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Analysis by capillary electrophoresis–laser-induced fluorescence detection of oligosaccharides produced from enzyme reactions

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Abstract

Six structurally similar, fluorescently labeled oligosaccharides were baseline resolved by capillary electrophoresis (CE); laser induced fluorescence (LIF) detection gave detection limits of 50 molecules for the oligosaccharides. A simple design of the LIF detector that incorporates the advantages of high sensitivity, stability and ease of operation is described. The system was used to monitor enzyme products formed during the incubation of yeast cells with α-D-Glc(1→2)α-D-Glc(1→3)α-D-Glc-O$(CH_2)_8$CONHCH$_2$CH$_2$NHCO-tetramethylrhodamine. This fluorescent trisaccharide is enzymatically hydrolyzed to fluorescent disaccharide, monosaccharide and the free linker arm that is used to conjugate the saccharides with the fluorophore tetramethylrhodamine.

1. Introduction

Oligosaccharides play important roles in many biological functions [1–3]. The formation of oligosaccharides results from a complex series of glycosyltransferase (synthetic) and glycosidase (hydrolytic) enzymatic reactions. Alternation of these enzyme activities can result in a change in the formation or amounts of the complete oligosaccharide chain. Such changes are associated with a number of diseases, especially cancer [4–8]. Assay of the activity of these enzymes and determination of the oligosaccharides are essential to an understanding of their roles in biology.

Conventionally, enzyme assays of glycosyltransferases have often been carried out by using radiochemical methods, gel-permeation, ion-exchange and affinity chromatography and enzyme linked immunosorbent assay [9–15]. Many of these assays involve tedious procedures and are consequently time consuming. In addition, these assays usually require the use of large amounts of sample and are applicable only to relatively high levels of enzyme.

Capillary electrophoresis combined with laser-induced fluorescence detection (CE–LIF) has been shown to be a powerful technique, which combines the efficient separation offered by CE and the exquisite sensitivity of LIF [16–21]. A minute amount of sample can be analyzed by CE–LIF because of the small sample volume requirement. Therefore, CE–LIF is an ideal technique for assaying enzyme activity in situations where the amount of substrate or enzyme is limited.

Previous work by our group [22] has demonstrated the capability of CE–LIF in assaying two enzymes, fucosyltransferase and α-fucosidase. Well characterized and purified enzymes were used to react with fluorescently tagged substrates

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under well defined conditions. CE was used to separate the substrate from the enzyme product and postcolumn LIF was followed to detect the fluorescent compounds. More recently, we have successfully used CE–LIF to moniotor biosynthetic transformations of a tetramethylrhodamine-labeled derivative of N-acetyllactosamine in crude microsomal extracts that were prepared from the cell line HT-29 (human colon adenocarcinoma) [23]. The technique allowed us to determine minor enzyme products in the presence of major amount of unreacted substrate.

In this work, we extended the application of CE–LIF to the determination of enzyme substrate and products in intact cells. Products formed from the α-glucosidase I and II reactions are monitored after a fluorescently labeled trisaccharide, α-D-Glc(1→2)α-D-Glc(1→3)α-D-Glc-O$(CH_2)_8$CONHCH_2CH_2NHCO-tetramethylrhodamine (-TMR) is incubated with *Saccharomyces cerevisiae* (baker's yeast). We also describe a simple design of an LIF detector using a postcolumn sheath flow cuvette and demonstrate the baseline resolution and sensitive detection of structurally similar oligosaccharides.

2. Experimental

2.1. Materials

Fluorescently labeled oligosaccharides were prepared as described previously [23,24]. Stock solutions including 0.1 *M* Na_2HPO_4 (Fisher Scientific, Nepean, ON, Canada), 0.1 *M* borate (Fisher Scientific), 0.1 *M* sodium dodecyl sulfate (BDH, Toronto, ON, Canada) and 0.1 *M* phenylboronic acid (Sigma, St. Louis, MO, USA), were prepared in deionized water (Barnstead NANOpure system) and filtered with a 0.2-μm pore size disposable filter (Nalgene, Cleveland, OH, USA). The electrophoresis running buffer was prepared by mixing these stock solutions to final concentrations of 10 m*M* each of Na_2HPO_4, borate, sodium dodecyl sulfate (SDS) and phenylboronic acid (pH 9.3). All other reagents were of analytical-reagent grade.

2.2. Capillary electrophoresis system

The electrophoresis was driven by a Spellman (Plainview, NY, USA) CZE 1000R high-voltage power supply. The separation voltage, injection voltage and injection time were controlled by a Macintosh Quadra 650 computer, with a program written in LabVIEW (National Instruments, Austin, TX, USA). Separation was carried out in a 42 cm × 10 μm I.D. × 144 μm O.D. fused-silica capillary (Polymicro, Phoenix, AZ, USA) at an electric field of 400 V/cm. The high-voltage injection end of the capillary was held in a Plexiglas box equipped with a safety interlock. The other end of the capillary was placed inside a sheath flow cuvette detector and was held at ground potential. For optimum separation, the running buffer was composed of 10 m*M* each of phosphate, borate, phenylboronic acid, and SDS at pH 9.3. The sheath fluid was identical with the running buffer and was gravity fed from a 250-ml wash bottle. The sheath fluid level was arranged to be 5 cm higher than the level in the waste reservoir. This arrangement maintained a smooth sheath flow through the cuvette.

2.3. Laser-induced fluorescence detection system

A postcolumn LIF detector using a sheath flow cuvette (Fig. 1) was constructed on an optical bread board (Newport, Irvine, CA, USA). A 1.0-mW helium–neon laser (Melles Griot, Nepean, ON, Canada) with a wavelength of 543.5 nm was used as the excitation source. The beam was focused with a 5× microscope objective (Melles Griot) into a sheath flow cuvette. The sheath flow cuvette was used as the fluorescence detection cell, as described elsewhere [16–18]. Briefly, the cuvette was constructed with high optical quality quartz, and had 1-mm thick walls, a 200-μm square inner chamber and a length of 2 cm. The quartz cuvette was mounted in a locally constructed stainless-steel body. A capillary was inserted into the cuvette such that the laser beam was illuminated approximately 20 μm below the tip of the capillary. The coating on the detector end of the capillary was removed to reduce any

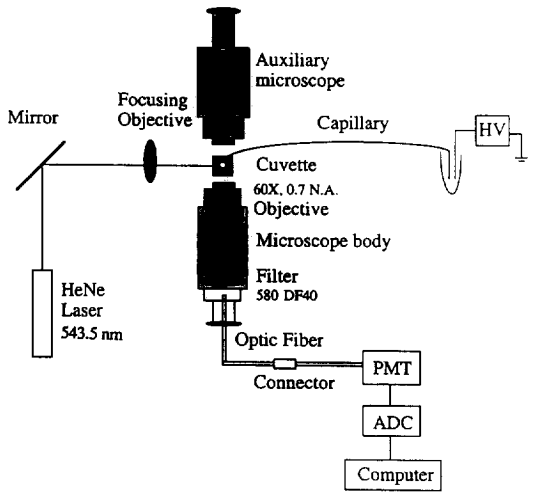

Fig. 1. Schematic diagram of a modified postcolumn laser-induced fluorescence detector for capillary electrophoresis.

possible scattering of laser light. Fluorescence was collected at right-angles with respect to the laser beam by using a high numerical aperture (0.7 NA), 60× microscope objective Model 60X-LWD (Universe Kogaku, Oyster Bay, NY, USA). The fluorescence was spectrally filtered with a band-pass filter (580DF40; Omega Optical, Brattleboro, VT, USA) and imaged on to a SELFOC fiber collimator (p-type; NSG America, Somerset, NJ, USA) that consisted of an optical fiber and two grin lenses, one on each end of the optical fiber [25]. The diameter of the grin lens was 2 mm. The fluorescence was propagated through the fiber collimator and was detected with an R1477 photomultiplier tube (PMT) (Hamamatsu, Bridgewater, NJ, USA). The output from the PMT was digitized by a National Instruments board in a Macintosh Quadra 650 computer.

It is necessary to align a tightly focused laser beam with a small-diameter sample stream so that the fluorescence passes through a high numerical aperture objective and is detected by the PMT. In the present optical design, the alignment can be optimized very easily and quickly. An auxiliary microscope was placed opposite the collection optic and used to assist the alignment. During the alignment process, the bulkhead connector (Radiant Communications, Plainfield, NJ, USA) between the two fiber collimators was disconnected. When light from a flashlight illuminates the end of the fiber collimator, light travels back through the optical system in a path that is the reverse of the fluorescence. A small, bright spot representing the image of the fiber collimator is projected through the collection optic. The auxiliary microscope is focused on this bright image of the fiber collimator. The position of the laser beam and the sheath flow cuvette are then adjusted to superimpose the projected image of the fiber collimator with the fluorescence from the sample stream. When the images superimpose, the system is in alignment. The sheath flow cuvette and the laser beam focusing objective are each mounted on a set of three-axis translation stages, and thus their positions can be adjusted with ease and precision. The system is compact and very stable.

2.4. Incubation of yeast cells and preparation of spheroplasts

Saccharomyces cerevisiae (baker's yeast, Fleischman) was grown on Sabouraud dextrose agar plates (Difco) at 37°C and then stored at 4°C. A typical colony was inoculated into 1 ml of sterile Sabauraud dextrose medium and grown at room temperature with shaking overnight (ca. 17 h). A subsample of 200 μl was transferred into a sterile microfuge tube and pelleted. Old medium was removed and fresh medium was added to the pelleted cells along with sterile filtered trisaccharide, α - D - Glc(1→2)α - D - Glc(1→3)α - D - Glc - O$(CH_2)_8$CONHCH$_2$CH$_2$NHCO - TMR, from a 10 m*M* stock solution. The final concentration of the trisaccharide substrate in the culture was 50 μM. Tubes were covered with aluminium foil and incubated at 25°C for 5 h with shaking. At the end of the incubation period, cells were pelleted and the supernatant was removed. A subsample of the supernatant was saved for analysis by capillary electrophoresis. Cells were then transferred to the surface of a 0.22 μm, 47 mm diameter cellulose filter

(Micron Separations) and washed under vacuum with ca. 500 ml of phosphate-buffered saline (PBS). Cells were washed off the filter into a test-tube with PBS and pelleted. A sample of the PBS supernatant was taken for capillary electrophoresis to determine if all free trisaccharide substrate had been removed. Control cells were prepared in the same manner except that no trisaccharide substrate was added.

The intact PBS-washed cells were used to generate spheroplasts with the following procedure [26,27]. Cells were washed twice with a high-ionic-strength buffer, 25 m*M* Tris–HCl (pH 7.5)–2.0 *M* sorbitol, and then incubated at 37°C in 40 μl of the same buffer containing 770 U per 100 μl of lyticase (*Arthrobacter luteus*, β1–3 glucanase; Sigma). After a 2-h incubation at 37°C, most of the cells were converted into spheroplasts, as determined by visual inspection of a sample under a microscope. Spheroplasts were lysed by adding a buffer containing 10 m*M* borate and 10 m*M* SDS and by sonicating for 1 h in a water-bath ultrasonic cleaner (Branson, Shelton, CT, USA). The sample was subjected to CE–LIF analysis.

2.5. Determination of oligosaccharides by CE–LIF

Samples were electrokinetically injected on to a 10 μm I.D. capillary typically by applying a 500–1000-V potential for 5 s. To reduce contamination, the capillary tip was first rinsed in a vial containing running buffer before the capillary was placed in the vial containing the buffer used for electrophoresis analysis. The separation was performed at room temperature. Peak identity in the electropherogram 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-elution of an analyte and the standard compound provided evidence that the reaction mixture contained that standard compound.

3. Results and discussion

Fig. 2 shows two electropherograms obtained from the analysis of a mixture of six fluorescently labeled sugars and the linker arm. Each component in the standard mixture was 10^{-10} *M* (for the top trace) and 10^{-9} *M* (for the bottom trace). The separation capillary (42 cm × 10 μm I.D.) has a total volume of 33 nl. Electrokinetic injection at 500 V for 5 s corresponds to the introduction of ca. 5.5 pl of the standard mixture. Therefore, ca. 350 (top trace) and 3500 (bottom trace) molecules of each compound

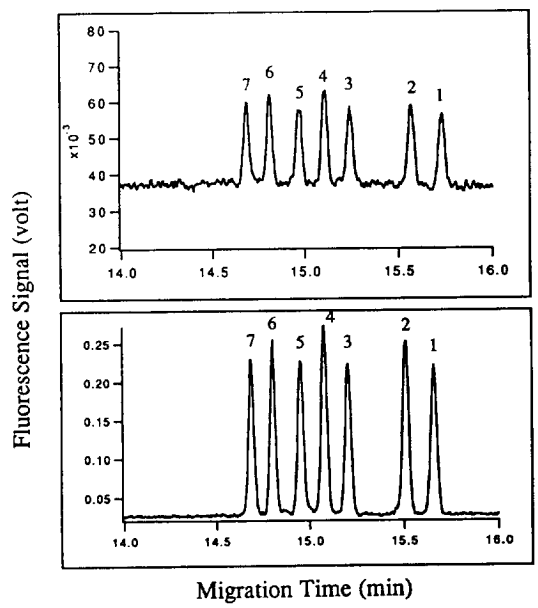

Fig. 2. CE–LIF of six fluorescently labeled saccharides (peaks 2–7) and the linker arm (peak 1). Capillary, 42 cm × 10 μm I.D. × 144 μm O.D.; running buffer, 10 m*M* phosphate–borate–phenylboronic acid–SDS; running voltage, 16.8 kV (400 V/cm); injection time, 5 s; injection voltage, 500 V; injection volume, 5.5 pl; concentration of each component, 10^{-10} *M* (top) and 10^{-9} *M* (bottom). Peaks: 1 = $HO(CH_2)_8CONHCH_2CH_2NHCO$-TMR (linker arm: -O–TMR); 2 = N-acetyl-β-D-glucosaminide-O–TMR (GlcNAc-O–TMR); 3 = βGal(1→3)βGlcNAc-O–TMR (Lewis C); 4 = βGal(1→4)βGlcNac-O–TMR (LacNAc); 5 = αFuc(1→2)βGal(1→4)βGlcNAc-O–TMR (H-Type II); 6 = βGal(1→4)[αFuc(1→3)]βGlcNAc-O–TMR (Lewis X); 7 = αFuc(1→2)βGal(1→4)[αFuc(1→3)]βGlcNAc-O–TMR (Lewis Y).

were injected for these analyses. The detection limit calculated according to Knoll's method [28] is ca. 80 yoctomol (8×10^{-23} mol), or 50 molecules of the TMR-labeled saccharides.

Compounds **3** and **4** are two disaccharide isomers, as are compounds **5** and **6**. The structural differences between these compounds are minor, especially after the saccharides have been labeled with the relatively large TMR fluorophore. The baseline resolution of these compounds shown in Fig. 2 demonstrates the excellent separation efficiency, which is achieved by using an optimized buffer solution containing borate, phenylboronic acid, phosphate and SDS. The combination of borate and phenylboronic acid is used to enhance the resolution; the complexation of sugar hydroxyl groups with borate [29,30] and phenylboronic acid enhances the mobility differences between the labeled saccharides.

Fig. 3 demonstrates an application of the CE–LIF system to the analysis of enzyme products generated in intact yeast cells. The top electropherogram was obtained from the injection of lysed spheroplasts, formed from cells incubated for 5 h with the trisaccharide α-D-Glc(1→2)α-D-Glc(1→3)α-D-Glc-$O(CH_2)_8$ $CONHCH_2CH_2NHCO$-TMR. Most of this substrate was converted into linker arm (L) and intermediate disaccharide (D) and monosaccharide (M). Prior to spheroplast formation, the yeast cells were thoroughly washed with PBS, and these compounds were not found to be present at detectable levels in the PBS wash solution. This indicates that the compounds detected after spheroplast lysis originating from inside the cells. Monitoring of the intact yeast cells, using confocal laser scanning microscopy, also confirmed the uptake of the fluorescent trisaccharide by yeast cells after an incubation period (results not shown). The trisaccharide substrate is stable in aqueous solution. The hydrolysis products observed arise from the sequential activity of α-glucosidase I and II inside the yeast, which act specifically on α-D-Glc(1→2) and α-D-Glc(1→3) linkages, respectively [31,32]. Using the present technique, we

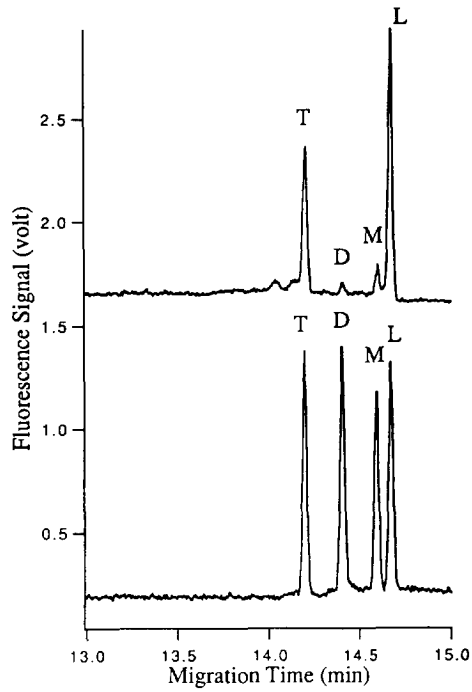

Fig. 3. Electropherograms obtained from the analysis of lysed yeast spheroplasts (top) and a standard solution containing 10^{-9} *M* of each component (bottom). Peaks: T = α-D-Glc(1→2)α-D-Glc(1→3)α-D-Glc-$O(CH_2)_8CONHCH_2CH_2NHCO$-TMR; D = α-D-Glc(1→3)α-D-Glc-$O(CH_2)_8CONHCH_2CH_2NHCO$-TMR; M = α-D-Glc-$O(CH_2)_8CONHCH_2CH_2$-NHCO-TMR; L = H-$O(CH_2)_8CONHCH_2CH_2$-NHCO-TMR.

are able to determine both the major and minor enzyme products in a single analysis.

The capability of the technique is not limited to the oligosaccharides described in this paper. The instrumentation and the general methodology presented here should be applicable to the assay of other enzymes and fluorescent substrates.

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