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Fluorophore-assisted carbohydrate electrophoresis (FACE) of oligosaccharides: efficiency of labelling and high-resolution separation

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Abstract

Reductive amination is a common technique for the derivatisation of reducing carbohydrates, thereby providing appropriate chromophores or fluorophores to overcome native detection deficiencies. Rarely, however, is the issue of labelling efficiency addressed for substrates larger than monosaccharides. Utilising a variety of radiolabelled synthetic maltooligosaccharides, we now present data on the APTS labelling efficiency for substrates up to an average degree of polymerization (dp) of 135. The labelling reaction was found to be highly reproducible and independent of average chain length between dp 3 and dp 135, with an average efficiency of 80%. Glucose (95%) and maltose (88%) were labelled more efficiently. In addition to this work, electrophoretic methodologies have been developed to aid the characterization of APTS-labelled oligosaccharide distributions across a wide range of chain lengths. Fluorescent imaging of polyacrylamide slab gels provides flexibility of gel format, and conditions that can be adapted to the resolution and quantification of short oligosaccharide populations (less than dp 30) or to enable the observation of polysaccharides. A capillary gel electrophoretic method was developed using laser–induced fluorescence (LIF) detection to fully resolve and quantify maltooligosaccharides up to approximately dp 100, a technique that finds particular use in the analysis of oligosaccharide distributions obtained from isoamylase debranching of the amylopectin component of starch. A comparison of data reproducibility across a range of chain lengths established the superiority of results obtained by the capillary gel electrophoretic method over a previously reported method involving DNA sequencer-mediated electrophoretic separation. © 1998 Elsevier Science Ltd. All rights reserved

Keywords: Reductive amination; APTS; Labelling efficiency; Slab gel electrophoresis; Oligosaccharide; Starch; Capillary electrophoresis

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1. Introduction

The analysis of carbohydrates is hampered by their lack of either chromophoric or fluorophoric functional groups and low extinction coefficients for both UV and fluorescent detection, thereby restricting commonly used detection to those using relatively insensitive mass responses. The consequence of these limitations is that many high-resolution separatory techniques such as electrophoresis and reversed-phase HPLC are not feasible without prior derivatisation of the analyte. Reductive amination has long been used as a simple means of derivatising the reducing end, introducing either a single fluorophore or chromophore. The introduction of a single label at the reducing end allows quantification on a molar basis, with facile conversion to a mass basis if the molecular mass of the carbohydrate is known. A further sophistication of the method is the use of a charged chromophore or fluorophore, which enables separation of the conjugates by electrophoretic methods. A variety of aromatic primary amines have been used for these applications [1–4], although most recent work has concentrated on the use of polysulfonic acid derivatives of monoamino-substituted aromatic compounds, such as 8-amino-1,3,6-naphthalenetrisulfonic acid (ANTS) [5–7] and 8-amino-1,3,6-pyrenetrisulfonic acid (APTS) [8–10]. These labels possess absorption and emission characteristics that suit a range of commercially available detection systems, and their multiple negative charges confer electrophoretic mobility. Both ANTS and APTS have found use in fluorophore-assisted carbohydrate electrophoresis (FACE) [4,5], slab gel electrophoresis [10] and capillary electrophoretic methods [6–9,11,12].

Reductive amination with charged fluorophores is well established for the qualitative analysis of short oligosaccharides [13–16], although comparatively few reports extend these methods to encompass longer oligosaccharides [10,17–19]. The reductive amination of glucose with APTS is outlined in Fig. 1. For short-chain oligosaccharides, such as those derived from glycoconjugates [commonly between a degree of polymerisation (dp) of 8 and 14], the principal issue is frequently the identification of the oligosaccharide fragment rather than its precise quantification. The efficiency of labelling short oligosaccharides with a range of primary amines has been described as being both high (>95%) and consistent [5,20] and has been assumed to either remain constant with increasing chain length, or at least remain reproducible from sample to sample, rendering general deductions possible. However, these determinations have focused on the labelling reactions of various monosaccharides, primarily because of the lack of a wide range of appropriate commercially available oligosaccharide standards. An example of this is the case of maltooligosaccharides, where no commercial sources of pure standards beyond maltoheptaose can be readily obtained.

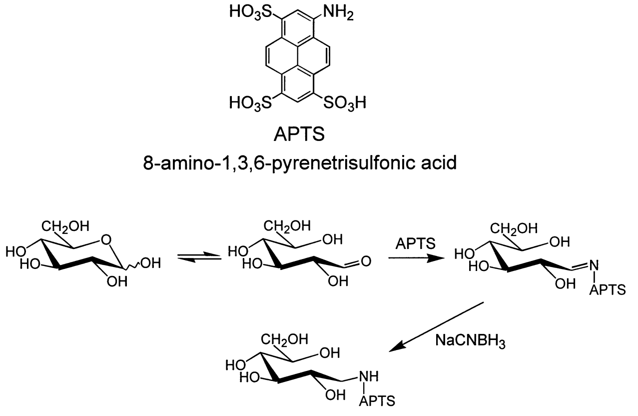

Fig. 1. Reductive amination of glucose with 8-amino-1,3,6-pyrenetrisulfonic acid (APTS).

The advantages in both separation and detection afforded by derivatisation with charged fluorophores raises the prospect of analysing complex mixtures of long oligosaccharides and ultimately polysaccharides. An example is the use of reductive amination with APTS as a method for the analysis of the oligosaccharides derived from debranched starches [10]. However, the quantitative analysis of such mixtures requires that either labelling efficiency remain constant with increasing chain length, or that efficiency changes in a predictable manner so that results can be normalised using defined correction factors.

In this paper we describe the further development of APTS-labelling as a method for the quantitative, reproducible and high-resolution analysis of oligosaccharides and polysaccharides. First, data are presented on the labelling efficiency of reductive amination with APTS across a range of maltooligosaccharides of defined chain lengths from dp 1 to dp 135. Secondly, electrophoretic procedures were developed for the separation and characterization of the APTS-labelled oligosaccharides that provide excellent resolution and sensitive and highly reproducible quantification.

2. Experimental

Synthesis of ^{14}C-labelled glucans.—Reaction mixtures contained 45 μmol of sodium citrate buffer (pH 7.0), 1.5 μmol of AMP, 0.3 μmol of maltoheptaose, and 75 μmol of [U-^{14}C]-glucose-1-phosphate (10.6 μCi/mL) in 1350 μL. The reaction mixture was incubated at 37 °C for 10 min before the addition of 150 μL of 10 mg/mL phosphorylase A (rabbit muscle; Sigma Chemical Co., MO, USA). The vial was gently inverted to mix all reagents, and the incubation was continued at 37 °C, with 300-μL aliquots removed at intervals of 40, 80, 120, 160 and 240 min after the addition of phosphorylase A. The aliquots were transferred to Eppendorf vials containing 100 mg of AG 501-X8 (D) mixed bed resin (Bio-Rad Laboratories, CA, USA) and incubated on a rotary inverter for 20 min at room temperature. The supernatant was removed and divided into approximately 5 nmol aliquots on the basis of reducing sugar analysis [21]. Aliquots were freeze dried for 18 h and stored at −70 °C until required. This procedure was used to obtain distributions with modal dp of 20, 31, 41, 53 and 77. A dp 135 distribution was obtained by reducing the maltoheptaose primer concentration to 60 μM and incubating the reaction mixture for 24 h. A dp 10 distribution was obtained by replacing maltoheptaose with maltotetraose and sampling the reaction after 20 min. The modal dp of these samples was determined by DNA sequencer-mediated slab gel electrophoresis [10,19] with the exception of the dp 77 and 135 distributions, in which the average dp was deduced from total sugar and reducing sugar assays [21].

Reductive amination with APTS.—Wheat starch (kindly provided by Dr. Ian Batey, CSIRO Division of Plant Industry, Sydney, Australia) was debranched as previously described [10], and a 5-nmol aliquot was determined by reducing sugar assay [21]. Pullulan molecular weight standards (obtained from Shodex, a division of Showa Denko K.K., Tokyo, Japan) were dissolved in deionized water and dispensed in aliquots containing 5 nmol of reducing ends, freeze dried, and labelled as below.

Aliquots of analyte containing 5 nmol of reducing ends were labelled in 200-μL microfuge tubes by the addition of 5 μL of a solution of 0.2 M APTS in aqueous glacial acetic acid (15%) and 5 μL of freshly prepared 1 M aqueous sodium cyanoborohydride. The reaction mixture was incubated at 37 °C for 15 h and diluted to a total volume of 100 μL with an electrophoresis sample buffer consisting of 6 M urea in 0.04 M boric acid and 0.04 M tris(hydroxymethyl)aminomethane (pH 8.6). The labelled oligosaccharides were stored at −70 °C until required.

Measurement of labelling efficiency by an anion-exchange resin adsorbtion technique.—APTS labelling reactions used for the determination of labelling efficiency were carried out as described above. After incubation at 37 °C for 15 h, the reaction mixture was diluted to a total volume of 1 mL by the addition of 40 mM Tris-borate buffer (pH 10.0) containing 6 M urea. An aliquot of 100 μL was removed for scintillation counting to determine the total ^{14}C per reaction. To the remaining solution was added 300 mg of AG1-X8 ion-exchange resin (Bio-Rad Laboratories, Hercules, CA, USA), and the vials were placed on a rotary inverter for 30 min in order to bind all APTS-labelled material and excess APTS. Fluorescence measurements obtained using a SLM-AMINCO spectrofluorimeter (Urbana, IL) operated by using SLM-AMINCO 8100, series 2, version 1.0 software, confirmed that all fluorescent material was bound to the ion-exchange resin. The supernatant was removed, and the resin was washed three times with 1 mL of 40 mM Tris-borate buffer (pH 10.0) containing 6 M urea, which proved sufficient to remove all unlabelled synthetic glucan. The washes were combined with the original supernatant, and an aliquot was removed for scintillation counting in 10 volumes of Emulsifier-safe™ high flash point economy LSC cocktail fluid (Packard Instrument Co., Meridian, CT) using a Beckman LS 3801 scintillation counter. Labelling efficiency was determined by subtracting the eluted ^{14}C (unlabelled glucan) from the total ^{14}C per reaction.

Measurement of labelling efficiency of dp 2 to dp 7 via fluorimager technique.—A substrate pool containing ^{14}C-maltose and approximately equimolar amounts of each maltooligosaccharide from dp 2 to dp 7 (Boehringer Mannheim GmbH, Germany) was prepared by weighing appropriate amounts of material as supplied. The concentrations of individual oligosaccharides was determined by integration of refractive index following HPLC through two HPX-42A carbohydrate analysis columns (Bio-Rad Laboratories, Hercules, CA) connected in series and held at 80 °C, using water as eluent with a flow rate of 0.6 mL/min. This analysis was conducted in order to correct for variable hydration states of the oligomers.

An aliquot of the substrate pool (containing 5 nmol reducing ends) was labelled as described above, and the labelling efficiency of maltose was determined by the ion-exchange resin method. The labelled mixture was electrophoresed through a 20% polyacrylamide gel that provided baseline resolution of individual oligomers (Fig. 2). Fluorescent imaging of the gel enabled all bands to be quantified in a reproducible manner as indicated by the low standard deviations in Table 2. Efficiencies of labelling of dp 3 to dp 7 were estimated by comparison of their fluorescent intensities to that of maltose, and adjusting the previously determined labelling efficiency of ^{14}C-maltose by these factors. These calculations were also based on the assumption that the fluorescent quantum yield of the label is independent of the size of the oligomer labelled.

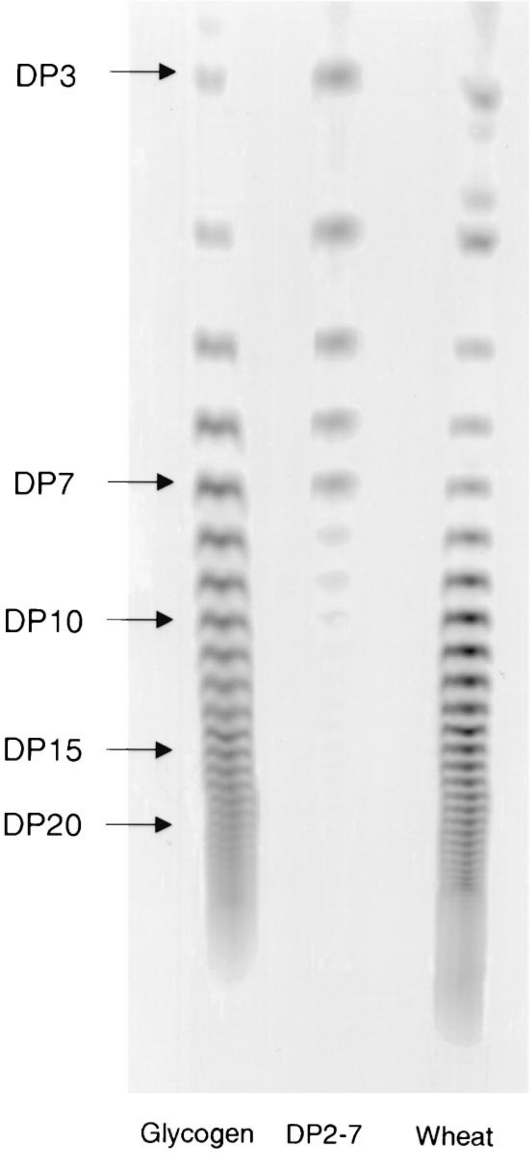

Fig. 2. Diagram of the fluorescent gel image obtained by electrophoresing APTS labelled debranched glycogen, an equimolar dp 2–7 mixture and debranched wheat starch on a 20% polyacrylamide gel in 1×TBE buffer for 4 h at 8 °C and a constant power of 75 W. The zone for dp 2 has electrophoresed beyond the top of the image.

Electrophoresis of APTS-labelled oligosaccharides in polyacrylamide slab gels.—Appropriate volumes (1–5 μL) were loaded into the wells of 4% or 20% polyacrylamide gels (19:1 ratio of acrylamide to *N*,*N*′-methylenebisacrylamide as cross-linker) which contained 8 M urea and were 32 cm in length and 0.5 mm thick. Slab gel electrophoresis was conducted on a Pharmacia Macrophor electrophoresis unit (Pharmacia Biotech, Uppsala, Sweden). Electrophoresis was performed at pH 8.0 using a polyacrylamide gel prepared in 89 mM Tris-borate containing 2 mM EDTA and 8 M urea. For 100 mL of gel mixture, 40 μL of TEMED and 400 μL of aqueous ammonium persulfate solution (10% w/v) were used to induce polymerisation. The running buffer was 89 mM Tris-borate containing 2 mM EDTA, and in the case of 4% polyacrylamide gels, electrophoresis was typically performed for 3–4 h at 8 °C allowing the voltage to vary up to a maximum of 3500 V at a constant power of 150 W, while for 20% polyacrylamide gels, electrophoresis was performed at 8 °C at a constant power of 75 W until the excess APTS reached the bottom of the gel (approximately 4 h).

Fluorescent imaging of polyacrylamide slab gels.—After electrophoresis, the slab gels were fluorescently imaged in a Molecular Dynamics Fluorimager that collects fluorescent data while scanning the gel with a low intensity argon-ion laser (488 nm). Data were collected in the normal sensitivity mode without the use of a filter and were processed using ImageQuant™ version 4.2 software (Molecular Dynamics, Sunnyvale, CA) that enabled the quantification of observed fluorescent bands. The instrument provided highly reproducible results that showed a linear response with increasing substrate loading over a wide dynamic range (results not shown).

Electrophoresis of APTS-labelled debranched wheat starch using a DNA sequencer.—Details of the separation of APTS labelled oligosaccharides using an Applied Biosystems 373A DNA sequencer (Perkin–Elmer Corporation, Applied Biosystems Division, Foster City, CA) have previously been reported [10]. In the case of the more recent 377

DNA sequencer (Perkin–Elmer Corporation, Applied Biosystems Division, Foster City, CA), the APTS labelling reaction mixture described above was further diluted with an electrophoresis sample buffer consisting of 6 M urea in 0.04 M boric acid and 0.04 M tris(hydroxymethyl)aminomethane (pH 8.6) by a ratio of 1:100 and a 1-μL aliquot loaded into the wells of uniform 10% polyacrylamide gels (37.5:1 ratio of acrylamide to *N*,*N'*-methylenebisacrylamide as cross-linker) containing 8.3 M urea. Electrophoresis was conducted using a buffer containing 0.089 M Tris base, 0.089 M boric acid and 0.002 M EDTA for a period of 5 h at 51 °C at a voltage of 0.55 kV (current 25.6 mA). Fluorescence data were collected and analysed using Genescan® software (version 2.1).

Capillary electrophoresis of APTS-labelled debranched wheat starch.—Separation was performed on a P/ACE 5510 instrument with the cathode on the injection side (reversed polarity) and monitored with a laser-induced fluorescence detector (Beckman Instruments, Fullerton, CA) with an argon-ion laser as the excitation source. The capillary used was a 50 μm diameter eCAP™ neutral coated capillary (obtained from the eCAP™ N-linked oligosaccharide profiling kit (Beckman Instruments, Fullerton, CA) along with the carbohydrate separation gel buffer) cut to a length of 47 cm (40 cm to the detector), and the sample was introduced by pressure injection of the diluted reductive amination reaction mixture, typically 2–5 s at 0.5 psi. The separating medium consisted of carbohydrate separation gel buffer, and separation was achieved using an applied voltage of 23.5 kV (current 14 mA) at a temperature of 25 °C.

3. Results

Optimisation of labelling procedure.—The labelling efficiency of APTS reductive amination was determined on the radiolabelled substrates using an ion-exchange adsorbtion method. The calculation of labelling efficiency was made by determining the percentage of the applied glucan that remained unbound as a function of the amount of glucan applied to the resin. Both free APTS and APTS-labelled glucan bound to available anion-exchange resins, suggesting that binding occurs through a combination of the strong anion-exchange binding mediated through the free sulfonic acid groups and the steric interactions of the planar dye with the support resins. Control experiments using radiolabelled glucans demonstrated that less than 2% of unlabelled (with APTS) glucan bound to the anion-exchange resins used over the range from dp 1 to dp 135. This method has the advantage that absolute measurements of labelling efficiency are obtained as opposed to the relative labelling efficiencies obtained in previous reports [10,18]. With respect to optimising the reductive amination procedure, previous work has defined the critical parameters to be APTS concentration, temperature and the pK_a of the acid catalyst [20,22,23]. For the present work, these parameters were revisited using the resin adsorbtion method outlined above, with radiolabelled glucose as substrate. The optimum reaction conditions (see Experimental) were confirmed to be identical to those outlined in a previous report [10]. Attempts to reproduce claims that significantly higher derivatisation yields could be obtained by using organic acid catalysts with a pK_a higher than acetic acid [22,23] failed to produce any significant yield enhancement.

Effect of chain length on labelling efficiency.—The objective of this research was to examine whether reductive amination labelling efficiency was independent of chain length over the range of maltooligosaccharides present in a typical debranched amylopectin. However, maltooligosaccharides of defined dp were not commercially available over this range (dp 3 to 100). [U^{14}C]-labelled glucose and maltose are commercially available, and efficiencies of labelling were determined using the radiolabelling procedure (see Experimental). The results are shown in Table 1. In the case of glucose, this result duplicates the findings of other groups who have previously shown that for sterically unencumbered monosaccharides, the labelling efficiency of reductive amination is virtually quantitative [5,20].

While maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose were available commercially as discrete oligomers, they are not available in [^{14}C]-labelled form, and a fluorimager protocol (see Experimental) was used to compare the relative efficiencies of labelling of an equimolar maltotriose to maltoheptaose series to the absolute efficiency determined for [^{14}C]-maltose (Table 1). As discrete oligomers longer than dp 7 were not commercially available, narrow distributions of [^{14}C]-labelled linear glucans of differing average chain lengths were generated by phosphorylase-A catalysed extension of either dp 4

Table 1
APTS labelling efficiency of malto-oligosaccharides

ACL of product	dp of primer	Synthesis reaction time (min)	Labelling efficiency (%)	Relative standard deviation[f]
1[a]			95.0[d]	0.8 (5)
2[a]			88.0[d]	1.6 (5)
3[b]			80.7[e]	1.6 (4)
4[b]			77.7[e]	0.9 (4)
5[b]			88.4[e]	2.1 (4)
6[b]			83.0[e]	1.7 (4)
7[b]			89.6[e]	0.9 (4)
10[c]	4	20	82.2[d]	3.5 (4)
20[c]	7	40	82.2[d]	4.1 (5)
31[c]	7	80	79.1[d]	3.4 (4)
41[c]	7	120	78.2[d]	7.7 (4)
53[c]	7	160	77.7[d]	1.8 (4)
77[c]	7	240	77.0[d]	2.0 (5)
135[c]	7	1440	80.7[d]	1.0 (3)

[a] [U^{14}C]-labelled glucose and maltose were obtained commercially.

[b] Maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose were obtained commercially.

[c] The modal dp 10 and dp 135 distributions were products of specific phosphorylase A extension reactions, while the remainder were produced by sampling a single elongation reaction of maltoheptaose.

[d] Measured by anion-exchange absorbtion of radiolabelled oligosaccharides.

[e] Estimated relative to the labelling efficiency of maltose by comparing relative fluorescent intensities.

[f] Values in parentheses indicate the number of replicates used for statistical analysis.

or dp 7 primers (see Experimental). The radiolabelling procedure for the determination of labelling efficiency was employed and the results are summarised in Table 1. An analysis of variance showed there was no significant difference in labelling efficiency over the range of dp 3 to dp 135, with the exception of dp 5 and dp 7. This may be a result of the indirect method for determining these figures. The average labelling efficiency was determined using the absolute values obtained for the glucans between dp 10 and dp 135, and it was found to be 80.0%.

Reproducibility of labelling and detection.—The issue of labelling reproducibility was addressed by comparison of a number of separate labelling experiments of debranched wheat starch, and the equimolar dp 2 to dp 7 mixture. First, the reproducibility of sample loading and fluorimager detection procedure was examined by analysing replicate samples from common labelling reactions. Comparison of the total fluorescent intensity across the entire distribution demonstrated that the technique has a relative standard deviation of loading and detection of only 5.46% and 6.76% for the dp 2–7 and debranched wheat starch samples respectively (detection experiments in Table 2). In further experiments, replicate labelling reactions were analysed in order to assess the total variation across an experiment (electropherogram not shown), with the relative standard deviation across the total fluorescent measurements similar to that of loading and detection determined above (labelling experiments in Table 2), indicating the reproducibility of the reductive amination procedure to be very high. Within these distributions of maltooligosaccharides, the quantification of discrete oligosaccharides was proved highly reproducible irrespective of the substrate used (Table 2).

Slab gel electrophoretic methods for the high-resolution analysis of APTS-labelled oligosaccharides.—We have previously reported a method for the chain length analysis of APTS-labelled oligosaccharides using a DNA sequencer to resolve and detect individual oligosaccharides over the range from dp 3 to dp 80 [19]. The DNA sequencer format is restrictive in the choice of variables such as gel composition, running time and well-to-read distance, and an alternative, more flexible system was developed in order to bypass these limitations. In this approach, a manual DNA sequencing apparatus was used to electrophorese samples through thin polyacrylamide slab gels. The gels incorporated high concentrations of urea, thereby providing denaturing conditions to reduce the effects of secondary structure on electrophoretic mobility. This method retains high-resolution slab gel electrophoresis as the core ingredient, but no longer relies on a DNA sequencer to provide a fixed laser induced fluorescence (LIF) detection system. Detection of the fluorescent oligosaccharides on the gel was accomplished using a Molecular Dynamics Fluorimager, in which the entire gel was scanned at 488 nm by an argon-ion laser source, and the resultant fluorescent emissions were captured and digitally recorded.

Analysis of APTS-labelled oligosaccharides of dp <20.—Electrophoresis of mixtures of linear maltooligosaccharides derived from debranched glycogen and wheat starch on 20% polyacrylamide gels showed that high-resolution separations of maltooligosaccharides could be obtained up to approximately dp 20 (Fig. 2). This resolution would prove sufficient for applications involving relatively short oligosaccharide distributions, such

Table 2
Fluorescent data as a measure of the reproducibility of detection and labelling efficiencies for reductive amination with APTS

Substrate	Experiment	Parameter measured	Average fluorescence units ($\times 10^6$)	Relative standard deviation [a]
dp 2–7	Detection [b]	Σ2–7 [d]	156.9	5.46 (4)
	Labelling [c]	Σ2–7 [d]	157.9	5.30 (4)
	Labelling	dp 2 [f]	27.4	5.53 (4)
	Labelling	dp 3 [f]	25.1	5.21 (4)
	Labelling	dp 4 [f]	24.2	4.68 (4)
	Labelling	dp 5 [f]	27.5	5.24 (4)
	Labelling	dp 6 [f]	25.8	6.00 (4)
	Labelling	dp 7 [f]	27.9	5.44 (4)
Debranched wheat starch	Detection	Σ6–30 [e]	58.0	6.76 (4)
	Labelling	Σ6–30 [e]	56.1	5.45 (4)
	Labelling	dp 7 [f]	1.8	7.01 (4)
	Labelling	dp 12 [f]	3.6	3.00 (4)
	Labelling	dp 21 [f]	0.6	6.30 (4)
Average relative standard deviation across all labelling experiments				5.38

[a] Values in parentheses indicate the number of replicates used for statistical analysis.
[b] Multiple loadings of an identical volume from a single labelling reaction.
[c] Identical volumes of replicate labelling reactions loaded.
[d] Sum of the fluorescence over the region containing dp 2 to dp 7 in a lane loaded with a labelling reaction containing equimolar amounts of dp 2 to dp 7.
[e] Sum of the fluorescence over the region containing dp 6 to dp 30 in a lane loaded with APTS-labelled debranched wheat starch.
[f] Fluorescence measurement for an individual band.

as those derived from debranched glycogen where very little material exists beyond dp 20 [10,19], or from glycoconjugates for example; however, a higher resolving technique is required for the analysis of samples such as those from debranched amylopectins.

Analysis of APTS-labelled oligosaccharides of dp >20.—By decreasing the concentration of the polyacrylamide gel to 4%, considerably longer oligosaccharides could be electrophoresed, albeit at the expense of lower resolution. Fig. 3 shows the gel image obtained upon electrophoresis of the APTS-labelled synthetic amylose distributions. Although the decrease in resolution does not allow individual oligomers to be distinguished other than for the very short oligomers at the top of the diagram, the separation is useful in allowing comparisons of chain length profiles that are based on a molar, rather than a mass basis. One advantage of this electrophoretic format is that longer distributions can be visualised than can be analysed via the DNA sequencer method (practical limit about dp 80), because the polysaccharide does not have to migrate through the length of the gel to a fixed point detector, but can be visualised if it enters the gel. A second advantage over the DNA sequencer technique is an electrophoresis time of only 3–4 h as opposed to 15 h.

Analysis of APTS-labelled pullulans.—Attempts to extend the scope of this methodology to the electrophoresis of polysaccharides were based on commercially available pullulan standards which are commonly used as molecular weight markers for high-performance size-exclusion chromatography. The pullulans possess a reducing end amenable to fluorescent labelling by reductive amination and a high degree of water solubility, thereby eliminating many of the solubility problems encountered with the analysis of amylose or amylose-like substrates [24]. The molecular structure of pullulan consists of repeating units of maltotriose linked to one another by α-(1→6) bonds, thereby creating a kinked rather than linear structure (Fig. 4). It was anticipated that the electrophoretic behaviour of the pullulan standards would reasonably approximate those of similarly sized linear amyloses, providing useful standards for method development and optimisation.

Partial resolution was obtained for a wide range of pullulan standards (dp 70–1300) under the same electrophoretic conditions described for the labelled synthetic amyloses (Fig. 4). Since all of the pullulan standards can be simultaneously observed on a single gel after an electrophoresis time of only 3–4 h, the method enjoys significant throughput benefits with respect to size-exclusion

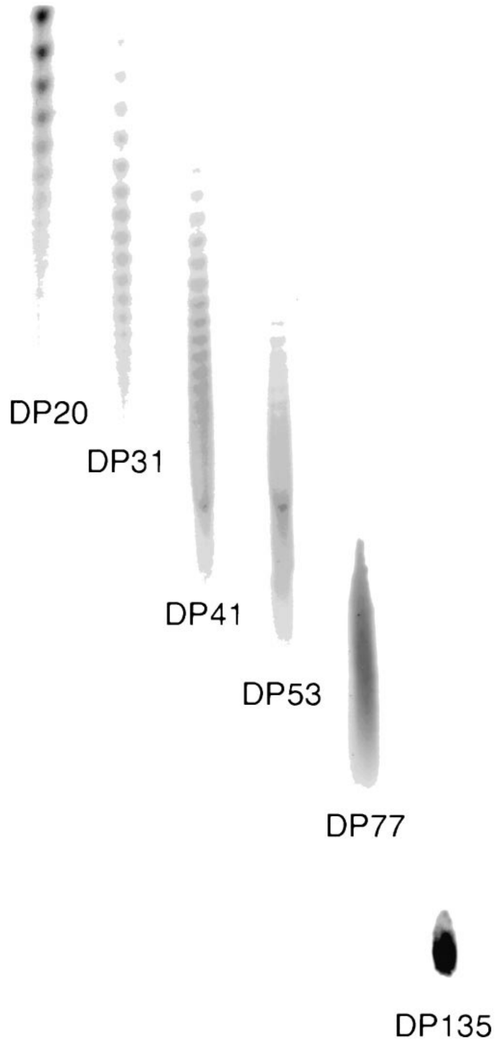

Fig. 3. Gel image depicting the separation of APTS labelled synthetic amyloses on a 4% polyacrylamide gel in 1×TBE buffer electrophoresed for 4 h at 8 °C while allowing the voltage to vary up to a maximum of 3500 V at a constant power of 150 W. The labels indicate the dp of the mid-point of the distribution by DNA sequencer mediated slab gel electrophoresis.

HPLC techniques. In addition, the stoichiometry of the APTS-labelling reaction permits quantitation of substrates on a molar basis. If the degree of polymerisation can be established, quantitation on a mass basis is also possible. This contrasts with most size-exclusion HPLC techniques that require the use of pullulan molecular weight standards to calibrate mass based detection systems in order to derive a molar result [25,26]. The separation efficiency and data generation of such techniques remains inferior to that described here, where the resolution is sufficient to observe discrete bands for the lower molecular weight pullulan structures differing by maltotriose increments.

Analysis of APTS-labelled oligosaccharides derived from wheat starch by capillary electrophoresis and DNA sequencer-mediated electrophoresis.— Capillary gel electrophoresis was investigated as an alternative separatory technique for debranched amylopectins, and the results demonstrated that high levels of resolution and reproducibility are obtained (Fig. 5). The resolution is at least comparable to that described for the DNA sequencer-mediated separation [10,19], and experiments on replicate electropherograms indicate that a higher degree of reproducibility of quantitative data is obtained (Table 3). The replicate analysis was also conducted on a more recent DNA sequencer than that on which the original data was obtained [10,19]. The newer instrument employs thinner gels (0.2 mm compared to 0.5 mm) and possesses greater resolving power. Analysis of the relative standard deviations obtained across a variety of ranges of debranched amylopectin chain lengths indicates that capillary gel electrophoresis possesses enhanced levels of reproducibility compared to both DNA sequencers, while as expected, the newer model DNA sequencer produces a slight improvement on the superceded one.

4. Discussion

The reductive amination of monosaccharides and short oligosaccharides with charged fluorophores have found widespread utility in the analysis of glycoconjugates and dextrins [2–6,14–19,27]. The method provides highly sensitive detection of the conjugate and, for short oligosaccharides, facile separation in polyacrylamide gels [3–5,14,28]. Several studies have compared relative labelling efficiencies for a range of target molecules and labelling conditions [4,5,10,18,19]. Our interest in this methodology is to facilitate the analysis of starch structure by enabling the comparison of complex distributions that are generated from starches by enzymatic digestions. In order for the reductive amination procedure to be useful for this purpose, labelling efficiencies must be either constant with increasing chain length, or must reproducibly change with chain length such that simple normalisation of the data can be performed. In this study, we have used radiolabelled glucose, maltose and oligosaccharides to determine absolute labelling efficiencies. We have shown that glucose is effectively quantitatively labelled (labelling efficiency

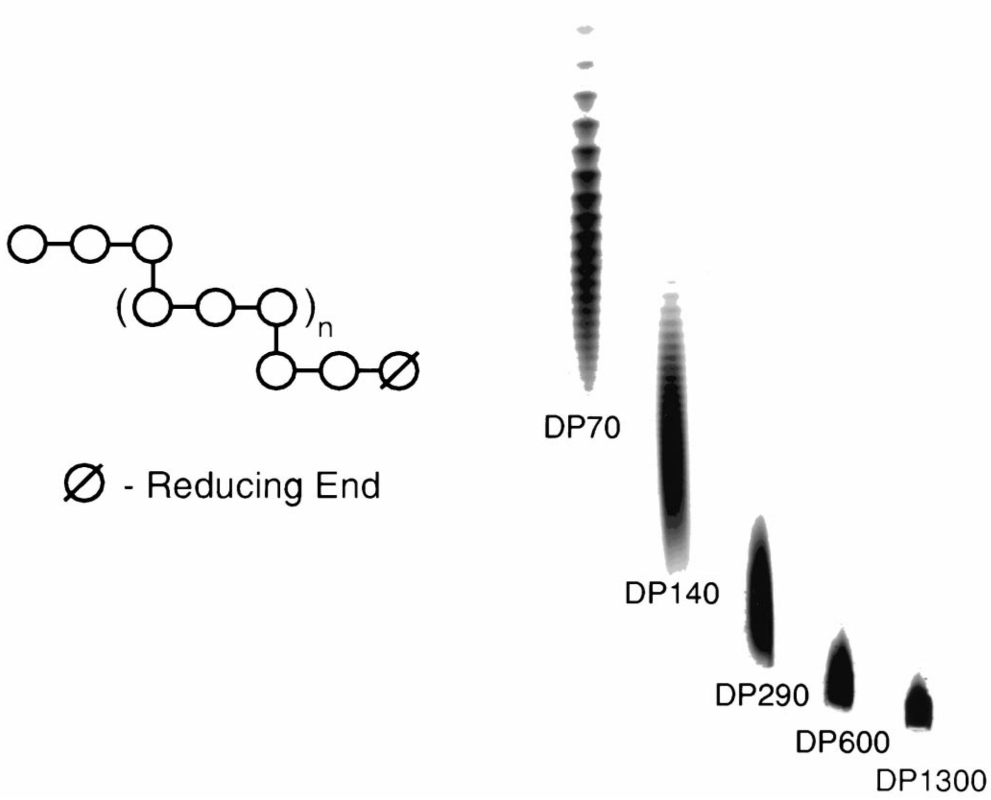

Fig. 4. The structure of pullulan and an electropherogram depicting the separation of APTS labelled pullulan molecular weight standard on a 4% polyacrylamide gel in 1×TBE buffer electrophoresed for 4 h at 8 °C and a constant power of 75 W. The dp shown is that calculated from the average molecular weight supplied for each pullulan standard.

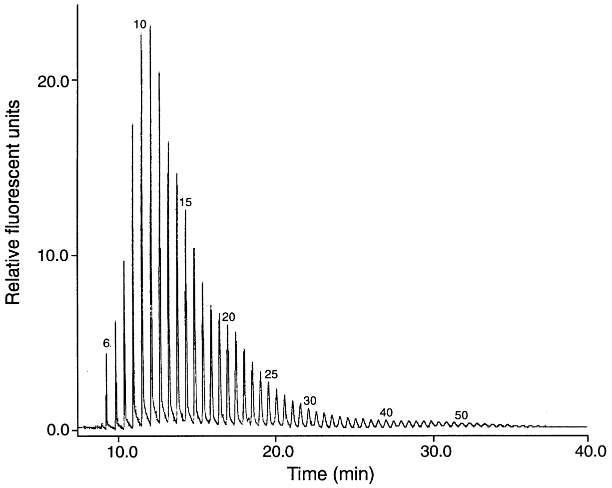

Fig. 5. Electropherogram obtained from capillary electrophoresis separation of isoamylase debranched wheat starch. The numbers indicate the degree of polymerisation. Separation conditions are contained in the experimental section.

Table 3
Comparison of average relative standard deviations across various chain length ranges for the mass responses obtained from various electrophoretic separations of APTS-labelled debranched wheat amylopectin

Method	Average relative standard deviation (n)[a]		
	dp 6–20[b]	dp 6–35[b]	dp 6–50[b]
373A sequencer	1.68 (9)	5.04 (9)	8.33 (9)
377 sequencer	1.48 (6)	3.61 (6)	5.94 (6)
CE	0.47 (3)	0.97 (3)	1.53 (3)

[a] Average relative standard deviation of the individual oligomers across the designated chain length range determined from the indicated number of replicate experiments.

[b] The ranges of dp 6–20, dp 6–35 and dp 6–50 contain approximately 59%, approximately 91% and approximately 98% of wheat amylopectin by mass, respectively, as determined by analysis of chain length profiles obtained from DNA sequencer-mediated electrophoresis and capillary gel electrophoresis.

> 95%), maltose is labelled slightly less efficiently (88%), and average chain lengths from dp 3 to dp 135 are labelled with an average efficiency of 80%. The chain length range over which labelling efficiency is constant encompasses the range of chain lengths found in a typical debranched amylopectin and, therefore, confirms the reductive amination procedure as a valuable method for the quantitative comparison of amylopectin chain length distributions.

While we have not investigated longer average chain lengths than dp 135, there is clearly no measurable drop off in labelling efficiency which would be predicted to preclude the use of reductive amination for the quantitative comparison of, for example, those oligosaccharides typically found in the amylose fraction of starch. However, this remains to be proven. Factors such as the solubility of the glucan in the acidic labelling reaction conditions may reduce the labelling efficiency at these chain lengths.

In this study we have also examined the reproducibility of reductive amination and quantification of the fluorescence intensity using a laser gel scanning system for visualising polyacrylamide gels. The efficiency of labelling was found to be highly reproducible, with an average relative standard deviation of all labelling experiments of 5.38% (Table 2). The reproducibility of labelling was maintained over the entire chain length distribution. In an analogous fashion, preliminary analysis using a capillary electrophoretic system with laser-induced fluorescence detection supported these results and achieved a very high level of reproducibility of oligomeric quantification over the entire profile (illustrated for dp 6–26 in Table 3). These results further confirm the utility of reductive amination derivatisation to permit quantitative comparisons of maltooligosaccharide distributions.

The slab gel electrophoretic methods we have employed for resolving chain length distributions can be chosen to suit the analysis of distributions of differing average chain lengths. Polyacrylamide gels (20% w/v) containing 8 M urea were used to provide baseline resolution of oligosaccharides from glucose to dp 14. Further discrete oligomers can be observed out to approximately dp 30, although baseline resolution is no longer maintained (Fig. 2). Imaging of these gels using laser excitation and detection provides a highly accurate and reproducible method of quantifying oligosaccharides within this size range. The flexibility of this format allows longer oligosaccharides to be resolved using polyacrylamide gels of 4% as highlighted by the separations of the synthetic glucans (Fig. 3). However, the decreased resolution obtained limits the observation of discrete oligomers beyond approximately dp 40, and provides only broad profiles beyond this range. Despite this, satisfactory resolution of pullulan molecular weight standards was achieved as an illustration of the potential extension of such a technique to the analysis of polysaccharides.

For the generation of quantitative data on individual oligomers, much higher resolution is required. Excellent resolution is obtained using either DNA sequencer-mediated slab gel electrophoresis [10,19] or capillary gel electrophoresis (Fig. 5) and permits the quantitation of oligomers up to limits of dp 80 [19] and dp 100, respectively (results not shown), thereby encompassing the range of oligosaccharide chain lengths found in the debranched amylopectin fraction of starches. Stefansson and Novotny [29] have previously reported the separation of 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) derivatised oligosaccharides from maize and potato amylopectin up to dp 70, but made no comment on the reproducibility of results.

In an attempt to define the reproducibility of these high-resolving methods, replicate analyses of APTS-labelled debranched wheat amylopectin were conducted, and the data were analysed across a variety of chain lengths on the basis of average relative standard deviation of the normalised mass response per oligomer (Fig. 5). While reproducibility for all instruments decreases with increasing chain length, presumably this is due to the concomitant decrease in response with chain length above dp 13. An analysis of chain lengths within the range of dp 6–20 will yield adequate results for many applications using any of the techniques. However, the capillary electrophoretic method is clearly superior when chain lengths of dp >20 are included in the analysis.

Table 3 shows the relative proportions of amylopectin mass contained within various chain-length ranges. While the DNA sequencers are capable of accurate analysis over the range dp 6–20, such as an analysis for amylopectin would be based on just 59% of the material. Clearly, such an analysis should be based on as wide a range as possible, to include the greatest proportion of amylopectin mass. For an analysis involving 98% of amylopectin mass, only the CE method can deliver an acceptable level of reproducibility across the range of chain lengths within dp 6–50.

A key advantage of the reductive amination technique is that a single fluorophore is attached per reducing end. In this study, we have shown that the efficiency of labelling of the reducing end does not alter significantly over the range from dp 3 to dp 135. In contrast, the response of pulsed amperometric detectors (PAD) does not remain constant or known over such a range. An analysis of amylopectin chain-length distributions has been reported using a high-performance anion-exchange chromatography system with pulsed amperometric detection (HPAEC–PAD) [30], but because response factors are known only up to dp 17 for maltooligosaccharides [31], and detector response increases steadily with increasing oligomeric chain length, molar quantitation of the results is not possible. To circumvent this problem, Wong and Jane [32] have recently introduced the use of a novel post-column enzymatic detector system to convert maltooligosaccharide peaks eluting from a high-performance anion-exchange system to glucose. This system also allows quantification of the data on an absolute basis, which is essential if significant differences in chain-length distribution are to be detected. The fact that similar results were obtained from both HPAEC methods mentioned above and the DNA sequencer-mediated electrophoresis of APTS labelled material [19], the PAD response factors may conceivably show only minor variation with increasing oligomeric chain length.

While we have not in this report analysed oligosaccharides other than linear α-(1→4) glucans or polysaccharides other than pullulan, reductive amination using charged fluorophores has been used widely as a means of profiling other oligosaccharides [17,18,27,33,34]. Quantification of the dependence of labelling efficiency on chain length for other oligosaccharides will be required to establish that the technique can be used to accurately determine the contributions of individual species to an overall chain length distribution. For short oligosaccharides (dp 1 to dp 3), our data suggest that significant differences in labelling efficiency may occur, a conclusion also reached by Klockow [18] for the reductive amination of maltooligosaccharides with ANTS. For longer oligosaccharides and polysaccharides, the stability and solubility of target polysaccharides in the labelling reaction, are likely to influence the labelling efficiency.

5. Conclusions

The profiling of oligo- and polysaccharides using electrophoretic separations driven by the charge and fluorescence conferred by labelling via reduction amination is providing an important advance in the ability of biologists and chemists to analyse complex carbohydrates. In this paper, we have demonstrated that for linear maltooligosaccharides, labelling is highly reproducible and is independent of average chain length between dp 3 and dp 135, with an average labelling efficiency of 80%. Glucose (95% efficiency) and maltose (88% efficiency) are more efficiently labelled. While caution should be exercised in extrapolating these results to polysaccharides of higher dp, or to polysaccharides composed of different monomeric units or linkages, these results suggest that labelling efficiency is unlikely to be highly sensitive to the degree of polymerisation for carbohydrates. In this paper, we have also demonstrated two systems offering high resolution of oligosaccharide mixtures while providing highly reproducible results.

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