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Note

Isotachophoretic determination of hyaluronate oligosaccharide-degrading enzyme activities

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The unbranched glycosaminoglycan hyaluronic acid (HA) consists of the basic disaccharide unit β-D-glycanopyranuronosyl-1→3-O-(2-acetamido-2-deoxy-β-D-glycopyranosyl)-1→4 and is initially hydrolysed by endoglycanohydrolases such as bovine testicular hyaluronidase (E.C. 3.2.1.35) and leech hyaluronidase (E.C. 3.2.1.36). The even-numbered oligosaccharides obtained are further degraded by exoglycanohydrolases such as β-N-acetylglucosaminidase (E.C. 3.2.1.30) and β-glucuronidase (E.C. 3.2.1.31), leading to odd-numbered oligosaccharides with terminal glucuronic acid (GlcUA) or N-acetylglucosamine (GlcNAc).

These defined oligosaccharides are useful tool for the detection and characterization of these enzymes. This paper presents an isotachophoretic method for the quantification and identification of HA oligosaccharide hydrolysis products. This method opens up the possibility of characterizing hyaluronidases, β-N-acetylglucosaminidases and β-glucuronidases with defined biogenic substrates without any sample pre-treatment. The method provides an easy and accurate quantification and high resolving power.

EXPERIMENTAL

Preparation of oligosaccharides

Disaccharide (βGlcUAl–3GlcNAc), tetrasaccharide (βGlcUAl–3βGlcNAc) and trisaccharide (βGlcNAcl–43βGlcNAc) were produced by digesting HA from umbilical cords with bovine testicular hyaluronidase[1] and β-N-acetylglucosaminidase-free β-glucuronidase from *Patella barbara*[2].

Disaccharide* (βGlcNAcl–4GlcUA), tetrasaccharide* (βGlcNAcl–4βGlcUAl-3)$_2$ and trisaccharide* (βGlcUAl–3βGlcNAcl–4GlcUA) were produced by using leech hyaluronidase, bovine spleen β-N-acetylglucosaminidase and β-glucuronidase[2].

The hydrolysis products were separated by chromatography on DEAE-Sephacel (formate form) with a linear gradient of formic acid[3]. The purity of the oligosaccharides was checked by high-performance liquid chromatography (HPLC)[4] and isotachophoresis.

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Instrumental

Analyses were performed with an LKB 2127 Tachophor (LKB, Gräfelfing, F.R.G.) in a 230-mm PTFE capillary with 7 mmol/l hydrochloric acid glycine–glycine/0.3% hydroxymethylcellulose as the leading electrolyte; 0.01 mol/l caproic acid was used as the terminating electrolyte. Detection was carried out at 254 nm and by thermal measurement. Quantification was effected by measuring the widths of thermal and UV peaks and referring them to a calibration graph. The injection volume was 1.5 μl and runs were made within 12 min at 12°C with a detection current of 50 μA.

Enzyme assays

Purified β-glucuronidase from *Patella barbara*[2], β-N-acetylglucosaminidase from bovine spleen[5] and bovine testicular hyaluronidase[1] were incubated at 37°C with 0.01 mol/l substrate in 0.005 mol/l acetate buffer (pH 4.0), then 1.5-μl aliquots were analysed in a time-dependent manner until total hydrolysis of the substrate oligosaccharides used was achieved.

RESULTS AND DISCUSSION

Some biogenic HA oligosaccharides show clear thermal step-height differences in the described isotachophoretic system (Table I, Fig. 1D). A differentiation of even-numbered disaccharides and tetrasaccharides with terminal reducing GlcUA or GlcNAc is impossible by isotachophoresis because there is no difference in charge or molecular size and, as a consequence, they do not differ in their mobility. In contrast, odd-numbered oligosaccharides, like trisaccharides, differ in their GlcUA/GlcNAc ratio and therefore in their net charge and show distinct isotachophoretic behaviour (Table I). Trisaccharide*, for instance, with two GlcUA per molecule with a higher net charge than the other trisaccharide, shows a higher mobility and consequently a lower thermal step height than trisaccharide. Surprisingly, GlcUA and trisaccharide, with a lower molecular size and higher net charge than tetrasaccharide, are separated inadequately from it as shown by thermal detection (Fig. 1D).

For enzyme assay of β-glucuronidases, β-N-acetylglucosaminidases and hyaluronidases, biogenic HA oligosaccharides are useful substrates when the following requirements are fulfilled. First, the substrate must be hydrolysed by the investigated

TABLE I

RELATIVE THERMAL STEP HEIGHTS OF BIOGENIC HYALURONATE

Saccharides with terminal reducing glucuronic acid are labelled with an asterisk.

Saccharide	*Thermal step height (%)*
Trisaccharide*	26
Glucuronic acid	28
Tetrasaccharide	28
Tetrasaccharide*	28
Disaccharide	36
Disaccharide*	36
Trisaccharide	48

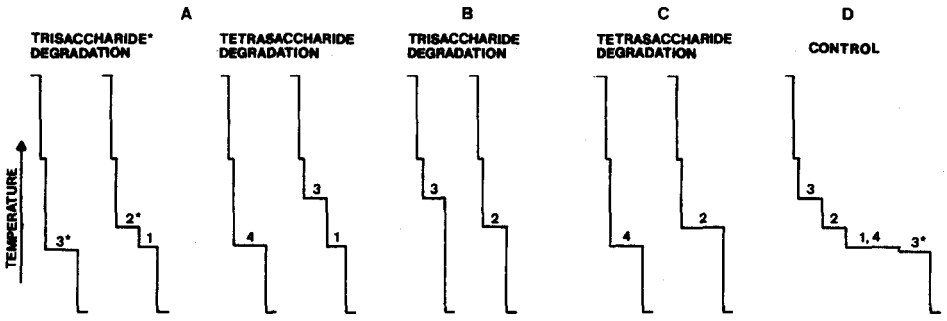

Fig. 1. Total degradation of hyaluronate oligosaccharides by (A) β-glucuronidase, (B) β-N-acetylglucosaminidase and (C) bovine testicular hyaluronidase monitored by isotachophoresis. Saccharides: 1 = glucuronic acid; 2 = disaccharide; 2* = disaccharide*; 3 = trisaccharide; 3* = trisaccharide*; 4 = tetrasaccharide.

enzyme and second, it must be degraded to hydrolysis products that are separable from the substrate and detectable by isotachophoresis. Useful substrate–enzyme combinations for enzyme characterization are as follows.

For β-glucuronidase determinations, trisaccharide* and tetrasaccharide are useful substrates: trisaccharide* → disaccharide* + GlcUA, tetrasaccharide → trisaccharide + GlcUA. In the trisaccharide* hydrolysis, disaccharide* and GlcUA are formed in equimolar concentrations. As can be seen in Fig. 1A, GlcUA is not well separated from trisaccharide* but the enzyme activity is determinable by the disaccharide* formed. In tetrasaccharide hydrolysis, tetrasaccharide and GlcUA are not separable and the enzyme activity is determined by the trisaccharide formed (Fig. 1A).

For β-N-acetylglucosaminidase activity detection, trisaccharide with terminal non-reducing GlcNAc is a useful substrate (Fig. 1B): trisaccharide → disaccharide + GlcNAc. In this case the disaccharide hydrolysis product and the substrate are detectable and both can be used for activity determination.

Hyaluronidase activity is measurable with tetrasaccharide as substrate: tetrasaccharide → 2 disaccharides. The substrate and the hydrolysis products are well separated by isotachophoresis (Fig. 1C). Further, the proof of hyaluronidases with HA as substrate is better by isotachophoresis than by HPLC. In HPLC problems can arise with the column system used for HA but not for HA oligosaccharides as substrates. The commonly used HPLC column systems for this separation are based on weak ion-exchange materials, which suffer from irreversible adsorption problems with the high-molecular-weight substrates. Further, the analysis takes longer, higher sample volumes are needed and the column lifetime is limited.

In contrast to chemical methods, isotachophoresis facilitates the simultaneous qualitative and quantitative detection of substrate and hydrolysis products. The small sample volumes, no sample pre-treatment and the short analysis time are advantages. Although this isotachophoretic system is not generally superior to HPLC systems, it is advantageous in the separation of disaccharide and trisaccharide, which is difficult by HPLC.

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