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Capillary electrophoretic examination of underivatized oligosaccharide mixtures released from immunoglobulin G antibodies and CTLA4Ig fusion protein

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Abstract

A procedure is presented for the separation of underivatized oligosaccharides by capillary electrophoresis (CE) with a phytic acid–borate buffer system. The presence of the phytic acid ion-pairing agent greatly increases resolution between oligosaccharides in the complex mixtures studied, which was demonstrated by the separation of oligosaccharides originating from various immunoglobulin G antibodies and CTLA4Ig, a biologic fusion protein. The conditions also resolve neutral oligosaccharides, usually a major CE limitation. High-performance anion-exchange chromatography with pulsed amperometric detection, a standard technique for oligosaccharide and sugar analysis, is used as a reference method to analyze some of the complex oligosaccharide mixtures.

Keywords: Oligosaccharides; Saccharides; Antibodies; Proteins

1. Introduction

Oligosaccharides are polymers comprised of typically 2–10 units. They are often found in nature associated with proteins through O-glycosidic or N-glycosidic bonds or associated with lipids through an O-glycosidic bond. Destruction of the glycosidic bonds yields a mixture of oligosaccharides. The resulting chemically heterogeneous sample presents a variety of analytical challenges, both from a separatory and detection point of view. Several methods have commonly been used to examine oligosaccharide mixtures, including ion-exchange chromatography with pulsed amperometric detection, derivatization followed by gas chromatographic analysis, and liquid chromatography with low-wavelength detection [1].

The examination of oligosaccharides cleaved from proteins for the purpose of establishing the suitability and reproducibility of the cell culture conditions for glycoprotein production was presented in a previous paper [2]. Although the previous procedure had the advantage of using only a few nanoliters of sample and an efficient analysis time of six minutes, the resolution limited the amount of analytical data obtainable, although sufficient for control (lot-to-lot

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variation) purposes. When the cost of commercial oligosaccharides and difficulties in acquiring even small volumes of oligosaccharides from biologic compounds is considered, the development of a capillary electrophoresis (CE) procedure (with nanoliter sample consumption) becomes a particularly worthwhile analytical endeavor.

In this investigation, CE was evaluated as a possible complimentary procedure to the conventional ion-exchange (high-performance anion-exchange chromatography–pulsed amperometric detection, HPAEC–PAD) procedure in the evaluation of oligosaccharide profiles. This paper presents high resolution procedures for the examination of underivatized complex oligosaccharide mixtures using CE with borate buffers modified with ion-pairing agents. The development of a procedure for the separation and detection of underivatized oligosaccharides is often preferential to derivatized protocols when the number and structure of analyte species is unknown. Stated simply, one is never certain that all species present in an uncharacterized oligosaccharide mixture have been derivatized and hence have been detected. Derivatized oligosaccharide protocols, however, usually offer greater sensitivity and to the extent that the resulting instrumental response is independent of the species derivatized, greater compositional quantitation. The CE results presented here are compared with HPAEC–PAD separations on the same molecules. The oligosaccharide mixtures were derived from a combination of standards, immunoglobulin G (IgG) antibodies, and an immunosuppressant fusion protein produced via recombinant techniques. It will be shown that relatively high selectivity in CE is achieved by addition of phytic acid (Fig. 1) to a borate buffer, thus offering a complementary analytical technique to HPAEC–PAD in many applications. In addition, the buffer system is successful in separating neutral oligosaccharides, a long-standing problem in CE analysis of complex mixtures.

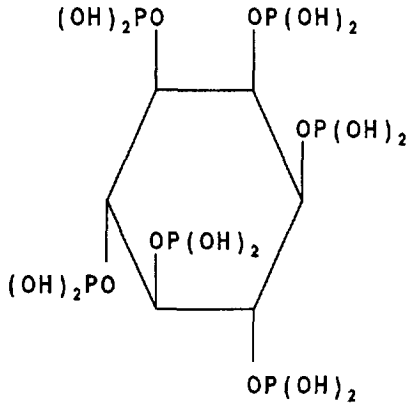

Fig. 1. The structure of phytic acid.

2. Experimental

2.1. Materials

Sodium borate crystals, 1-heptanesulfonic acid, and phytic acid (indostol hexanephosphoric acid) were obtained from Sigma (St. Louis, MO, USA). 1-Hexanesulfonic acid was purchased from Eastman Kodak (Rochester, NY, USA), and 1-octanesulfonic acid and 1-pentanesulfonic acid were from Aldrich (Milwaukee, WI, USA). Sodium hydroxide (10 *M*) was obtained from Fisher Chemical (Fair Lawn, NJ, USA). 2′-Fucosyllactose (2′-FL, Cat. No. AD-02001), LS-tetrasaccharide a (LSTa, Cat. No. AD-01017), 3′-sialyllactose (3′-SL, Cat. No. AD-02012), and disialyltetraose (DST, Cat. No. AD-1022), human milk- and urine-derived O-linked oligosaccharides, were obtained from Oxford GlycoSystems (Abingdon, UK), see Table 1. Additionally, the following oligosaccharide mixtures were obtained from Oxford GlycoSystems: (1) complex mixture A containing asialo-galactosylated biantennary (NA2); asialo-, galactosylated biantennary, core-substituted with fucose (NA2F); asialo-, galactosylated biantennary with bisecting N-acetyl D-glucosamine (NA2B); asialo-, galactosylated biantennary, core-substituted with fucose and bisecting N-acetyl D-glucosamine (NA2FB), Cat. No. RP2501; (2) complex mixture B containing asialo, agalacto-, biantennary (NGA2); asilo-, agalacto-, biantennary, core substituted with fucose (NGA2F); asialo-, agalacto-, biantennary (NGA2B); and asialo-, agalacto-, biantennary, core-substituted with fucose and with bisecting N-acetyl D-glucosamine (NGA2FB), Cat. No. RP2502; (3) hydrazinolysis-released oligosaccharides from

Table 1
Neutral and sialylated O-glycosidic linked oligosaccharides derived from human milk and urine

Species Number	Name	Abbreviation	Structure	Sialylation
1	2'-fucosyllactose	2'-FL	Fucα1-2Galβ1-4Glc	none
2	LS-tetrasaccharide a	LSTa	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 3 | NeuNAcα2	monosialylated
3	3'-sialyllactose	3'-SL	NeuNAcα2-3Galβ1-4Glc	monosialylated
4	disialyltetraose	DST	NeuNAcα2-3Galβ1-3GalNAc 6 | NeuNAcα2	disialylated

mouse, rat, sheep, and human IgG glycoproteins (Cat. Nos. LB-004, LB-006, LB-007, and LB-005).

Water from a Milli-Q filtration system (Waters Assoc., Milford, MA, USA, 18 MΩ) was used in the preparation of the buffer and sample solutions.

2.2. CE methods and procedures

A Beckman P/ACE 2100 CE system (Palo Alto, CA, USA) controlled by an IBM PS/2 with either P/ACE software and a Microsoft Windows interface or Beckman System Gold software was used throughout this study. Pretreated fused-silica capillaries [75 μm I.D., 50 cm effective length measured to the optical window, with a total length of 57 cm, Beckman Instruments (Palo Alto, CA, USA)] were used for all CE experiments. The electropherograms were monitored at 200 nm with a data collection rate of 10 Hz. The capillary cartridge temperature was maintained at 35°C. Samples were injected in the low-pressure mode for 5 s. Electropherograms were redrawn using Microsoft Excel. All buffers were filtered prior to use through a 0.45-μm filter.

HPAEC–PAD measurements were made with a Dionex PAD-II, (PAD with gold working electrodes) with a Dionex Advanced Gradient Pump and a Dionex CarboPac PA-1, 250×4 mm column (Dionex, Sunnyvale, CA, USA). The mobile phase was a ternary gradient which yielded a 60-min total run time, and the analysis was performed at ambient temperature. Integration was accomplished with a Dionex Advanced Computer Interface (ACI) with AI-450 software.

3. Results and discussion

The purpose of this study was to evaluate CE as a possible complementary procedure to the more established ion-exchange (HPAEC–PAD) method in the assessment of the purity of oligosaccharide mixtures. The method optimization studies were performed on a general borate-additive system to attempt to identify a single method which would possess sufficient specificity with standard oligosaccharides to warrant analysis of biotechnologically produced samples. Borate buffers for CE have commonly been used in sugar separation studies [4,5], and it is assumed that borate–sugar complexes are formed that increase the net charge and hence ionic mobility of the species formed. These complexes absorb at 190–200 nm more readily than sugars unassociated with borate

thus yielding a more sensitive assay [3]. In our original work, two CE procedures were developed for the separation of sialic acid containing species: a running buffer containing 52 m*M* sodium borate, 1 *M* TMAPS (trimethylammonium propanesulfonate), pH 9.35 at 35°C for the separation of neutral species or a 35 m*M* sodium borate, 130 m*M* boric acid, pH 8.35 buffer at 35°C [2]. That work concluded that the separation between neutral species is enhanced by increased borate concentrations whereas sialylated species are not separated as well at higher borate concentrations. The relative abundance of neutral and sialylated species for the complex sample therefore dictates different optimal separation conditions.

To generalize the separation of oligosaccharide mixtures, a variety of additives were tried individually: ion-pairing agents (e.g. TMAPS, phytic acid or hexanesulfonic acid), sodium dodecyl sulfate (SDS), as well as a combination of additives. The rational for using ion-pairing agents is to reduce possible interactions with the capillary wall and possibly form complexes with the analytes. For example, phytic acid, improves the resolution of peptides due to the decrease in the electroosmotic flow and also ion-pairing effects with the analytes [6].

Commercially available mixtures of oligosaccharides released from human, sheep, rat, and mouse IgG molecules were first analyzed using HPAEC–PAD and CE. The purity and structural integrity of the oligosaccharides were assessed by HPAEC–PAD and are shown in Fig. 2 for comparison with the CE electropherograms [7]. The structural identify of the individual oligosaccharides was unknown but assumed to be prototypical. Next, CE buffer conditions (pH, borate concentration, and ion-pairing agent concentration) were varied to optimize the separation of the oligosaccharides released from the IgG species. Fig. 3 shows the separation of oligosaccharides released from human IgG using 50 m*M* sodium borate and 15 m*M* phytic acid at various pH values. The two highest pH values (9.4 and 10.2) gave similar results. The separation at pH 9.4 gave better resolution of the early-migrating oligosaccharides, while the separation performed at pH 10.2 resolved more of the later-migrating species. Longer migration times and higher current values resulted at pH = 10.2. Using a similar borate–phytic acid buffer, a standard mixture of four oligosaccharide species

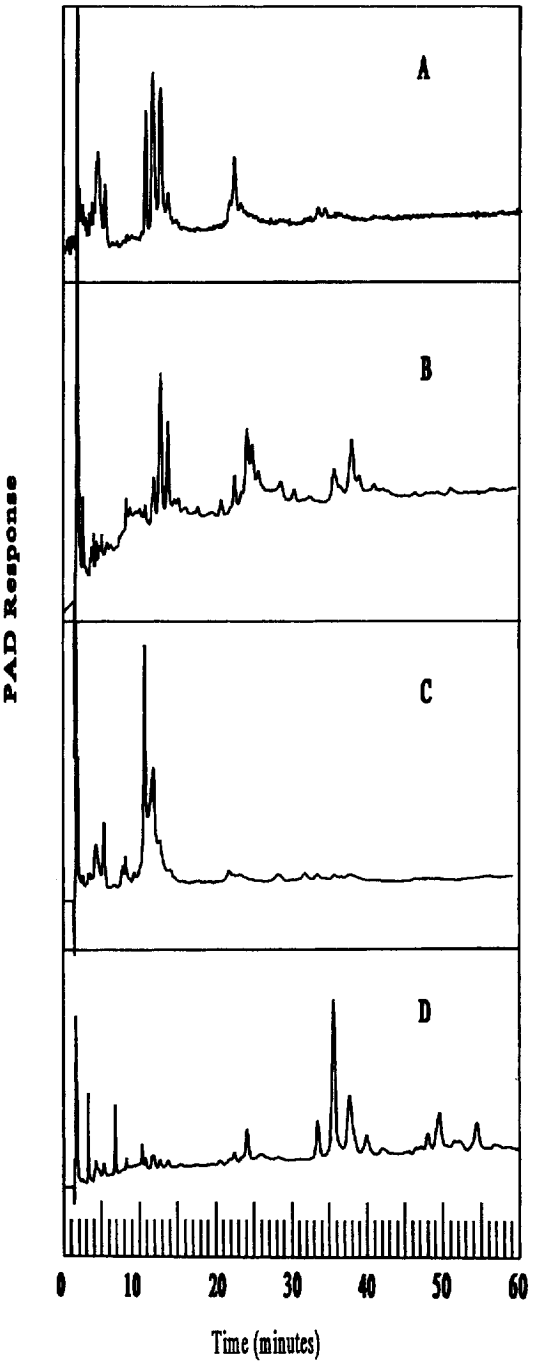

Fig. 2. Separation of various IgG released oligosaccharides by HPAEC–PAD: (A) rat; (B) sheep; (C) mouse; (D) human [7].

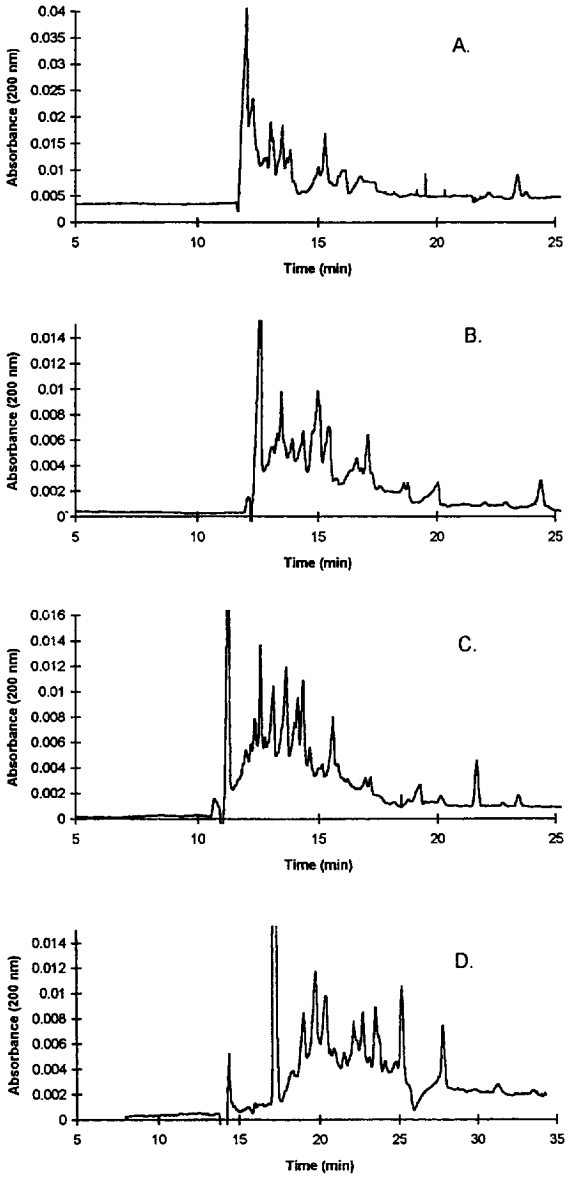

Fig. 3. CE Separation of oligosaccharides released from human IgG at various buffer pH values: (A) pH 8.5; (B) pH 9.1; (C) pH 9.4; (D) pH 10.2. Other conditions: 50 m*M* sodium borate; 15 m*M* phytic acid; a constant current of 130 μA; 35°C capillary temperature.

(2′-FL, LSTa, 3′-SL, and DST) were shown to separate by increasing sialic acid content, i.e., 0, 1, or 2 sialic acids present in the molecule, Fig. 4.

Fig. 5 shows the effect of borate concentration on the separation of the oligosaccharides released from

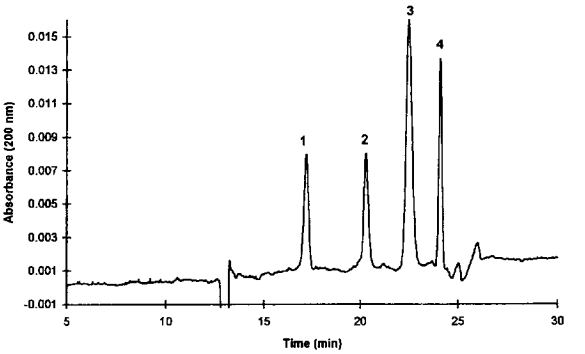

Fig. 4. CE separation of four neutral and sialylated O-glycosidic linked oligosaccharides derived from human milk and urine: (1) 2′-FL; (2) LSTa; (3) 3′-SL; (4) DST. Conditions: 30 m*M* phytic acid; 50 m*M* sodium borate; pH 9.4; constant current 130 μA; 35°C capillary temperature.

human IgG at pH 9.4 using 15 m*M* phytic acid. No attempt was made to determine the precise structure of the oligosaccharides detected. The 50 m*M* sodium borate buffer provided adequate resolution in a reasonable amount of time (under 24 min). Increasing the borate concentration (75 or 100 m*M*) gave similar separation patterns, but more components appeared to be resolved using 100 m*M* sodium borate. At elevated concentrations of borate, the current was substantially higher, but the separation of some of the later migrating species seem to be better resolved vs. the 50 m*M* separation (Fig. 5). These results with the sodium borate concentration are again in agreement with our original findings that the separation between neutral species is enhanced by increased borate concentration where as sialylated species are not as well separated [2].

In an attempt to study the separation of neutral species, two other complex mixtures were examined (complex mixtures A and B). A series of ion-pairing agents (hexane-, heptane-, pentane-, and octanesulfonic acid) were evaluated in conjunction with sodium borate at pH 9.4. Similar profiles were obtained for all of the sulfonic acid buffer systems; however, the current generated in the capillary was not the same for all additives, increasing the time required for some separations. Some loss in resolution was observed with the sulfonic acid ion-pairing agents for complex mixture B (e.g., pentanesulfonic acid) yet maintaining the same general

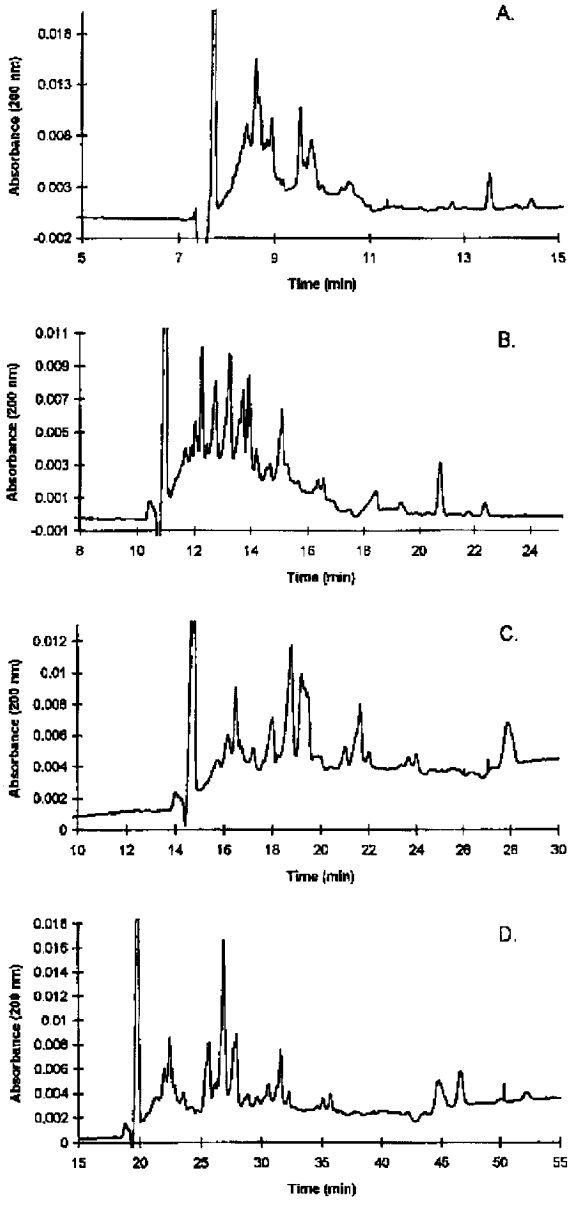

Fig. 5. CE separation of oligosaccharides released from human IgG at different sodium borate concentrations: (A) 25; (B) 50; (C) 75; (D) 100 m*M*. Other conditions: 15 m*M* phytic acid; pH 9.4; a constant current of 130 μA; 35°C capillary temperature.

profile (Fig. 5A) as compared to the phytic acid buffer system. The buffer in our original paper with TMAPS (procedure 1) gave an almost identical separation to the sulfonic acid additives, Fig. 6A. Fig. 5C is the separation of the neutral complex

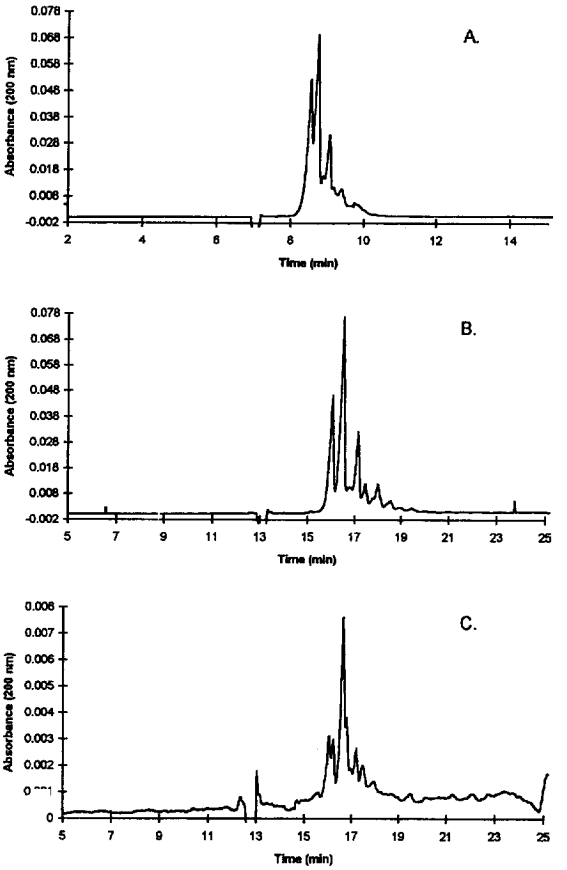

Fig. 6. Separation of neutral N-linked oligosaccharides (complex mixture B (NGA2, NGA2F, NGA2B and NGA2FB) from sheep IgG using (A) 30 m*M* pentane sulfonic acid or (B) 30 m*M* phytic acid. (C) separation of complex mixture A (NA2, NA2F, NA2B and NA2FB) using 30 m*M* phytic acid. All other conditions as in Fig. 4.

mixture A using 30 m*M* phytic acid and 50 m*M* sodium borate at pH 9.4. Other additives were tried to further improve the separation of the oligosaccharides. Addition of sodium cholate (a bile salt detergent), SDS, or a combination of detergents and ion-pairing agents to the borate buffer system did not improve the resolution of the neutral oligosaccharides (data not shown).

Fig. 7 shows the separations of the commercially available mixtures of N-linked neutral oligosaccharides released from human, sheep, rat, and mouse IgG, using the borate–phytic acid buffer. Complex electropherograms were obtained for all of the IgG released oligosaccharide species and show correla-

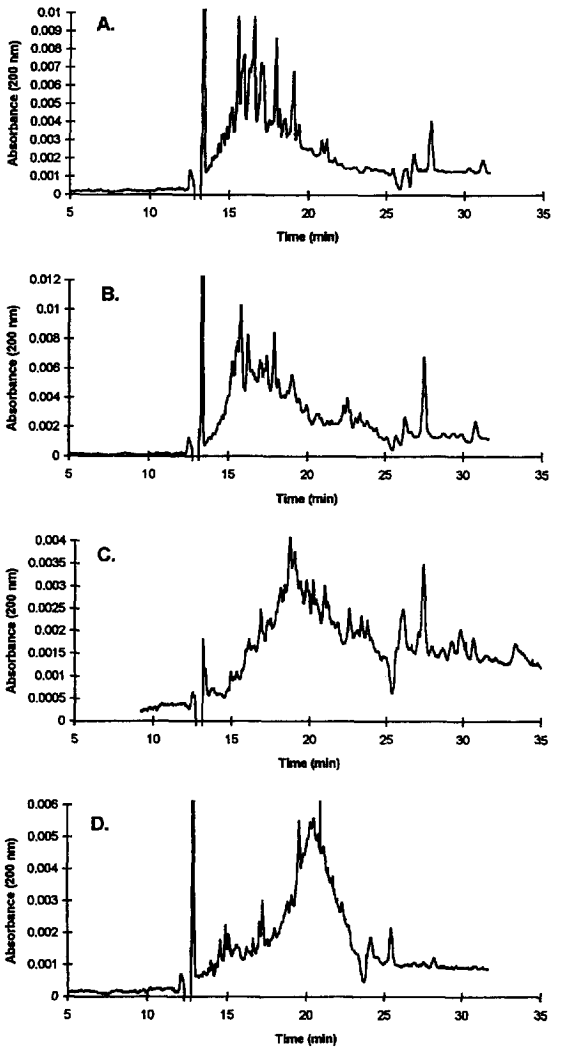

Fig. 7. Separation of various IgG released oligosaccharides by capillary electrophoresis: (A) rat; (B) sheep; (C) mouse; (D) human. Conditions: 30 m*M* phytic acid; 50 m*M* sodium borate; pH 9.4; a constant current of 130 μA; 35°C capillary temperature.

tion to the HPAEC–PAD chromatograms shown in Fig. 2. Maximum resolution was obtained when the current, rather than the potential, was maintained at a constant value. The current value was relatively large (ca. 130 μA) compared to conventional analysis, but typical for carbohydrate separations. It is not apparent why phytic acid contributes to the high resolution of the oligosaccharide mixtures other than by decreasing the electroosmotic flow. Examination of the structure of phytic acid and the oligosaccharide–borate complexes suggests the possibility of particularly selective phytic acid–complex interactions, but this hypothesis was not investigated in the current study.

It is tempting to compare the analytical results presented in Fig. 2 and Fig. 7 to attempt to correlate the oligosaccharide separations. Unfortunately, the mechanisms of CE separation for oligosaccharides are presently not well understood and the interpretation of HPAEC–PAD examinations is often complex. Additional complications include the presence of O-linked species and the relatively large CE time interval that, for example, monosylated species may take to elute or migrate. These cautions having been set forth, it is nonetheless instructive to compare the two procedures in a qualitative fashion. Noting again that oligosaccharides elute in the order of increasing sialylation, certain observations are possible. Arbitrarily assign 17, 21, and 24 min as "typical" or average CE migration times for neutral, mono, and disialylated oligosaccharide families and similarly, 11, 23, and 40 min for the corresponding HPAEC–PAD elution times. Note that within a charged group, the actual peak time varies within several min; for an example of monosialylation variation, see Fig. 4; for neutral species variation, see Fig. 6. If we now examine Fig. 7A, we might describe the oligosaccharide mixture as containing mostly neutral species by CE with very little mono or disialylated species present. This is in agreement with Fig. 2A, analysis of the same sample by HPAEC–PAD. In a similar fashion, CE Fig. 7B depicts multiple neutral species in abundance, with significant and multiple mono and disialylated species. An interesting aspect of Fig. 7C is the presence of multiple, relatively abundant disialylated species; Fig. 7D depicts an almost totally neutral and monosialylated mixture. This informal analysis, and more importantly, the use of CE to compare oligosaccharide mixtures, requires highly reproducible migration times. Other important validation variables, such as the limit of detection of various species are very useful if the identity of the components are known. Reproducibility of migration times and method development variables must, of course, be evaluated for each system under study. Hence, given the current knowledge of the two analytical techniques, the comparison of results

appear to be at least possibly consistent considering the very simplistic model employed.

To demonstrate the utility of the developed CE procedure for the analysis of biotechnologically produced samples, an underivatized oligosaccharide mixture of a recombinant fusion protein, CTLA4Ig, was examined by CE and HPAEC–PAD. CTLA4Ig is a homodimer containing 356 amino acids and has a molecular mass of ca. 92 000. The theoretical primary structural mass is ca. 78 000 with the remainder of the mass contained in carbohydrate attachments, the structure of which are not known at this time. Oligosaccharides released from CTLA4Ig were evaluated under normal and thermally stressed conditions (Fig. 8). The thermally stressed sample was used as a simple measure of specificity, i.e., the degraded sample is expected to present an electropherogram distinctly different than the unstressed sample. For comparison, the HPAEC–PAD chromatogram of an unstressed sample is shown in Fig. 9. Four regions are seen in the HPAEC–PAD chromatogram. A complex pattern is observed in the electropherogram of CTLA4Ig released oligosaccharides. The thermally stressed sample displayed a different pattern of peaks than the unstressed sample indicating selectivity with respect to oligosaccharide composition. Two different lots of oligosaccharides released from CTLA4Ig were also analyzed and yielded similar profiles by CE and HPAEC–PAD. The neutral, mono, and disialylated regions depicted in the CE electropherogram (Fig. 8) or the HPAEC–PAD chromatogram (Fig. 9) may be qualitatively

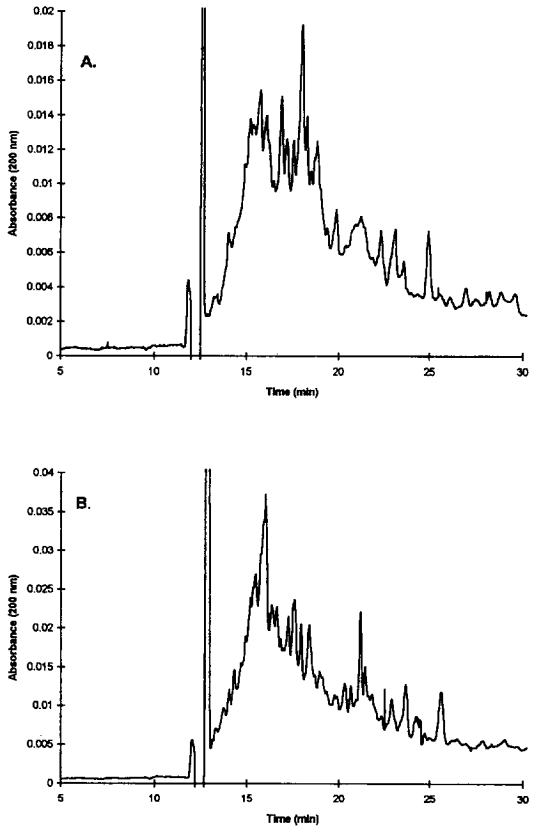

Fig. 8. Separation of oligosaccharides released from CTLA4Ig: (A) CTLA4Ig; (B) sample thermally degraded at 100°C for 30 min prior to analysis. Conditions: 30 m*M* phytic acid; 50 m*M* sodium borate; pH 9.4; constant current 130 μA; 35°C capillary temperature.

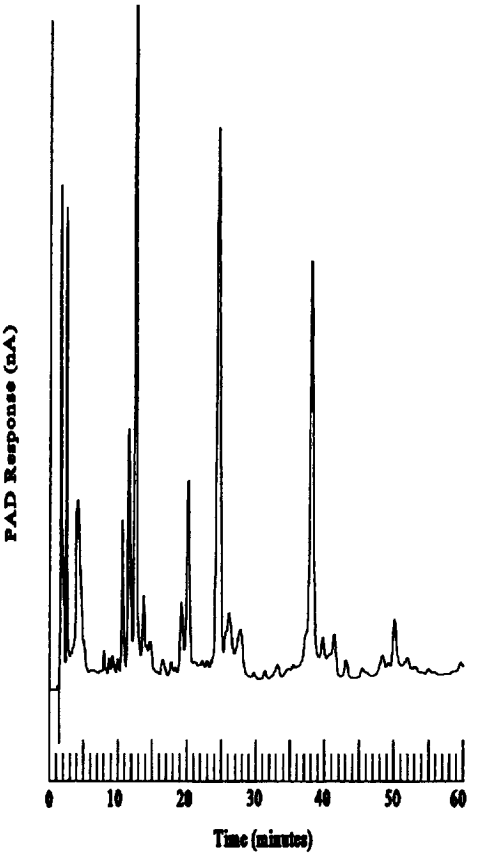

Fig. 9. Separation of oligosaccharides released from CTLA4Ig, as analyzed by HPAEC–PAD [7].

examined as presented for the IgG released oligosaccharides described above.

To the extent that CE and HPAEC–PAD represent orthogonal analytical procedures, selectivity differences are to be expected; additionally, the apparent concentrations of the various oligosaccharides may be dissimilar due to the intrinsic sensitivities of ultraviolet detection (CE) and electrochemical detection (HPAEC–PAD). Nonetheless, a comparison of Fig. 8 and Fig. 9 (refer to the average migration times previously discussed), both indicate that both procedures characterize CTLA4Ig-derived oligosaccharides as being predominantly neutral. Both procedures indicate the presence of a single major disialylated species. Both procedures yield at least three mono-sialylated species, however, there are apparent selectivity and sensitivity differences, with the CE procedure representing more species present.

4. Conclusion

The purpose of this paper was to present a CE procedure that with a borate-based buffer increased the resolution between oligosaccharides relative to previous separations, particularly in complex and neutral mixtures. Using the standard borate system, albeit at a relatively high pH, several ion-pairing agents were evaluated in the separation buffer. Phytic acid was chosen and shown to facilitate the separation of neutral oligosaccharides, a difficult analytical task. The final system was shown to be useful for oligosaccharide mixtures released from IgG antibodies and a biotechnologically produced protein. The results of these assays were shown to be consistent with HPAEC–PAD chromatograms of the same complex mixtures. Hence, it appears that a variety of oligosaccharide mixtures may be characterized with the advantage of a short run time and nanoliter sample consumption that are characteristic of CE.

Acknowledgments

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