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DEGRADATION OF CHONDROITIN 4-SULPHATE BY TRIFLUOROACETOLYSIS: ISOLATION OF OLIGOSACCHARIDES FROM THE CARBOHYDRATE–PROTEIN LINKAGE REGION

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ABSTRACT

Oligosaccharides from the linkage-region tetrasaccharide, β-D-Glc*p*A-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Xyl*p*, of chondroitin 4-sulphate were isolated after trifluoroacetolysis. The oligosaccharides were purified by ion-exchange chromatography and paper chromatography and subjected to sugar and methylation analysis and g.l.c.–m.s. The recovery of linkage-region oligosaccharides was ~45% after trifluoroacetolysis, calculated according to the D-xylose present in the chondroitin 4-sulphate preparation. The following structures were identified: β-D-Gal*p*-(1→4)-D-Xyl*p*, β-D-Gal*p*-(1→3)-D-Gal*p*, β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Xyl*p*, β-D-Glc*p*A-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Xyl*p*.

INTRODUCTION

In mammalian proteoglycans, most polysaccharide species are linked to the core proteins by an *O*-glycosylic linkage between D-xylose and serine[1]. The D-xylose residue is part of a distinct linkage-region tetrasaccharide that has the structure β-D-Glc*p*A-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Xyl*p*. For the chondroitin sulphate proteoglycans of cartilage, this structure was established by enzymic degradation of the proteoglycans to glycopeptides followed by acid hydrolysis and characterisation of the released fragments[1–4].

Using trifluoroacetolysis, it is possible to isolate *N*- and *O*-glycosylically linked oligosaccharide chains in glycoproteins[5–7]. The glycosylic bonds are stable due to the electron-withdrawing character of the *O*-trifluoroacetyl groups introduced in the sugar moiety[8–12]. We now report on a new procedure for the isolation of the oligosaccharides from the carbohydrate–protein linkage region of chondroitin sulphate. The method is based on cleavage of the carbohydrate–protein linkage and degradation of the polysaccharide chains by trifluoroacetolysis[13].

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EXPERIMENTAL

Materials. — Chondroitin 4-sulphate was prepared from bovine nasal septa as described[2]. β-D-Gal*p*-(1→4)-D-Xyl*p* and β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Xyl*p* were gifts of Professor B. Lindberg and had been synthesised as described[14].

General methods. — G.l.c. was performed with a Perkin–Elmer 3920 gas chromatograph equipped with a flame-ionisation detector. Separations were performed on (*a*) an SE-30 W.C.O.T. glass-capillary column (25 m × 0.25 mm) (Scientific Glass Engineering Inc., U.S.A.) at 180–330° (for permethylated oligosaccharide alditols), and (*b*) a glass column (2 m × 0.5 cm) packed with 3% of ECNSS-M on Chromosorb Q (Applied Science Laboratories Inc., U.S.A.) at 200° (for alditol acetates). G.l.c.–m.s. was performed on a Varian MAT 311A instrument fitted with the appropriate column. The spectra were recorded at 70 eV, with an ionisation current of 3 mA and an ion-source temperature of 120°. The spectra were processed by an on-line computer system (Spectrosystem 100, Varian MAT).

Analytical methods. — Total hexose was determined by a colorimetric method[15]. Sugar analysis was performed by g.l.c.[16] and m.s.[17] after hydrolysis with aqueous 90% formic acid at 100° for 5 h followed by hydrolysis with 0.25M sulphuric acid at 100° for 18 h. Methylation analysis was performed as previously described[18].

Trifluoroacetolysis. — Chondroitin 4-sulphate (50 g) was heated in a mixture of trifluoroacetic acid (TFA) and trifluoroacetic anhydride (TFAA) (1:50, 1500 mL) at 100° for 48 h in a sealed vessel (*caution*: corrosive mixture under pressure). The black mixture was cooled to room temperature and concentrated to dryness, and a solution of the residue in methanol (500 mL) was concentrated to dryness. The residue was then treated with aqueous 50% acetic acid (500 mL) for 4 h at room temperature, and the mixture was concentrated to dryness. A portion (1%) of the residue was reduced with sodium borodeuteride (100 mg) and then permethylated[19], and the product was analysed by g.l.c.–m.s. The remainder of the material was dissolved in water (200 mL) and passed through columns (60 × 5 cm) of Dowex 50 (H^+) and 2 (OAc^-) resins. The desalted solution was concentrated to dryness and the residue was fractionated by preparative paper chromatography (ethyl acetate–water–acetic acid, 3:1:1). The fractionation was monitored by g.l.c.–m.s. of each fraction after reduction with $NaBD_4$ and permethylation. The fraction containing β-D-Gal-(1→3)-β-D-Gal*p*-(1→4)-D-Xyl*p* was eluted from the paper and passed through a column (20 × 2 cm) of Sephadex G-25 eluted with water. The fractions containing the trisaccharide were combined and lyophilised. The yield of the trisaccharide was 50 mg.

RESULTS

Trifluoroacetolysis was applied to two chondroitin 4-sulphate preparations (6.85 and 50 g). Analysis by g.l.c. of the alditol acetates revealed 0.24% of D-xylose in each preparation. The recoveries of β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Xyl*p*

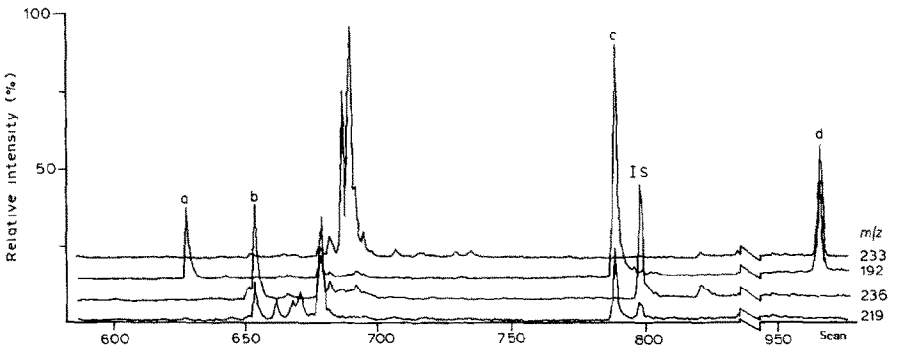


Fig. 1. Multiple ion-tracing in g.l.c.–m.s. of permethylated oligosaccharide alditols (peaks *a*–*d*) released by trifluoroacetolysis of chondroitin 4-sulphate; I.S., internal standard (maltotriose).

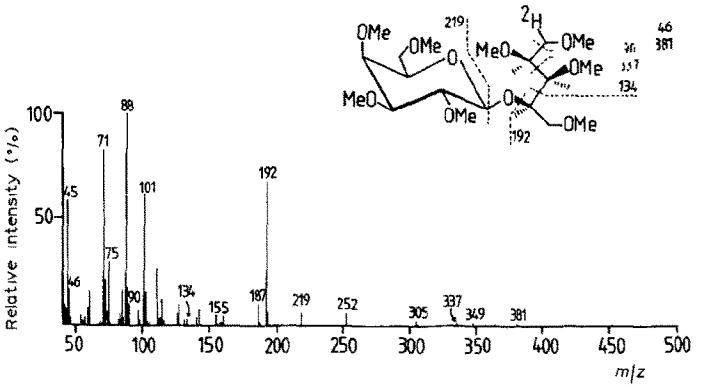


Fig. 2. Mass spectrum and some important primary fragments of the component in peak *a* (Fig. 1).

after the two trifluoroacetolyses were 19 and 14%, respectively. The values were calculated from the analysed yield of D-xylose. Total recoveries of the linkage-region oligosaccharides were 46 and 40%, respectively.

G.l.c.–m.s. of the reduced and permethylated products of trifluoroacetolysis gave the ion chromatogram shown in Fig. 1. The ion tracing for four of the major components (peaks *a*–*d*) indicated that they were derived from the carbohydrate–protein linkage region of the polysaccharide.

The material in peak *a* gave a mass spectrum (Fig. 2) characteristic[20] of a Hex*p*-(1→4)-Pentitol-*1-d*. The intensity ratio of the A_1-, A_2-, and A_3-fragments can be used to differentiate the *gluco* configuration from the *manno* and *galacto* configurations[21]. The mass spectrum (Fig. 2) indicated that the terminal non-reducing sugar was galactose. Since the only hexose and pentose constituents of chondroitin 4-sulphate are D-galactose and D-xylose, respectively, it was concluded that peak *a* contained permethylated D-Gal*p*-(1→4)-D-Xylitol-*1-d*. The proposed structure was further supported by the observation that the retention time of the compound in g.l.c. was the same as that of authentic β-D-Gal*p*-(1→4)-D-Xyl*p* which

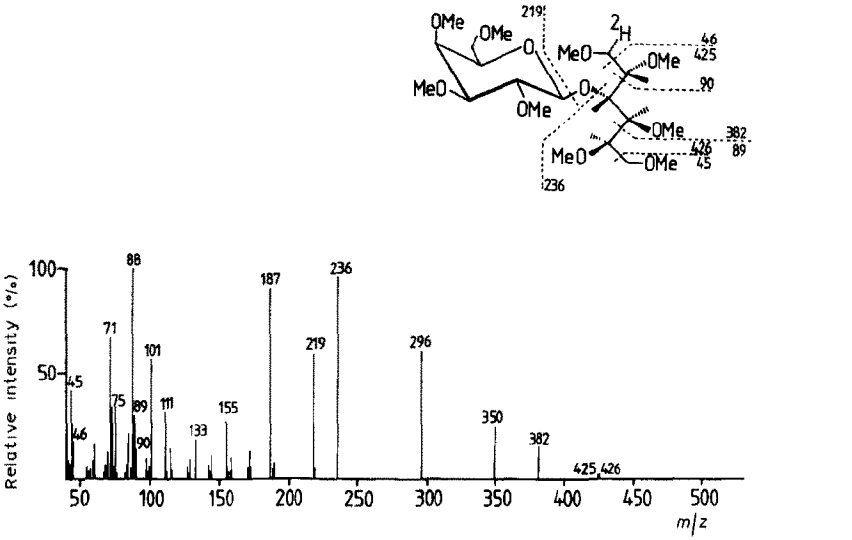


Fig. 3. Mass spectrum and some important primary fragments of the component in peak *b* (Fig. 1).

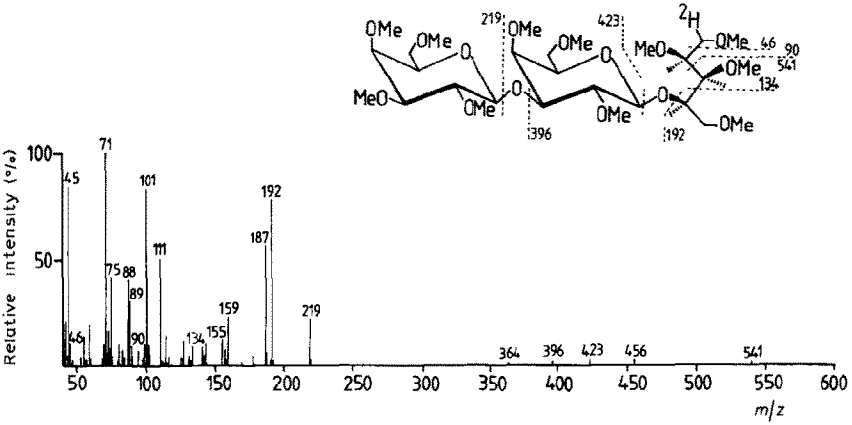


Fig. 4. Mass spectrum and some important primary fragments of the component in peak *c* (Fig. 1).

had been reduced and permethylated. The g.l.c. analysis further suggested that the glycosidic linkage was β, since separation of the α and β anomers would probably have occurred.

The product in peak *b* gave a mass spectrum consistent with a permethylated Hex*p*-(1→3)-Hexitol-*1-d* (Fig. 3). Since the only hexose present in chondroitin 4-sulphate is D-galactose and two β-linked residues of this sugar are present in each polysaccharide molecule, the isolated disaccharide was assumed to be permethylated β-D-Gal*p*-(1→3)-D-Galactitol-*1-d*[21].

The mass spectrum of the major peak (peak *c*, Fig. 1) was typical of a permethylated Hex*p*-(1→3)-Hex*p*-(1→4)-Pentitol-*1-d* (Fig. 4). This spectrum was

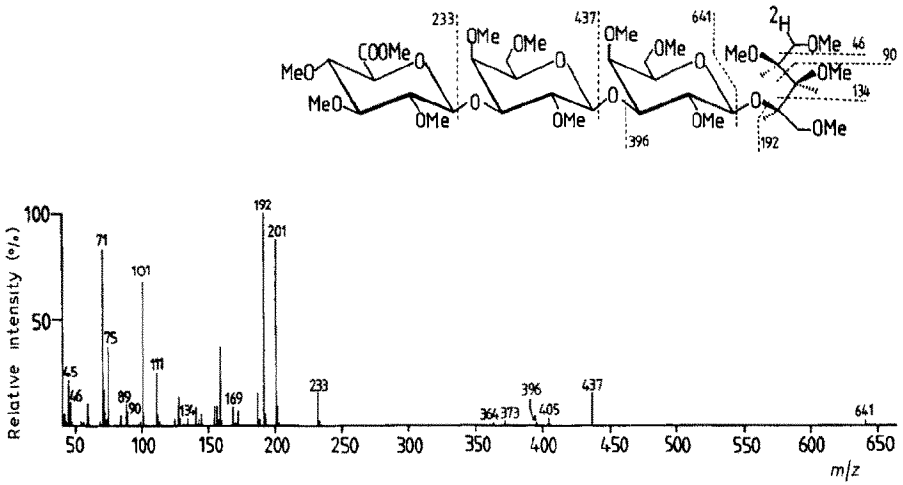


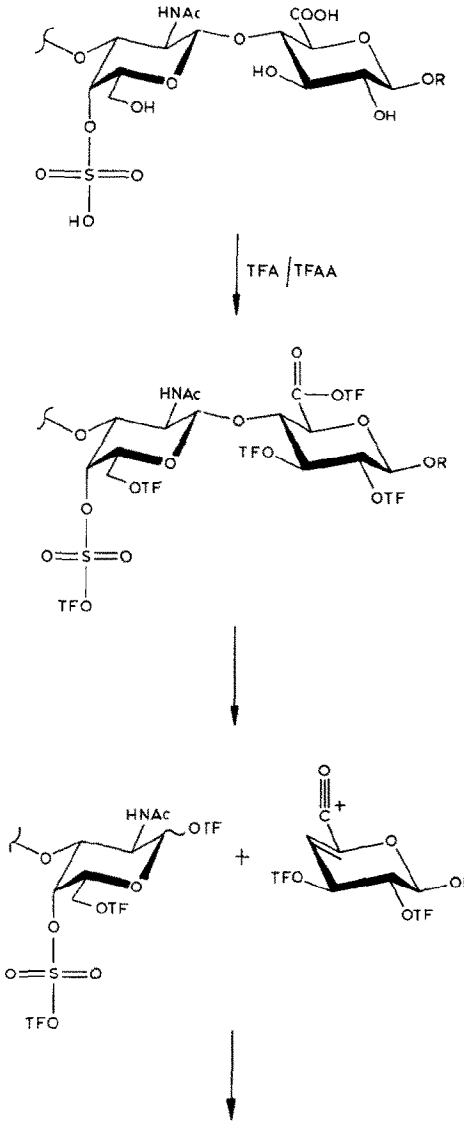
Fig. 5. Mass spectrum and some important primary fragments of the component in peak *d* (Fig. 1).

identical to that observed for a reduced and permethylated sample of authentic β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Xyl*p*. Identity of the oligosaccharide in peak *c* with the derivative of the authentic trisaccharide was further indicated by the finding that their retention times in g.l.c. were the same.

The largest compound (peak *d*, Fig. 1) showed a mass spectrum characteristic of a permethylated Hex*p*A-(1→3)-Hex*p*-(1→3)-Hex*p*-(1→4)-Pentitol-*1*-*d* (Fig. 5), indicating that it represented the entire linkage-region tetrasaccharide and was the permethylated derivative of β-D-Glc*p*A-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Xylitol-*1*-*d*. The intensity ratios of the A-fragments of hexuronic acids show the same relationship as for neutral hexoses and indicated[21] a terminal non-reducing D-Glc*p*A.

The response of *m/z* 233 at scan 680 in the ion chromatogram (Fig. 1) corresponds to degradation products of the polysaccharide chains under trifluoroacetolysis.

The major product of the trifluoroacetolysis was further characterised as follows. The bulk (99%) of the reaction mixture after removal of the trifluoroacetyl groups was desalted and subjected to preparative p.c., yielding 50 mg of trisaccharide [β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Xyl*p*] from 50 g of chondroitin 4-sulphate. Monosaccharide composition was analysed by g.l.c. of the alditol acetates and showed D-galactose and D-xylose in the molar ratio 2:1. The fraction containing the trisaccharide was pure as determined by g.l.c.–m.s. after reduction with $NaBD_4$ and permethylation. Methylation analysis of the reduced trisaccharide gave 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,4,6-tri-*O*-methyl-D-galactose, and 1,2,3,5-tetra-*O*-methyl-D-xylitol-*1*-*d* in equimolar proportions.



Degradation products + β-D-Gal-(1→3)-β-D-Gal-(1→4)-D-Xyl

R = →3)-β-D-Gal-(1→3)-β-D-Gal-(1→4)-D-Xyl→

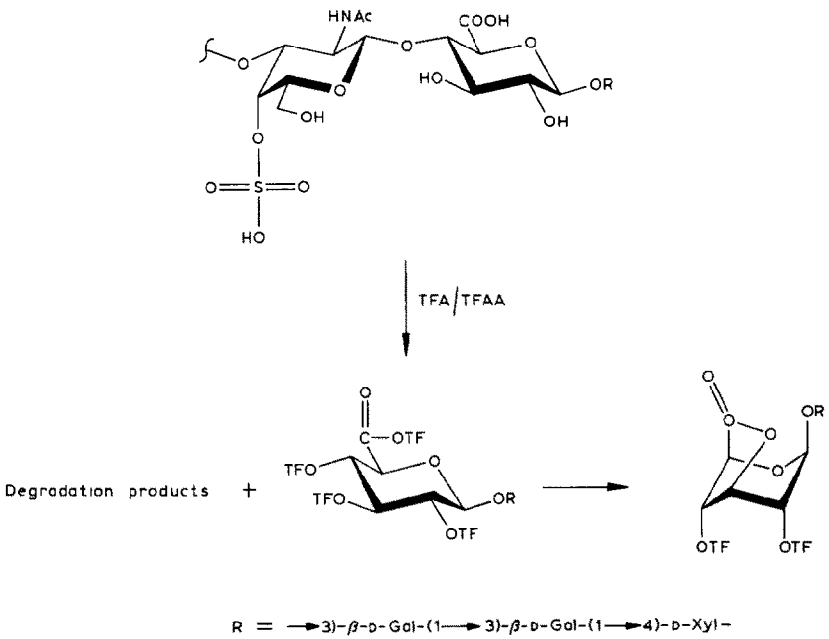
Scheme 1

DISCUSSION

Trifluoroacetolysis of chondroitin 4-sulphate releases the oligosaccharide chains from the protein core by acid-catalysed elimination[6] after degradation of the protein by transamidation[5]. The disaccharide repeating-unit, →3)-β-D-Gal*p*NAc4SO_4^--(1→4)-β-D-Glc*p*A-(1→, is degraded by solvolysis of both the glycosidic linkages. The glycosidic linkage of the 4-sulphated D-Gal*p*NAc residue is cleaved due to lack of protection by *O*-trifluoroacetyl groups, and the glycosidic linkage of the D-Glc*p*A residue is cleaved after acid-catalysed elimination of the substituent at position 4 (Scheme 1).

However, if the 4-sulphated D-Gal*p*NAc residue is solvolysed, a non-reducing terminal D-Glc*p*A residue is generated, which, to a large extent, is stable towards trifluoroacetolysis because of lactone formation[13] (Scheme 2).

The trisaccharide β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Xyl*p* is essentially stable after being pertrifluoroacetylated because of the electron-withdrawing effect of the *O*-trifluoroacetyl groups[6,8]. The formation of a small proportion of β-D-Gal*p*-(1→3)-D-Gal (peak *b*, Fig. 1) may be ascribed to degradation by elimination reactions[6]. However, the presence of β-D-Gal*p*-(1→4)-D-Xyl*p* (peak *a*, Fig.1) among the reaction products was not expected and cannot readily be explained by degradation of the trisaccharide. Although it is possible that some polysaccharide chains contained an incomplete linkage-region with only one D-galactose residue, previous



R = →3)-β-D-Gal-(1→3)-β-D-Gal-(1→4)-D-Xyl-

Scheme 2

quantitative analyses of glycopeptides from the linkage region indicate that such incomplete structures do not exist in normal cartilage[3].

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