25,26] and derivatization with suitable chromophores or fluorophores [11–13,24,27,28]. LIF detection of derivatized oligosaccharides provides the best detection limits [14–21]. In the present work, we chose to label the oligosaccharides with TMR because of the high molar absorptivity and quantum yield, giving intense orange fluorescence at an excitation wavelength of 543.5 nm from an inexpensive green He–Ne laser. We use a sheath flow cuvette as a post-column detection cell and we optimize the fluorescence collection system. As a result, an excellent detection limit is obtained. Figs. 2 and 3 show electropherograms obtained from the CE–LIF analyses of approximately 10^{-20} moles (10^{-9} *M* concentration, 10 pl injection), or 6000 molecules, of each oligosaccharide. These results are consistent with our previously reported detection limits of approximately 80 yoctomoles (8×10^{-23} moles), or 50 molecules of the TMR-labeled saccharides [21].

The compounds shown in Figs. 2 and 3 may be either substrates (LacNAc and Type I) or potential products of enzymatic reactions catalyzed by glycosyltransferases and glycosidases. The excellent detection limit and resolution makes the CE–LIF ideal for the detection of minor enzymatic products in the presence of a potential excess of substrate.

Fig. 4 demonstrates an application of the CE–LIF system to the determination of enzyme products in the presence of unreacted substrate. The assay involved the incubation of A431 cells with a substrate and the CE–LIF analysis of the contents of the cell samples. TMR-labeled βGal(1→3)βGlcNAc, a disaccharide derivative, was used as the substrate. After a 20 h incubation of the substrate (25 μ*M*) with A431 cells, several products are detected in the

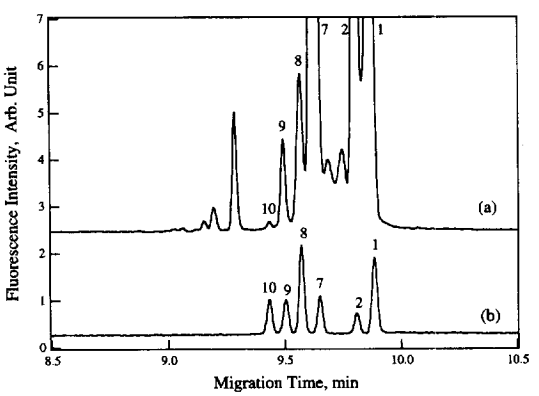


Fig. 4. Electropherograms obtained from the CE–LIF analyses of (a) A431 cells after incubation with βGal(1→3)βGlcNAc-O-TMR for 20 h and (b) a standard containing $5\cdot10^{-10}$ *M* each of βGal(1→3)βGlcNAc-O-TMR (the substrate), Le^a, Le^b, and GlcNAc, and 10^{-9} *M* of H-type I and the linker arm. A 40-cm capillary (10 μm I.D., 144 μm O.D.) was used with a 16-kV running voltage for the electrophoretic separation. A mixture containing 10 m*M* each of phosphate, borate, phenylboronic acid and SDS (pH 9.3) was used as the running buffer. Peak identities are shown in Table 1.

cells (Fig. 4a). On the basis of their migration times, compared with those of standards (Fig. 4b), these compounds are identified as H-type I, Le^a, Le^b, GlcNAc and the linker arm. The identification of these compounds was supported by analyzing the sample with co-injected individual standards; the spiked standard compound co-migrated with the analyte in the sample.

The formation of H-type I, Le^a and Le^b was further confirmed by additional CE–LIF analyses of the sample after the sample was treated with hydrolysis enzymes. Fucosidases from human placenta and almond meal were chosen because we have demonstrated their specificity for the hydrolysis of H-type I, Le^a and Le^b. After the same cell sample as shown in Fig. 4a was incubated with the fucosidases for 36 h at 37°C, CE–LIF analyses of the fucosidase-treated samples showed that the peaks at the migration time corresponding to H-type I, Le^a and Le^b disappeared, confirming the identity of these products in the original cell sample. The formation of these compounds following the incubation of βGal(1→3)βGlcNAc with A431 cells is expected, since both glycosyltransferases (synthetic enzymes) and glycosidases (hydrolytic enzymes) are common in mammalian cells. The action of glycosyltransferases in the cells is expected to produce H-type I, Le^a and Le^b from βGal(1→3)βGlcNAc. Glycosidases acting on the acceptor produce GlcNAc-TMR and the free linker arm (HO-TMR). The substrate βGal(1→3)βGlcNAc can also be degraded by lysosomal enzymes in the cells.

The enzyme reaction products in the cell samples are stable at 4°C for more than 130 days, confirmed by replicate CE–LIF analyses of the cell samples after a period of storage. No difference was observed on comparing the CE–LIF results from the fresh sample with those from the sample stored at 4°C for 130 days. Sample solution diluted in CE running buffer is also stable at room temperature for over 43 days in a dark room.

The predominant species in the cells is the unreacted substrate. Because of the excellent CE separation, the excess substrate in the sample does not interfere with the determination of the minor enzyme reaction products. Several minor products are unidentified because of a lack of fluorescently labeled standards. We suspect that some of these unidentified species are derived from the action of other glycosyltransferases (e.g. α-sialyltransferases and glucuronosyltransferases), since these enzymes have also been found in mammalian systems [3,4].

LIF technology has shown a single molecule detection limit [29–31]. However, CE–LIF has not been widely used for the analysis of real samples at analyte levels below the femtomolar level. This is primarily because of the problems involved in fluorescent labeling of trace levels of analyte in the presence of a sample matrix, namely non-quantitative derivatization and poor selectivity of the labeling. A unique feature of the present assay is that it does not require the fluorescent labeling of the analytes in the sample. Instead, the fluorescent labeling of the standard oligosaccharide compounds, as shown in Table 1, was carried out in neat solutions at m*M* concentrations and therefore was much easier than attempting to label trace levels of analytes in the sample. Furthermore, quantitative labeling is not a requirement in the present study as would be the case if analytes in the sample are to be labeled. In the present study, the oligosaccharides were aminated and then reacted with the N-hydroxysuccinimide ester of TMR. The reaction products were purified by using a series of preparative chromatography procedures, and their identities were confirmed by using mass spectrometry and nuclear magnetic resonance spectroscopy. These TMR-labeled compounds were then used for the studies of enzymatic transformation. When typical glucosyltransfereases and glycosidases acted on the TMR-labeled substrate, e.g., βGal(1→3)βGlcNAc-O-TMR, the TMR label remained attached on the enzymatic reaction products that were subsequently detected by CE–LIF. Other hydrolysis products that did not contain TMR label were not detected. The excellent detection limit and resolution demonstrated in this paper make the CE-LIF technique a powerful tool for the monitoring of enzymatic transformations of fluorescently labeled oligosaccharides in in vitro studies.

Acknowledgments

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