THE PRESENCE OF A HEPARAN SULPHATE PROTEOGLYCAN IN OVINE BRAIN

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1. Introduction

Although the presence of heparan sulphate has been established in brain tissue from a variety of sources [1–3], relatively little is known about the molecular organisation of this glycosaminoglycan in the central nervous system. Heparan sulphate is known to be synthesised by glial cells [4] and the accumulation of this glycosaminoglycan has been reported in certain mucopolysaccharidoses [5]. Structural studies on heparan sulphate have shown this glucosamineglycan to resemble heparin in that it contains a repeating disaccharide unit consisting of glucosamine and glucuronic acid [6]. Unlike heparin it varies in degree of *N*- and *O*-sulphated residues [7]. Biosynthesis studies have shown that the glycosaminoglycan chains are assembled attached to a polypeptide backbone [8]. However, heparan sulphate from brain tissue has always been considered to exist as discrete glycosaminoglycan chains. We believe that the reported presence of these free chains is a result of the disruptive methods of isolation adopted by previous workers [1–3,9].

Recently we have shown the existence of a chondroitin sulphate proteoglycan from ovine brain using a non-disruptive method of extraction [10]. We have extended this study further and report here that heparan sulphate also exists covalently bound to a protein moiety.

2. Materials and methods

Fresh sheep brains (500 g portions) were freed from meninges, serous membranes and blood vessels and defatted by repeated extraction with chloroform/methanol (3:1,v/v). The lipid-free material was suspended in 0.1 M citrate, pH 3.1 and gently stirred to remove contaminating proteins. After centrifugation the residue was extracted by gentle stirring with 4 M guanidinium chloride in 0.05 M Tris–HCl, pH 7.5, 48 h at 4°C. The extract was collected and dialysed. On removal of the guanidinium chloride an insoluble residue formed (residue B) which was collected by centrifugation. This residue was repeatedly extracted with 0.1 M citrate, pH 5.0 and then with 0.4 M citrate, pH 5.0. The supernatant was collected (fraction Bn) and chromatographed on DEAE-cellulose (Whatman) in a linear salt-gradient (0–1 M NaCl) in 0.4 M citrate, pH 5.0. The fractions collected were pooled, dialysed against 1 M potassium acetate, pH 7.0 and further chromatographed on Sepharose 4B (Pharmacia). Hexuronic acid- and protein-containing fractions were dialysed and then precipitated by the addition of ethanol and potassium acetate (fraction Bns).

Amino acid content was determined on a Joel JLC BAH analyser. Protein was estimated from total amino acid concentrations and by the microbiuret method [11]. Hexosamines were measured after hydrolysis (4 M HCl for 7 h at 100°C) by amino acid analysis and the modified Elson-Morgan reaction [12]. *N*-Sulphated hexosamines were determined by the nitrous acid oxidation procedure [13]. Hexuronic acid was measured by the carbazole reaction [14]; xylose, galactose and sulphate were determined colorimetrically [15–17]. Anticoagulant activity was estimated by modification of the methods described by Sharp et al. [18].

Alkali-induced β-elimination was carried out in the presence of disulphite ions [19]. The solution was then desalted on Sephadex G-10 and the products

were separated by ion-exchange on DEAE-cellulose using a NaCl elution gradient as previously described. Glucosaminoglycans were degraded with nitrous acid by mixing a 5 mg sample with equal vol. (2 ml) 5% $NaNO_2$ and 33% acetic acid for 5 h at 25°C. After neutralisation the oxidation products were chromatographed on Sephadex G-25. Chondroitinase ABC (Sigma) digestion was carried out as described by Yamagata et al. [20]. Infrared spectra were obtained with KBr discs in a Unicam SP 200 spectrophotometer.

3. Results and discussion

It appeared from ion-exchange and gel chromatography on 4% agarose (fig.1) that the heparan sulphate preparation behaved as a monodispersed species in which hexuronic acid- and protein-containing material migrated as a single peak. This fraction (fraction Bns) accounted for 0.8% of the total weight of lipid-free dry brain.

Fraction Bns was found to be resistant to chondroitinase ABC, and the only amino sugar detected was glucosamine. The carbohydrate moiety was highly sulphated, having a sulphate to glucosamine molar ratio of 1.8:1. The molar proportion of hexuronic acid and glucosamine were found to be approximately equal which suggests that the glucosaminoglycan chains had not undergone any extensive damage during isolation (see table 1).

It is known that nitrous acid reacts only with *N*-sulphated hexosamine residues to form 2,5-anhydro-D-mannose [21]. When the preparation was treated with nitrous acid two major fractions were eluted from a Sephadex G-25 column, fractions *X* and *Y* (fig.2). Fraction *X*, which was not retained by the column, was found to contain protein. However no anhydromannose derivative was detected by the indole reaction. On amino acid analysis of this fraction only glucosamine was detected, with a glucosamine to hexuronic acid molar ratio of 0.9:1. This suggests that fraction X contains a small proportion of repeating dissacharides in which the hexosamine residues are resistant to nitrous acid. The fraction retained by the column (fraction *Y*) was found to contain anhydromannose equivalent to 70% of the total hexosamine present in the original Bns preparation (fig.2). This implies that a large proportion of the heparan sulphate chains is composed of *N*-sulphated glucosamine. In addition infrared analysis of the untreated Bns fraction revealed the presence of a strong absorption band in the region of 1240 cm^{-1} which is characteristic of *N*-sulphated substitution [22]. However this absorption was not as strong as that obtained from a sample of commercial heparin.

A study of the anticoagulant activity showed that the sample was biologically active; 15 IU/mg compared to an activity of 150 IU/mg for a heparin sample. This value is in keeping with results obtained by other workers for highly sulphated heparan-sulphate species [23].

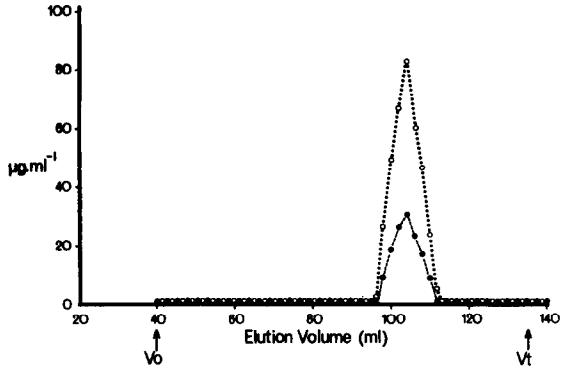

Fig.1. Elution profile of proteoheparan sulphate chromatographed on Sepharose 4B column. Fractions were assayed for protein (•) and hexuronic acid (○). V_o and V_t of the column are indicated by arrows.

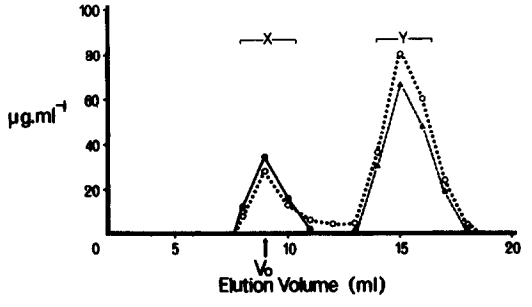

Fig.2. Gel filtration on Sephadex G-25 of the proteoheparan sulphate preparation (fraction Bns) after nitrous acid treatment. Fractions were analysed for hexuronic acid (○); 2.5-anhydro-D-mannose (▲); protein (•). Procedures used are given in Materials and methods.

Table 1
Amino acid analysis and chemical composition of proteoheparan sulphate (fraction Bns)

Amino acid	Untreated sample	Alkali-treated sample
Cysteic acid	–	37
Hydroxyproline	–	–
Aspartic acid	110	108
Threonine	69	73
Serine	220	182
Glutamic acid	102	105
Proline	20	23
Glycine	182	176
Alanine	69	73
Valine	63	58
Cysteine	–	–
Methionine	–	–
Isoleucine	21	20
Leucine	45	43
Tyrosine	11	13
Phenylalanine	9	10
Lysine	36	32
Histidine	10	12
Arginine	33	35

Chemical composition

Protein	Glucosamine	Xylose	Galactose	Sulphate (K^+ salt)	Hexuronic acid: Glucosamine (molar ratio)
12	26	0.47	1.2	31	1.2:1

Amino acid contents are expressed as residue/1000 residues and are corrected for hydrolytic losses. Chemical composition values are expressed as percentages of dry ash-free sample.

The amino acid composition and total chemical composition of the proteoheparan sulphate is reported in table 1. Treatment of the protein moiety with alkali produced a 17% decrease in serine content with the formation of an equivalent amount of cysteic acid. There was no decrease in threonine which implies that the polysaccharide chains are linked to a specific serine residue within the protein backbone. Neutral sugar content revealed a galactose to xylose molar ratio of 2.1:1. This indicates that the potential reducing ends of the glycosaminoglycan chains have not undergone degradation during the isolation procedure. Furthermore the amount of serine lost during alkali-induced β-elimination accounted for 0.45% of the total preparation. This value is in agreement with the amount of xylose present in the preparation (table 1). Therefore, it would appear that a Ser–Xyl–Gal–Gal linkage region is present, which is characteristic of most proteoglycans [24].

The molecular weight of heparan sulphate has been estimated from a number of sources and values ranging from $3–170 \times 10^3$ have been reported [23,25]. This particular preparation was found to have a glycosaminoglycan chain weight of about 32×10^3 as estimated from xylose concentrations. However, fraction Bns was found to be excluded from Sephadex G-100 and when further chromatographed on 4% agarose (fig.1), a K_{av}-value of 0.67 was estimated [26]. Under the high ionic strength of the eluting buffer used, repulsive forces would be minimised and so the carbohydrate moiety would be expected to adopt a random-coil conformation.

In conclusion we postulate that fraction Bns consists of a multi-chain complex in which heparan

sulphate is covalently bound to a common protein backbone. To date the possible function of this macromolecule in vivo has not been established since this appears to be the first time that a heparan sulphate proteoglycan has been isolated from brain tissue.

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