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Carbohydrate Research 258 (1994) 1–9

CARBOHYDRATE RESEARCH

Resolution of the branched forms of oligosaccharides by high-performance capillary electrophoresis

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(Received October 22nd, 1993; accepted in revised form December 19th, 1993)

Abstract

The heterogeneous nature of most polysaccharides found in nature includes distribution in molecular weight, primary sequence, and branching. The analytical methodology used in the characterization of these structural aspects must ensure high separation efficiency and selectivity. This paper reports on the high-performance capillary electrophoresis (HPCE) separation of branched forms of oligosaccharides as well as some variants in the primary structure. Oligosaccharide maps were obtained after selective debranching using isoamylase, laminarinase, and cellulase enzymes. The samples investigated were α-D-glucans (amylose, amylopectin, and pullulan) and β-D-glucans (exemplified by lichenan). The solutes were separated as fluorescent derivatives with 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) and detected by laser-induced fluorescence at 514 nm using a He/Cd laser (excitation at 325 nm). The number of theoretical plates was in excess of one million per meter. Baseline resolution of oligosaccharides with a degree of polymerization ~ 70 was obtained within 15 min using borate buffer as the electrolyte.

1. Introduction

Polysaccharides are found most often as polydisperse compounds with a distribution in molecular weight and primary structure. Starch, for example, consists of amyloses and amylopectins. True amyloses are linear (1 → 4)-α-D-glucans with a degree of polymerization (dp) up to several thousands. Amylopectins (AP) are known to consist of hundreds to thousands of short amylose chains with dp of 6–100 glycosyl residues interconnected by (1 → 6) linkages within clusters [1,2]. These branches are formed by starch branching enzyme [3] or Q-enzyme (EC

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SSDI 0008-6215(94)00003-X

2.4.1.18). Different techniques have been applied to characterize such disperse polymers and to estimate the degree of branching, including photometry, periodate oxidation, polarimetry, osmometry, radiometry, and enzymatic analysis [4].

The branched chains can be liberated selectively by the action of debranching enzymes and then later subjected to further structural analysis through size-exclusion chromatography (SEC) with differential refractometer and small-angle laser light-scattering photometry [2]. Individual chains, however, are not resolved by this technique and the average values are used to describe chain-lengths, number of chains, etc. High-performance anion-exchange chromatography with pulsed amperometric detection, under alkaline conditions, has considerably improved the separation of saccharides and other polyols. Using this approach, isoamylolysates of the amylopectins were resolved up to dp of ~ 60, in less than 40 min [5]. However, the branched forms were not resolved. Ammeraal et al. [6] reported resolution of the branched forms of a waxy maize β-limit dextrin, but the baseline separation was not achieved for components with dp > 20.

During the past several years, high-performance capillary electrophoresis (HPCE) with laser-induced fluorescence detection has developed into a powerful tool for the separation of glycoconjugates [7–13]. Combined with suitable fluorescence-tagging procedures, highly fluorescent and charged derivatives of various oligosaccharides can be formed and separated in electric fields, and detected at the attomole level [8]. The resolving power of HPCE allows the separation of complex carbohydrate mixtures [13], including sugar enantiomers [14]. Electrolyte composition, tag charge, and capillary wall treatment are the fundamental parameters influencing separation efficiency, and by proper utilization, glycoconjugates with minute variations in molecular structure can be resolved [13].

This paper reports on the separation of highly complex oligosaccharide mixtures obtained by a selective hydrolysis of amyloses, amylopectins, lichenan, and pullulan polysaccharides, using debranching enzymes. Complex glucan chains with numerous residual branches can potentially be assessed using this oligosaccharide mapping procedure. An example is given with a baseline separation of an intact amylose sample with dp close to 70. All runs were characterized by very short analysis times.

2. Experimental

Chemicals.—All polysaccharides and enzymes used in this study were obtained from Sigma (St. Louis, MO). Sigma also supplied all buffer chemicals, except boric acid (Malinkrodt Inc., Paris, KY). Acrylamide and ammonium persulfate were purchased from Bio-Rad Laboratories (Richmond, CA). Sodium cyanoborohydride was obtained from Aldrich (Milwaukee, WI) and the fluorogenic reagent, 8-aminonaphthalene-1,3,6-trisulfonate (ANTS), was a product of Molecular Probes, Inc. (Eugene, OR).

Column preparation.—Various lengths of fused silica capillaries (Polymicro Technologies, Phoenix, AZ) of 50 μm i.d. (187 μm o.d.) were used as the

separation columns. They were coated with a linear layer of polyacrylamide according to a slightly modified version of the Hjertén method [15]: The new capillary was treated with 0.1 M NaOH for 1 h and rinsed with water and MeOH. γ-Methacryloxypropyltrimethoxysilane (10 μL dissolved in CH_2Cl_2 containing 0.02 M AcOH) was then coupled to the silica wall during 60 min, under nitrogen pressure. The capillary was then rinsed with MeOH and water, and 4% (w/w) acrylamide solution, containing 1 μL/mL of *N,N,N',N'*,-tetramethylethylenediamine (TEMED) and 1 mg/mL ammonium persulfate, was then passed through the capillary under nitrogen pressure for 30 min. Finally, the capillary was rinsed with water and dried under a stream of nitrogen.

Sample preparation and enzymatic treatment procedures.—The polysaccharides were derivatized through Schiff-base formation between the aromatic amine of ANTS and the aldehyde form of a sugar, followed by reduction to give a stable product. The reagent concentration was 20–50 mM in 3% (w/w) AcOH [16]. The mixtures were subsequently heated for 60 min at 90°C in the presence of 0.1 M sodium cyanoborohydride. The samples prepared through this procedure are stable and can be stored for months at −20°C before analysis.

Debranching by isoamylase.—A polysaccharide (2–4 mg) (amylopectins, amyloses, or pullulan) was dissolved in acetate buffer (0.025 M, pH 3.5) containing 80 U of isoamylase (EC 3.2.1.68, from *Pseudomonas amyloderamosa*). The samples were incubated for 3 h at 37°C.

Enzymatic cleavage of lichenan.—1 mg of lichenan (*Cetraria islandica*) was incubated with 1 U of laminarinase (EC 3.2.1.6, from *Penicillium* species) and 4 U of a commercial cellulase preparation (EC 3.2.1.4, from *Trichoderma viride*) for 3 h in 0.05 M acetate buffer, pH 5.2 (37°C). The reaction was stopped by boiling the incubation mixture for 10 min. After derivatization with ANTS, the mixture was analyzed by direct injection into the capillary system.

Apparatus.—A home-built instrumental setup for capillary electrophoresis/laser-induced fluorescence has been described earlier [17]. A high-voltage power supply (Spellman High Voltage Electronics, Plainview, NY) capable of delivering 0–40 kV was employed. On-column fluorescence detection was accomplished with a Model 56X He/Cd laser (Omnichrome, Chino, CA) as the excitation source, operating at 325 nm, while fluorescence was measured at 514 nm for 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) derivatives.

The samples were introduced into the capillary of the instrument by a hydrodynamic technique.

3. Results and discussion

α-D-Glucans.—The electrophoretic mobility of an analyte in an electric field is basically governed by the charge-to-mass ratio. For oligomers with identical or similar molecular weights but different primary structures, a charge difference usually has to be imposed on the molecules in order to achieve separation. The potential of variation within a given number of sugar units is immense and, hence,

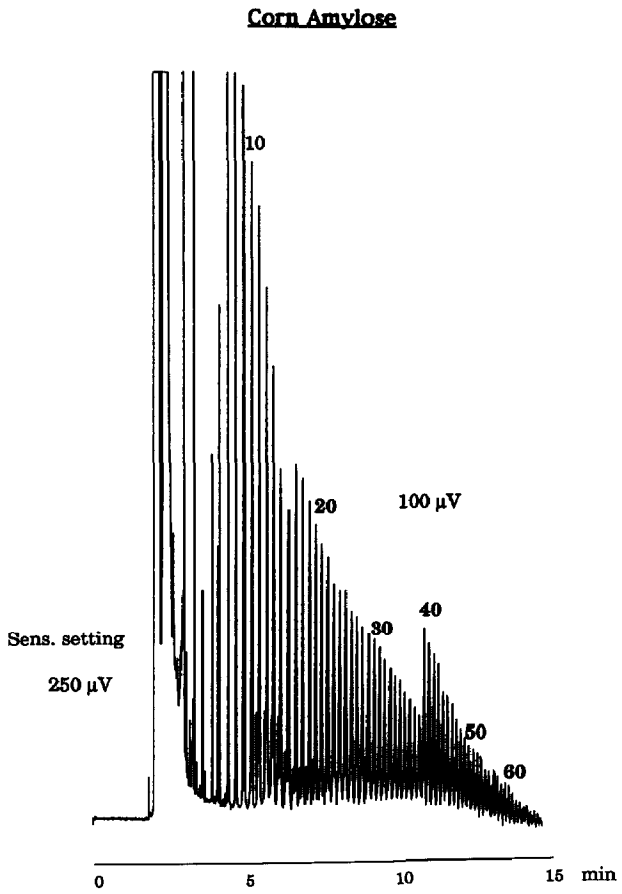

Fig. 1. Separation of the corn amylose oligomers. Conditions: 0.2. M borate-Tris (pH 8.65); −500 V/cm (18 μA) and 35 cm effective capillary length. The numbers refer to the degree of polymerization.

puts extremely high demands on the analytical methodology used. Boric acid, added to the electrolyte buffer, has often been used for the purpose of increasing the selectivity due to its discriminating complexation behavior with miscellaneous carbohydrate structures [18]. In the separation of a corn amylose sample, careful adjustment of the borate concentration revealed minor peaks, presumably due to branched chains (Fig. 1), using 0.2 M borate-Tris (pH 8.65) as the buffer. While the earlier minor components are well separated from the major oligomers, above ~ dp 20, their presence is observed as a slight leading or tailing at the base of the peaks due to comigration. The effect of borate complexation on selectivity between the oligomers with similar structures decreases with increasing size of the oligomer. This behavior is due to the large number of charges already imposed on the molecule. Further increase in the concentration of boric acid did not improve the separation selectivity. However, baseline resolution of the amylose chains with dp

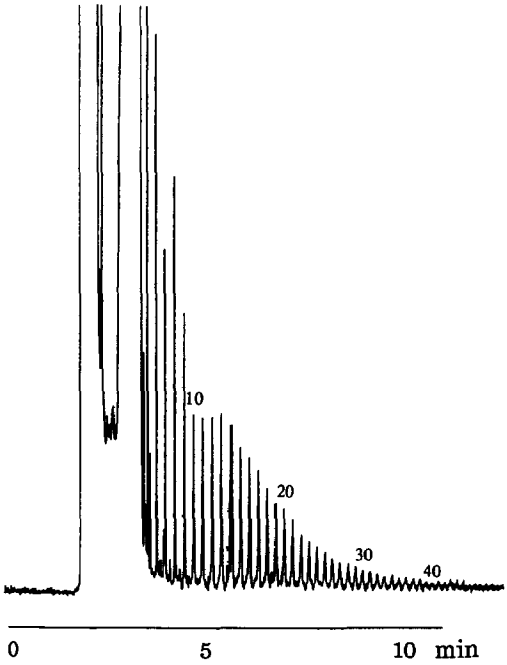

Fig. 2. Separation of the corn amylose oligomers after debranching with isoamylase. Conditions as in Fig. 1.

close to 70 was achieved in just 15 min. Tentative evidence for the branched nature of these chains was obtained through their disappearance after incubation with the debranching enzyme, isoamylase (Fig. 2).

Amylopectin, a branched and high molecular weight component of starch, varies among different plant species and may change throughout the season. Information on the size of the molecule can be obtained through size-exclusion chromatography and laser-light scattering measurement [2]. However, in order to assess the structural organization of the molecule, the chains must be selectively hydrolyzed by isoamylase treatment. The chains thus obtained are subsequently separated, and the amount and degree of polymerization of the individual oligomers must be determined. The chains are classified as A, B, and C depending on their position relative to the reducing end and whether they carry chains themselves [19]. The oligosaccharide maps from the isoamylolysates of corn and potato amylopectins are presented in Figs. 3A and B. A large number of branched species, mostly present at low concentration, are separated here for the first time, and further visualized in an expanded part of the electropherogram in Fig. 3A. Both amylopectins exhibit a species-specific and polymodal distribution for linear as well as branched chain lengths.

Pullulan is a linear, water soluble polymer comprised mostly of (1 → 6)-α-D-maltotriose [(6)-α-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 →] and some (1

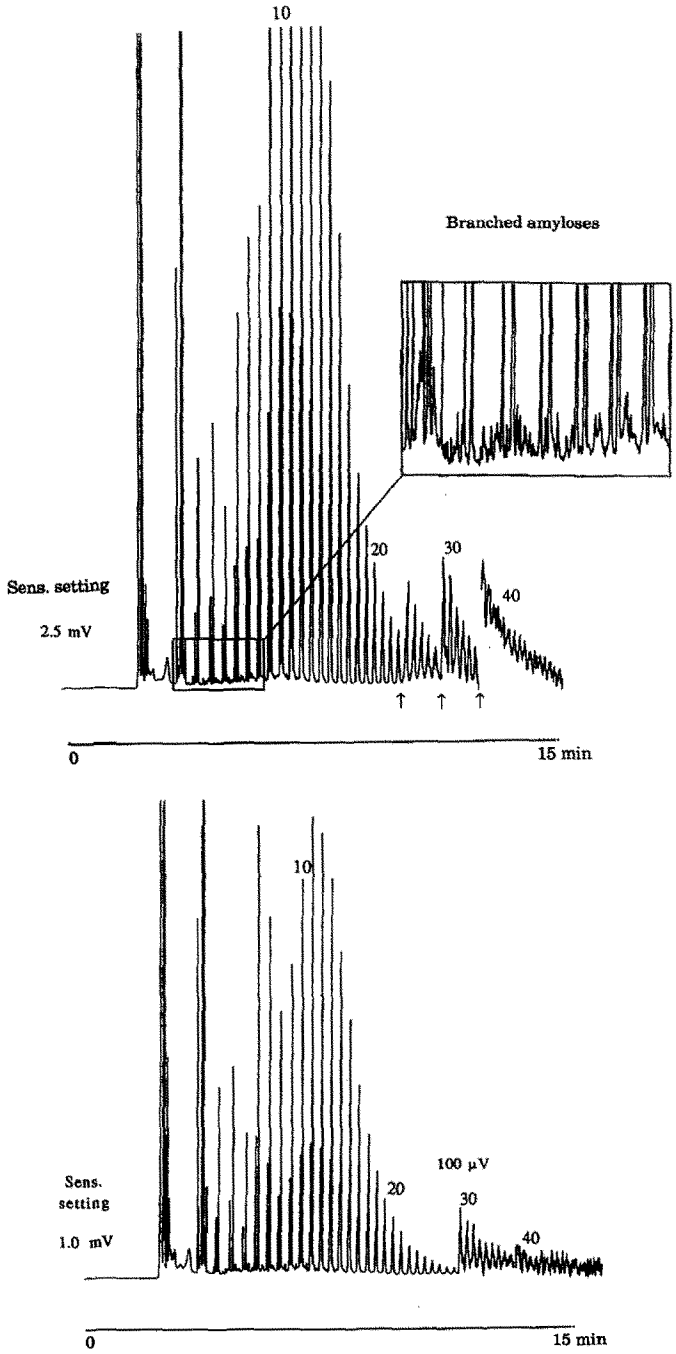

Fig. 3. Isoamylolyzates of (A) corn amylopectin and (B) potato amylopectin. Conditions as in Fig. 1, except for 65 cm effective length of the separation capillary. The symbol ↑ used in (A) indicate changes in sensitivity setting from 2.5 to 1.0 mV, 250, and 100 μV, respectively.

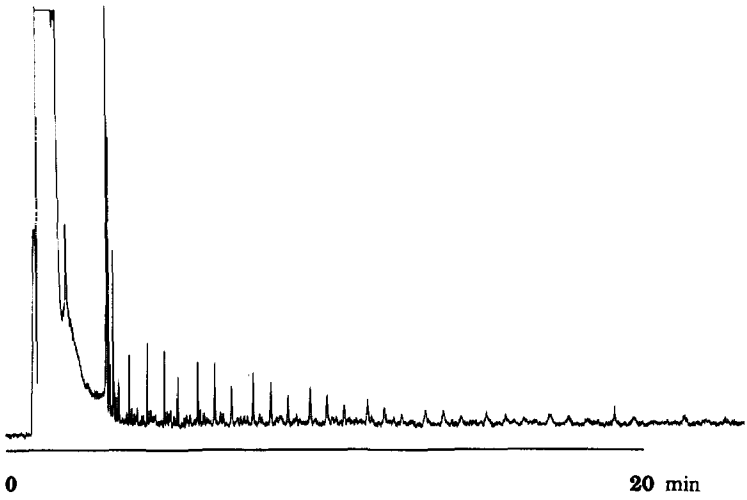

Fig. 4. Oligosaccharide map of pullulan after enzymatic cleavage with isoamylase. Conditions as in Fig. 1.

→ 6)-α-D-maltotetraose repeating units [20]. The oligosaccharide map of pullulan, after enzymatic cleavage with isoamylase, is shown in Fig. 4. A periodic pattern of three peaks with decreasing size, probably reflecting the amount of maltotetraoses present in the oligosaccharides, can be seen in the electropherogram. Depending on the sequences of maltotriose and maltotetraose, a multitude of oligomers with the same number of glucose units can be obtained.

β-D-Glucans.—Many polysaccharides found in seaweed and other plant organisms are β-D-glucans. They exhibit large variations in size, monosaccharide composition, and degree of branching. Recently, they have been under biomedical investigation because of their roles in diseases of bacterial [21], viral [22], fungal [23], and parasitic origin [24]. Lichenan is a good example of a (1 → 3),(1 → 4)-β-D-glucan containing (1 → 4)-β-D-glucopyranosyl residues as an intrachain group [25]. Separation of the water-soluble part of the sample is shown in Fig. 5, using 0.2 M borate-Tris as the buffer electrolyte. Its solubility decreases with increasing size of the glucan, but the sample can be totally solubilized at elevated temperatures (i.e., during the enzymatic treatment, as shown below). An incomplete separation of polydisperse oligomers with apparently very similar molecular structures resulted in broad peaks (Fig. 5). In the separation of laminarin, a β-(1 → 3)- and β-(1 → 6)-D-glucan, optimum separation selectivity was achieved using 50 mM morpholinoethanesulfonate (MES) and 25 mM tris(hydroxymethyl)aminomethane (Tris) as the electrolyte buffer [14], at pH 5.95. This is in contrast to the lichenan sample, emphasizing the importance of the proper choice of an electrolyte buffer to achieve maximum selectivity. The polydispersity of the lichenan sample was further investigated by enzymatic hydrolysis with laminarinase (a β-(1 → 3)-glucose-hydrolyzing enzyme), and the result of the hydrolysis is shown in Fig. 6. The

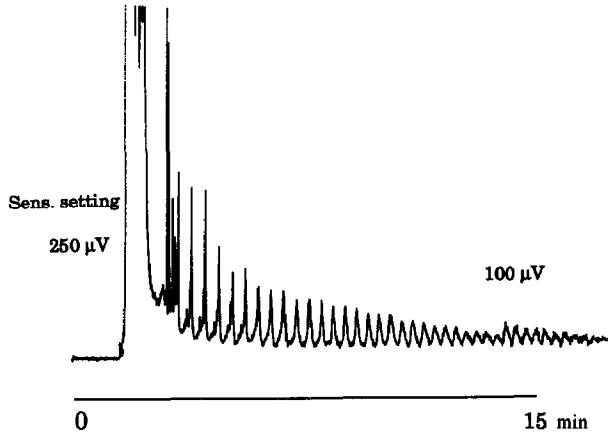

Fig. 5. Separation of the water-soluble oligomers of an intact lichenan sample. Conditions as in Fig. 1.

separated oligomers now appear to migrate in "clusters". The distance between each "cluster" is approximately equivalent to an increase in chain-length by 3–4 glucose units and, hence, mirrors the ratio of maltotriose and maltotetraose units in the polysaccharide. Incubation with a commercial preparation of cellulase from *Trichoderma viride* (another β-(1 → 3)-glucose hydrolyzing enzyme) totally hydrolyzed the sample within minutes (data not shown).

In conclusion, we have shown that high-resolution of complex and branched oligosaccharides can be obtained by open tubular capillary electrophoresis after selective hydrolysis using debranching enzymes. The oligosaccharide maps contain

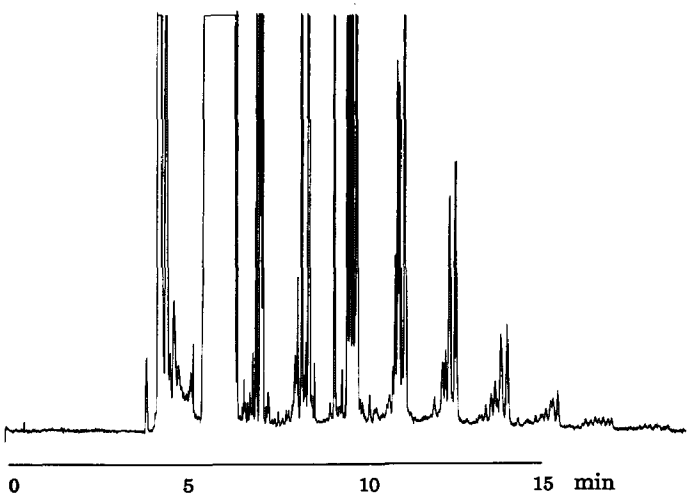

Fig. 6. Oligosaccharide map of lichenan after laminarinase treatment. Conditions as in Fig. 1. Effective length, 65 cm.

qualitative and quantitative information due to the ability to perform direct hydrodynamic injection and because of the inherently high efficiency and peak capacity of the separation principle. We are currently undertaking separation of larger glycoconjugate molecules (such as glycogens of different origin) using debranching enzymes for the oligosaccharide mapping procedures and separations of the intact molecules in entangled polymer matrices and pulsed electric fields [25].

Acknowledgements

This work was supported by Grant no. 24349 from the National Institute of General Medical Sciences, U.S. Department of Health and Human Services, and a grant-in-aid from Astra/Hässle (Mölndal, Sweden). One of us (MS) has been a recipient of fellowships from The Sweden–American Foundation, the foundation "Stiftelsen Blanceflour Boncampagni-Ludovisi, född Bildt", and The Swedish Academy of Pharmaceutical Sciences.

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