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Proteoglycans from the gum exudate of myrrh

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The watersoluble gum fraction of myrrh (40% w/w), has been found to comprise of a heterodisperse mixture of proteoglycans with dominating amounts of uronic acid rich polymers. The major fraction (70% w/w) was a proteoglycan ($M_r \sim$ 200 000) with protein content of 10% and Hyp as the main amino acid (40%). Structural investigations using mild acid hydrolysis, Smith degradation and alkaline hydrolysis demonstrated that two different types of sugar chains are linked to the protein-core. These comprise either of short chains of arabinose or of slightly branched chains composed of galactose and 4-*O*-methyl-glucuronic acid. Both structures are linked to the protein moiety via Hyp. As the galactose is substituted at C4 with 4-*O*-methyl-glucuronic acid, which is substituted at C2 with galactose, for this specific structure the term "4-*O*-methyl-glucurono-galactan" is proposed. It has not yet been reported in any other gum described so far.

Gum myrrh containes furthermore arabino-3.6-galactan–protein fractions with an average molecular weight of about 70 000 and a protein amount of 31 and 16%, respectively.

It can not be concluded whether the different types of proteoglycans represent the naturally occurring diversity of polymers or whether they derive from degradation processes during isolation or storage of the commercial sample of myrrh, as it is used in the purview of the European Pharmacopea.

INTRODUCTION

Myrrh is a commercially used resin of different Commiphora species, mostly of *Commiphora molmol*, Engler, Burseraceae, obtained after injuring the bark of the small trees, growing wild in Somalia, Jemen and Arabia. It is mainly used in toothpastes and tinctures for the treatment of gingivitis and is composed of three main constituents: (i) the essential oil (2–10%); (ii) the EtOH soluble resin (25–40%); and (iii) the watersoluble gum (30–60%) (Tucker, 1984).

Despite the fact that myrrh is one of the oldest remedies, especially in oriental medicine, little is known about its exact chemical composition. Mainly for the presence of essential oil, it has been widely used until today for the treatment of inflammatory diseases of the mucosa of mouth and throat, as well as for its desinfecting and astringent properties.

Concerning the structural properties of its main constituent, the gum, it is only known that it contains Ara, Gal and 4-*O*-Me-GlcA (Hough *et al.*, 1952). Based on these results, gum myrrh has been estimated as an AGP-type gum A according to the classification of Stephen (1980).

The aim of this study was to gain more detailed information about this polymer fraction, about its homogeneity and the basic structural details.

EXPERIMENTAL

Myrrh resin was purchased from Caesar and Loreriz, Hilden, FRG, in German Pharmacopoca (DAB 10) quality without further indication of the precise botanical origin. All chemicals were of analytical grade quality. DEAE–Sephacel, DEAE–Sephadex A 25, Sephadex G 25 and Superose^{TM}6 and 12 were purchased from Pharmacia, Biogel P6 and P12 from Biorad.

General methods

Total carbohydrate, uronic acid and protein were assayed by the anthrone (Morris, 1948), *m*-hydroxybiphenyl (Blumenkrantz & Asboe-Hansen, 1973) and Lowry methods (Lowry *et al.*, 1951) respectively, using neutral standard sugars, GalA, and bovine serum albumin as the respective standards. Hydroxyproline was assayed by the Leach test (Leach, 1960).

Amino acids were analysed with the aid of Biotronic LC 5001, column BTC 2710 (3.2 × 385 mm), bed height 210 mm, coil temperature 133°C, using ninhydrine for detection. For this analysis the samples (2–5 mg) were

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hydrolysed under vacuum in a sealed tube with 6 M hydrochloric acid for 24 h at 110°C. After hydrolysis the insoluble components were strained and hydrochloric acid was removed by evaporating twice, diluting with water under reduced pressure at 40°C.

The molecular weight distribution of the proteoglycans was estimated by GPC on Superose^{TM}6 with the aid of standard pullulans (Shodex).

Polysaccharides were hydrolysed with 2 M trifluoroacetic acid (TFA) at 121°C for 1 h (Albersheim *et al.*, 1967). After removing TFA, sugars were converted into their corresponding alditol acetates by the method of (Blakeney *et al.*, 1983). GLC was performed at 207°C using a Varian 3500 gas chromatograph with FID on a glass capillary column (0.25 mm × 25 m) packed with 3% of OV 225, using He as carrier gas. The molar ratio of the neutral sugars was calculated from the peak areas and molecular weight of the corresponding alditol acetates. The molar ratio of uronic acid and neutral sugars was calculated from the uronic acid content.

Reduction of acidic sugars was done in the presence of carbodiimide using $NaBD_4$ (Taylor & Conrad, 1972).

Isolation of gum myrrh

Myrrh was ground in a mortar to a particle size of 1–2 mm and pre-extracted by successive percolation with CH_2Cl_2, C_2H_5OAc and MeOH until the extract was colourless to remove the lipophilic components. The dried pre-extracted powder was stirred with water as a 5% suspension at 20°C for 6 h. The crude gum was obtained by pouring the water extract into a five-fold volume of 99% EtOH at 4°C. The precipitate was dissolved in water, dialysed against water (M_r CO = 3500) and freeze-dried (crude gum A).

In a second experiment, myrrh was reduced to small pieces as mentioned above and stirred for 6 h with water at 20°C without pre-extraction. Further isolation processing was done as in the above procedure (crude gum B).

Fractionation of gum myrrh

Purification and fractionation was carried out on DEAE–Sephacel (PO_4^{3-}, pH = 6.0). The crude gum B was subsequently eluted with water, a four-step phosphate-buffer gradient (0.13, 0.26, 0.33, 0.39 M) with linear end (0.39–1 M) as shown in Fig. 1 and finally 0.2 M NaOH.

Pronase treatment

The proteoglycans (25 mg/ml) were incubated with pronase E (Serva) in 0.15 M tris-acetate buffer pH 7.8, 1.5 mM in calcium acetate (Spiro, 1976). The enzyme was added initially in an amount equal to 1% of the

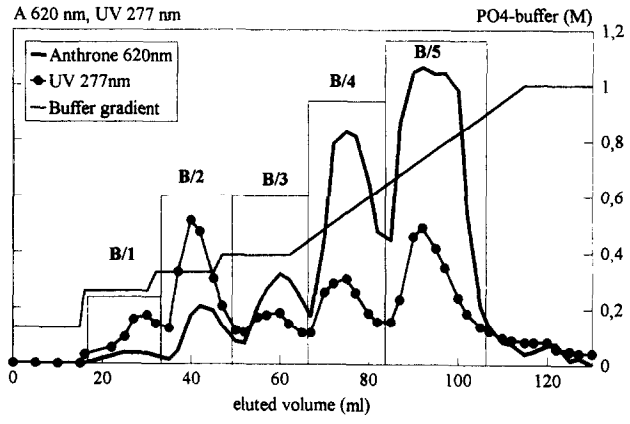

Fig. 1. Ion exchange chromatography and fractionation of Gum B on DEAE–Sephacel with a four-step phosphate-buffer gradient (0.13, 0.26, 0.33, 0.39 M) with linear end (0.39–1 M).

weight of the proteoglycan and again at 24 and 48 h intervals in amounts equal to 0.5% of the substrate weight. The incubation was carried out at 37°C for 72 h with gentle shaking. After freeze drying the material was dissolved in 5 ml 0.1 M pyridine acetate buffer pH 5.0 and the insoluble material was removed by centrifugation. The supernatant was separated by GPC on Sephadex G 25 with 0.1 M pyridine acetate buffer pH 5.0 as eluent.

Mild acid hydrolysis

The proteoglycan fractions were subjected to hydrolysis with increasing strength, using TFA at concentrations of 0.05 and 0.5 M at 100°C for 1 h. The resulting partially degraded products were separated by GPC on Biogel P2, which had been calibrated using pullulanes (Shodex) stachyose and glucose.

Methylation analysis of acidic proteoglycans

Premethylation and methylesterifying carboxylgroups
The sample (1 mg) was stirred in a small amount of water (5 µl) in a screw-cap vial for 30 min, and then added to a 10-fold excess of dry DMSO and stirred for another 3 h. The first methylation was performed with 500 µl of dansyl-K and 365 µl of CH_3-J, stirred for 3 h with 365 µl of CH_3-I. The sample was kept stirring overnight. While cooling the sample, 5 ml of water and a few crystals of $Na_2S_2O_3$ were added to remove the excess of methyliodine and nitrogen was bubbled through the solution until it became uncoloured and clear. The solution was dialysed for 24 h against water (M_r CO = 1000) and freeze-dried.

Reduction of uronic acid
This procedure was done according to Stevenson (Stevenson *et al.*, 1988). The premethylated sample was reduced with 1 ml of $Li(Et)_3BD$ (4 M in tetra-

hydrofuran). After 4 h the reduction was quenched by adding 20 µl aliquots of HAc until foaming stopped. TM solvents were removed in a stream of nitrogen. Again the sample was dialysed.

Methylation of the reduced sample

The premethylated and deutero reduced polysaccharides were methylated according to Harris *et al.* (1984) by dissolving in 200 µl dry DMSO. Methylation was done twice with Dimsyl–K/CH_3I in the ratio 60/15 µl and 200/150 µl successively. The methylated samples were hydrolysed, the products reduced with $NaBH_4$, followed by acetylation.

The resulting partially methylated alditol acetates were analysed by GLC–MS on a Hewlett-Packard GC 5890 A with a bonded phase fused Silica capillary column SP 2380 (0.2 µm film, 0.25 mm × 30 m).

NMR-analysis

^{1}H-NMR was performed on a Bruker WM at 250 MHz and 297 K with D_2O as internal standard (δ = 4.67 ppm).

^{13}C-NMR was run at 297–307 K at 62.89 MHz with TMS as internal standard. Samples were dissolved in D_2O.

Identification of aldobiuronic acids

Gum B was hydrolysed with 2 M TFA in a heating block at 121°C for 1 h. After removal of TFA the hydrolysate was separated by GPC on Biogel P2. Fractions were monitored for carbohydrate (Morris, 1948) and uronic acid (Blumenkrantz & Asboe-Hansen, 1973).

Alkaline hydrolysis

A portion of proteoglyan (~ 100 µg Hyp) was heated in 0.5 ml 0.2 M $Ba(OH)_2$ at 105–110°C for 18 h in a heating block (Lamport, 1984). After neutralisation to pH 7.0 with conc. H_2SO_4 and centrifugation, the products were recovered by freeze-drying of the supernatant solution. The hydrolysed samples were fractionated on Biogel P6 with water as eluent (Fig. 3) and by IEC on DEAE–Sephadex A 25 (pH = 6.0) with a linear imidazol–buffer gradient (0.01–1 M) as shown in Fig. 5. Fractions were monitored for carbohydrate (Morris, 1948), uronic acid (Blumenkrantz & Asboe-Hansen, 1973) and for Hyp content (Leach, 1960).

Smith degradation

Fraction B/5 (15 mg) was degraded with 10 ml 0.05 M $NaIO_4$ for 48 h in the dark (Blaschek *et al.*, 1987). After destroying the excess of periodate the solution was dialysed and freeze dried. Reduction was performed with 5 ml 0.4% $NaBH_4$ during 15 h at room temperature. After neutralisation with 50% acetic acid, dialysis and lyophilisation, the resulting fragments were hydrolysed with 1 ml 0.5 N TFA during 15 h at room temperature.

RESULTS

Isolation of the water-soluble compounds and identification of the constituents

In the previous work on the structure of gum myrrh (Hough *et al.*, 1952), organic pre-extraction of the resin was applied due to the major amount of terpenoids present in the resin. With reference to this previous work, a pre-extraction of the resin was done by successive treatment with organic solvents prior to the extraction with water (crude gum A). In parallel, a check of the necessity of this step was performed by omitting the pre-extraction procedure and extracting directly the resin with water (crude gum B).

After pre-extraction with lipophilic agents the water extract of the remaining yellow–brown powder yielded 39.1% of a crude gum (A). It was composed of protein (16.4%) and large amount of carbohydrate with Gal, Ara and uronic acid as the main components besides small amounts of Rha, Man and Glc, respectively.

After reduction with $NaBD_4$ the reduced compound could be identified by GC–MS according to retention time and fragmentation pattern (Doares *et al.*, 1991) as 4-*O*-methyl-glucose, labelled with deuterium at C6, proving the identity of the native uronic acid as 4-*O*-methylglucuronic acid. The endogen methyl group in position 4 was proved additionally by inethylation with CD_3I, and by the respective signal in NMR-analysis.

The presence of this uronic acid has been described in other gums, for example mesquite (Aspinall & Whitehead, 1970) or gums from acacia species (Gammon *et al.*, 1986). Otherwise it is widely distributed as a component of 4-*O*-methylglucuronoxylans (Shimizu *et al.*, 1978; Yoshida *et al.*, 1990).

No other UA could be detected in the crude gum or its subtractions.

Crude gum B, obtained after direct extraction with water in a yield of 40.3%, showed a similar composition in the carbohydrate moiety as crude gum A, and also the protein content was comparable (16.8 %).

The molecular weight distributions of both crude gums were identical, but heterogenous in a wide range from 40.000 to 300.00 d with a maximum at 120 000–130 000.

These results demonstrated the similarity of gums isolated either with or without organic pre-extraction. Thus it was chosen to continue work with gum type B due to the simplicity of the procedure and the higher originality of the sample resulting from less thermal stress and minor chance of chemical alteration of the gum by the pre-extraction procedure.

Fractionation

Five acidic fractions were obtained by DEAE–Sephacel chromatography of gum B, applying a phosphate-buffer gradient (Fig. 1).

UV-detection at 277 nm showed coelution of protein in all carbohydrate containing fractions. This was a strong indication for the existence of covalent linkages between carbohydrate and protein. Table 1 shows the yield, the protein- and the carbohydrate content and also the buffer concentration of fraction B/1–B/5.

The acidic fractions B/4 and B/5 represented the dominating part of the gum with a yield of 75% of the total eluted material. They were eluted at a buffer strength of 0.55 and 0.78 M, respectively. All fractions were analysed for their sugar and protein composition except B/1 for its minor amount and its high protein- and minor sugar content.

The sugar composition of fraction B/2–B/5 was determined both with and without proceeding reduction of the 4-*O*-methylglucuronic acid with $NaBD_4$.

As a result the galactose content increased in all fractions after deutero reduction of the 4-*O*-methylglucuronic acid, but without any galactose being deutero marked. This can be explained as the result of the reduction of 4-*O*-methylglucuronic acid and therefore a better susceptibility to complete hydrolysis of what would otherwise form aldobiuronic acids. Table 2 shows the sugar composition of fraction B/2–B/5 after reduction of the uronic acid.

After reduction of the 4-*O*-methylglucuronic acid the ratio of Gal:4-*O*-Me-GlcA was approximately 1:1 in the two main fractions B/4 and B/5.

The fractions B/2–B/5 differed markedly in their Ara content, which decreased from 25.8% in B/2 to 7.7% in fraction B/5. The protein content and the amino acid composition also differed markedly as shown in Table 1 and Table 3.

In the main fractions B/4 and B/5, Hyp was found as the dominating amino acid followed by Ser and Ala. The fractions with a higher protein content (B/1–B/3) showed Asn, Gly, Ala, Gln and Leu as main amino acids, while Hyp was present in smaller amounts.

Table 1. Characteristics of the proteoglycans: yield, protein- and carbohydrate content and elution buffer strength of the fractions B/1–B/5

Fraction	Yield (%)	Protein (%)[a]	Sugar (%)[a]	PO_4^- buffer (M)
B/1	1.8	49.4	15.4	0.26
B/2	4.7	31.1	32.9	0.33
B/3	7.7	15.9	54.9	0.39
B/4	18.4	12.6	69.9	0.55
B/5	25.6	10.1	70.6	0.78

[a]Weight %.

Table 2. Sugar composition (mol%) of fraction B/2–B/5 from myrrh gum after uronic acid reduction

Fraction	4-*O*-Me-Glc[a]	Rha	Ara	Man	Gal	Glc
B/2	27.2	1.9	25.8	4.2	35.5	5.5
B/3	38.8	0.5	17.5	2.1	39.0	2.4
B/4	45.6	0.6	12.5	0.9	39.1	1.3
B/5	47.2	0.8	7.7	0.6	43.0	0.9

[a]Deutero reduced 4-*O*-Me-GlcA.

Table 3. Amino acid composition (mol%) of the fractions B/1–B/5 of gum myrrh

	Fraction				
Amino acid	B/1	B/2	B/3	B/4	B/5
Hyp	2.7	8.3	16.9	32.4	38.8
Asp	12.1	11.3	9.6	5.0	3.7
Thr	5.8	5.1	5.0	4.9	4.6
Ser	7.7	6.7	11.0	13.0	13.0
Glu	7.6	7.0	8.2	6.6	6.6
Pro	6.2	6.3	4.8	4.2	3.7
Gly	9.0	8.7	6.7	3.6	2.7
Ala	8.6	8.6	9.0	8.9	8.6
Cys	0.9	0.1	—	—	—
Val	7.3	6.6	4.4	2.4	1.7
Met	0.5	0.3	0.6	0.4	0.4
Ile	6.1	5.6	3.8	2.7	2.1
Leu	8.3	8.1	7.1	6.1	5.7
Tyr	3.5	3.2	2.7	1.9	1.6
Phe	5.3	4.9	3.2	1.8	1.3
Lys	2.7	3.2	2.9	2.8	2.2
His	1.9	3.5	1.6	1.3	1.4
Arg	3.3	1.1	2.2	1.5	1.4

A correlation between molecular weight and uronic acid content could be seen: The most acidic fraction B/5 had the highest M_r of 200 000. Fraction B/4 had an average M_r of 120 000, B/3 67 000, B/2 42 000, and B/1 had two maxima, at 42 000 and 16 000 respectively.

For the main fractions B/3–B/5, it could be shown to be polydisperse over a broad range of M_r but homogeneous by means of their composition of constituents. Therefore, no further purification of the above fractions was performed prior to detailed analysis procedures.

Methylation analysis

Complete methylation of acidic polysaccharides can be quite difficult in the presence of uronic acids, especially when they are substituted in position C4 (Björndal *et al.*, 1970; Shimizu, 1981), and when the compound is of a large M_r, resulting in several problems like under-methylation or β-elimination.

From the gathered data concerning UA-content, identity and the large M_r, it was necessary to reduce the acidic polysaccharide fractions prior to premethylation.

The fractions B/2–B/5 were methylated as described for the analysis of Rhamnogalacturonan type II (RG II)

(Stevenson *et al.*, 1988). After the sample had been dissolved in DMSO and traces of water, the premethylation was done resulting in methylesterification of the carboxyl groups of the uronic acid. For reduction of these carboxy groups, $LiEt_3BD$ was used. The reduction was found to be complete (> 95%) when checked by the biphenyl method. The second step of methylation, the perinethylation, gave a complete methylation of the free hydroxyl groups and the dideutero groups of the reduced carboxyl function.

In Table 4 the results of this methylation for the proteoglycans B/2–B/5 are listed.

All fractions contained 4-*O*-methylglucuronic acid in terminal or 2-linkage. Gal was shown to be 3.6-branched or 3- and 4-substituted; the proportion of these three linkage types varied in the different fractions. Ara appeared in all fractions in the furanose form only. For fraction B/2 almost 75% of Ara was terminal; the other fractions had more than the half of Ara in terminal position. The main fractions B/4 and B/5 did not differ markedly in their composition (Table 3), but in their ratio of linkages. While the ratio of terminal Ara: 2-linked Ara (1, 3:1) and terminal UA: 2-linked UA (1:3) was almost identical in these two fractions, the amount of 3-linked Gal (including 3.6-branched Gal) was higher in fraction B/4.

The size of sugar chains can theoretically be determined by the ratio of terminal to glycosidically linked sugars. The ratio found in the fractions B/2–B/5 indicated the presence of short oligosaccharides with a dp of 3–7 sugars. Considering the high molecular weight of these fractions, together with their marked protein content, a covalent binding of these short oligosaccharides to a protein is the only explanation for this finding.

Table 4. Sugar linkages (mol%) after methylation in the main fractions B/2–B/5 of gum myrrh

Sugar-	Linkage	B/2	B/3	B/4	B/5
Rha	1-	1.6	—	—	—
Ara (f)	1-	20.3	10.7	6.5	4.3
	1.2-	5.0	4.8	5.4	3.6
	1.3-	2.5	2.9	—	—
	Σ	27.8	18.4	11.9	7.9
4-*O*-Me-Glc[a]	1-	17.6	19.5	11.1	9.2
	1.2-	11.7	20.2	30.1	34.7
	Σ	29.3	39.7	41.2	43.9
Gal	1-	0.5	0.8	—	—
	1.3-	8.8	8.0	7.0	3.9
	1.4-	8.4	13.0	25.3	36.2
	1.6-	2.7	2.5	1.8	—
	1.3.6-	18.0	17.9	12.7	8.2
	Σ	38.3	41.9	46.9	48.2
Man	1-	1.1	—	—	—
	1.2-	2.0	—	—	—
	Σ	1	3.0		

[a]Deutero reduced 4-*O*-Me-GlcA.

In order to confirm this, it was necessary to degrade the macromolecule by several methods (Lamport, 1980), and to investigate the carbohydrate and protein moieties and the covalent binding site.

Pronase degradation

Pronase E is widely used for the removal of protein in cellwall components (Selvendran, 1975), but this enzyme is known to be not suitable for Hyp rich glycoproteins (Fincher *et al.*, 1974; Neukom, 1976). Extensin (Fry, 1988) and AGP are known to be resistant against proteases (Jermyn, 1980; Fincher *et al.*, 1983) because of their "Wattle Blossom" structure: the protein core is shielded by the voluminous polysaccharide chains against the enzyme (Fincher *et al.*, 1983).

Incubation of the crude Gum B with pronase E for 18 h (Asamizu and Nishi, 1979) did not result in a removal or a compositional change of the protein moiety.

Variation of the degradation procedure, as developed for certain glycoproteins (Spiro, 1976), were applied, but the protein moieties of the fractions B/2–B/5 were not affected in amount or composition. This result indicates that the protein moiety in Gum B is not a suitable substrate for pronase degradation, due to its high Hyp content and its sterical protection against enzymatic digest.

Partial acid hydrolysis of the carbohydrate moiety

The effect of hydrolysis with increasing TFA-concentration on M_r and the sugar composition was investigated for fractions B/2–B/5.

Fraction B/2

B/2 consisted of 27.8% Ara (terminal-, 2- and 3-linked), 29.3% 4-Me-GlcA (terminal and 2-linked) and 38.3% Gal (3-, 4-, 6-linked and 3.6-branched) (Table 5).

After hydrolysis with 0.05 N TFA and separation by GPC on Biogel P2, 40% of Ara and traces of Gal had been cleaved. The second hydrolysis procedure using 0.5 N TFA resulted in a complete removal of Ara. The ratio of UA:Gal remained unchanged. The methylation analysis of the residual fraction B/2a is shown in Table 5.

After the second hydrolysis step Ara was totally removed, thus increasing the respective amounts of Gal and 4-*O*-Me-GlcA. The relative amount of 6-linked Gal increased, while correspondingly the relative amount of 3.6-branched Gal decreased to about half of its former share in total Gal amount. The relative moieties of 4-linked Gal, 3-linked Gal and uronic acid remained unchanged.

These observations lead to the conclusion that Ara must be linked via C3 to every second 3.6-branched Gal.

Table 5. Sugar linkages (mol%) of the fractions B/2 and B/3 after partial hydrolysis with 0.5 N TFA

Sugar-	Linkage	B/2	B/2a	B/3	B/3a
Rha	1-	1.6	—	—	
Ara (f)	1-	20.3	—	10.7	—
	1.2-	5.0	—	4.8	—
	1.3-	2.5	—	2.9	—
	Σ	27.8	—	18.4	—
4-*O*-Me-Glc[a]	1-	17.6	18.6	19.5	18.3
	1.2-	11.7	15.3	20.2	23.5
	Σ	29.3	33.9	39.7	41.8
Gal	1-	0.5	—	—	—
	1.3-	8.8	14.5	8.2	12.1
	1.4-	8.4	16.8	13.1	17.5
	1.6-	2.7	17.2	2.5	11.1
	1.3.6-	18.0	19.7	17.9	17.5
	Σ	38.3	66.1	41.9	58.2
Man	1-	1.1	—	—	—
	1.2-	2.0	—	—	—
	Σ	3.0			

[a]Deutero reduced 4-*O*-Me-GlcA.

Since 75% of the Ara was linked terminally and about twice as much Ara-molecules were released compared to the amount of 6-linked Gal, which originated from 3.6-branched Gal, part of Ara seems to be directly linked to protein (see Section 3.8).

The stoichiometrical ratio of the described findings lead to the proposal of a partial structure, showing major oligosaccharide sidechains with terminal 4-*O*-Me-GlcA, 1,3- and 1,4- linked Gal, and 1.3.6- branched Gal with one half of it linked to terminal Ara. The possible arrangement of these compounds is given in Fig. 2.

Fraction B/3

After the first hydrolysis step (0.05 N TFA), 45% of Ara was removed. The second hydrolysis (0.5 N TFA) cleaved nearly all Ara residues. The remaining fraction B/3a contained 4-Me-GlcA and Gal only. The relative amount of 6-linked Gal increased from 6 to 19%, compared to a decrease of 3.6-branched Gal from 43 to 30% (Table 5). The proportions of the other sugar linkages did not change significantly.

This demonstrates that every third 3.6-branched Gal contained terminal linked Ara in position 3. With an Ara content of 18% in Fraction B/3, 40% of this Ara was detectable as a substituent of Gal. For 60% of the Ara no substitution neither to the uronic acid nor to C3 or C4 of Gal could be found, indicating that this amount of Ara is linked directly to the protein core.

Considering this finding, fraction B/3 of the crude gum consists of a proteoglycan with at least two different carbohydrate moieties linked to the protein core: arabinans as short chains or monomer units and a galactan bearing 4-*O*-Me-GlcA, Gal and Ara in its sidechains.

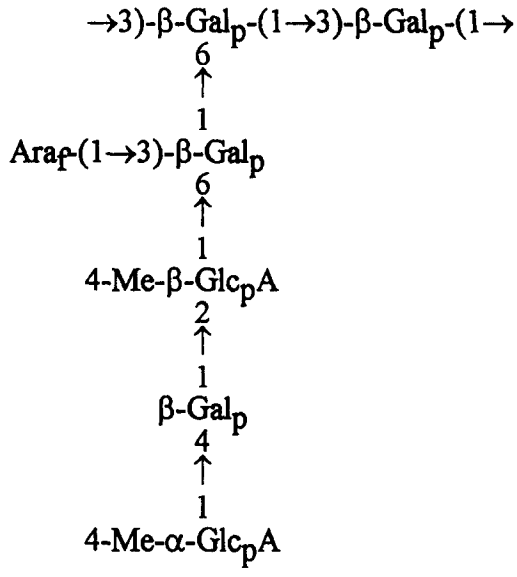

Fig. 2. Proposed partial structure of fraction B/2.

Fractions B/4 and B/5

The fractions B/4 and B/5 were the major portions of gum myrrh after IEC separation. They contained 12.5 and 7.7% Ara respectively (Tables 2 and 4). After partial acid hydrolysis and subsequent GPC, no Ara could be detected in the higher molecular weight fraction. Nevertheless, this did not affect the methylation results of the degraded polymers (B/4a, B/5a) (results not given). This could indicate a direct linkage of Ara to the protein (see Section 3.8).

Smith degradation

The main fraction B/5 was subjected to a sodium periodate treatment (Blaschek *et al.*, 1987) for 48 h. After reduction and hydrolysis, the remaining polymer yielded 23% of the original amount. Sugar analysis showed 4.8% Ara, 31.8% 4-Me-GlcA and 62.3% of Gal. Comparison with composition of B/5 (see Table 2) showed a major loss of uronic acid.

The protein content increased from 5.3 to 14.5%; the amino acid composition of the protein moiety remained constant.

However, the methylation analysis of fraction B/5smith showed an incomplete degradation: 15% 4-linked Gal were detected (although this is a labile linkage type for periodate oxidation). 2-Linked 4-Me-GlcA decreased from 34.7 to 7.6%, though it should stay resistant against Smith degradation due to the methylgroup in position 4.

In a new attempt for a Smith degradation, reaction time was prolonged up to 7 days (Stephen *et al.*, 1990). After dialysis (M_w CO $<500\,000$), no fragments could be detected, indicating that fraction B/5 had completely been degraded.

A structure of alternating uronic acid connected to 4-linked Gal in form of aidobiuronic acids could have led

to a degradation to disaccharides, which were removed during dialysis. Due to the lack of material, no further Smith degradation experiments could be performed. The data suggest that in this fraction a core consisting of 3- or 3.6-Gal must be missing, and an alternating arrangement of 4-Me-GlcA and Gal is most probable.

Identification of aldobiuronic acids

The occurrence of aldobiuronic acids in gum myrrh has been reported earlier (Hough *et al.*, 1952; Jones & Nunn, 1955). For the identification of these structures in the present gum B was hydrolysed with 2 M TFA and the resulting products were separated by GPC on Biogel P2.

Besides monosaccharides two fractions with a higher molecular weight ($M_r \sim 4000$) were found. These fractions contained 4-*O*-methylglucuronic acid and Gal as the only sugars. Analysing the reduced fractions a complete hydrolysis could be achieved and the amount of 4-Me-Glc and Gal was found to be equimolar.

Both fractions eluted as brown and oily fractions containing coeluted protein moieties which could explain their chromatographical behaviour. The protein composition was equal to that in crude gum B.

Methylation analysis of the two fractions showed that 4-*O*-Me-GlcA only appeared in terminal linkage, whereas the Gal moiety showed linkage via C4 and C6 with only minor quantitaties of terminal Gal.

The amount of 4-Gal, 36.7 and 39.0% respectively, was about six times higher than the amount of 6-Gal with 5.3 and 8.1%.

These results indicate the presence of disaccharides in gum B, probably linked to a protein moiety not removable by the chosen hydrolysis method (as could be expected from the results of alkaline hydrolysis). Methylation analysis characterised these disaccharides as aldobiuronic acids, 4-*O*-(4-*O*-methyl-D-glucuronosyl)-D-galactose and 6-*O*-(4-*O*-methyl-D-glucuronosyl)-D-galactose respectively, in a molar ratio of about 6:1.

Alkaline hydrolysis

Alkaline hydolysis was performed with crude gum B and with its major fraction, B/5.

Crude gum B

Hot alkali can be used for a selective cleavage of peptide linkages and sensitive *O*-glycosidic linkages of certain sugars to a protein core. Linkages between sugars and sugar–Hyp are more stable and usually remain uncleaved (Lamport, 1980).

The crude myrrh gum B was hydrolysed with 0.2 M $Ba(OH)_2$ for 18 h. Fractionation of the hydrolysate on Biogel P6 is shown in Fig. 3.

After hydrolysis gum B (M_r 40 000–200 000) was degraded to lower molecular weight fractions (B/a.h.1–B/a.h.4) with a yield of 60% of the original gum B. Fraction B/a.h.1–B/a.h.4 yielded 14.4, 41.5, 28.5 and 15.6% of the found gum respectively. Their molecular weights were determined as 6600, 3900, 1800 and 400–700, respectively. In all fractions Hyp could be detected. Quantification of Hyp was done both colorimetrically (Leach, 1960) and by amino acid analysis. It was shown that during treatment with hot alkali racemisation of the natural occurring *cis*-L-Hyp to *trans*-L-Hyp had occurred (Lamport & Miller, 1971; Gammon *et al.*, 1986) The two epimers differed in their retention time and were found to be equimolar. No other amino acids were detectable.

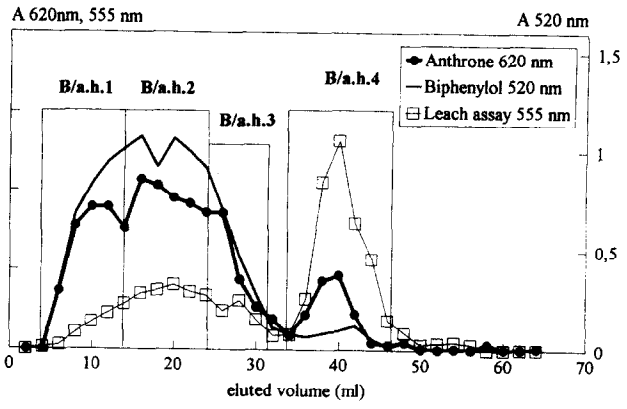

Fig. 3. Gel permeation chromatography and fractionation of alkaline hydrolysate of Gum B on Biogel P6.

The composition of the fractions are shown in Table 6.

The fractions B/a.h.1–B/a.h.3 differed markedly in their Ara-content, which, with the exception of fraction B/a.h. 1, was significant lower than the amount of Ara in the gum B (16.6%). Fraction B/a.h.4 consisted mainly of Ara.

The main fraction B/a.h.2 was rechromatographed and separated by IEC (Fig. 4). Fraction a.h.2A could not be quantified, a.h.2B had a yield of 18% and the main fraction a.h.2C 48%. The main fractions a.h.2B and a.h.2C contained 4-Me-GlcA and Gal as the dominant sugars besides traces of Ara. All fractions

Table 6. Composition of the fractions B/a.h.1–4 (mol%) including Hyp content

Fraction	4-*O*-Me-GlcA	Rha	Ara	Man	Gal[a]	Glc	Hyp
B/a.h.1	40.7	—	12.9	0.4	41.7	0.6	0.3
B/a.h.2	43.7	—	4.6	1.1	42.3	0.3	3.9
B/a.h.3	39.4	3.6	18.4	3.6	38.2	1.2	5.9
B/a.h.4	—	—	65.2	4.2	4.3	0.6	25.6
B/a.h.2B	45.5	1.4	5.4	0.7	39.4	1.0	6.0
B/a.h.2C	44.2	—	1.5	1.3	47.5	1.7	3.6

[a]Due to the presence of aldobiuronic acids (not quantified) the amount of Gal may be underrepresented.

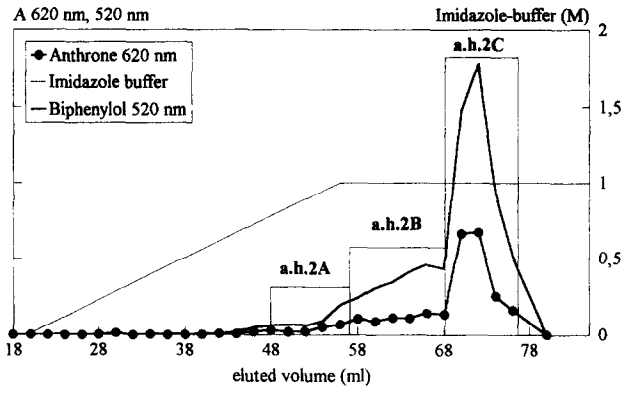

Fig. 4. Ion exchange chromatography of fraction B/a.h.2 on DEAE–Sephadex A 25 with a linear imidazol-buffer gradient (0.01–1 M).

contained Hyp. The amount of 3.6% Hyp in the main fraction a.h.2C gives a Hyp/sugar ratio of 1:25. The M_r of this fraction was about 4000 and agreed with the theroretical molecular weight derived from the above data.

Linkage analysis of B/a.h.1, B/a.h.2C, B/a.h.3 and B/a.h.4 is shown in Table 7.

Fraction B/a.h.1 contained 37% 4-Me-GlcA in terminal and in 2-linkage in a ratio of 1:4. More than the half of the Gal was found 3-linked and 3.6-branched. This fraction could possibly be a galactan with a main chain of 3-linked Gal and side chains containing Ara-, uronic acid- or aldobiuronic acid residues linked via C6 of the Gal and could consist of carbohydrate residues belonging to the proteoglycans B/2 and B/3 (see partial hydrolysis of these fractions).

Fraction B/a.h.2C showed no branched sugars. It consisted of 4-*O*-Me-GlcA and Gal in the ratio 1:1 and therefore is very likely to be composed of different aldobiuronic acids.

With 4.6% of terminal and 95% of linked sugars the theoretical chain length can be calculated as an average dp of 22 sugars. Since the fraction contained Hyp (3.6%) and no Ara, an *O*-glycosidic linkage between Gal and Hyp is the only possible binding site of the carbohydrate moiety to the protein. 4-*O*-(4-*O*-methyl-D-glucuronosyl)-D-galactose has been identified as the dominating aldobiuronic acid in the gum, and thus it can be concluded that this fraction is mainly composed of this aldobiuronic acid. These results lead to a structure similar to the one shown in Fig. 5.

Fraction B/a.h.3 consisted of 43% 4-Me-GlcA, with 19% of it terminally linked and 81% 2-linked. 75% of the Gal was 4-linked, the rest being 3-linked and 3.6-branched in an equimolar ratio. The amount of 8.3% terminal sugars pointed to a theoretical dp of approximately 12, and agreed with the M_r of $\sim$ 1800 found for this fraction. The Hyp content of 6% was in correspondence with these data.

Fraction B/a.h.4 contained 94% Ara, one third of which was terminally linked and indicated an average theoretical chain length of three molecules. Another third of the total Ara-amount showed 2-linkage, the rest consisted of 3- and 5-linkages. With a Hyp content of 25% for this fraction, a structure can be proposed showing in average three Ara-residues linked to Hyp.

Because of the minor amount of this fraction, no further data concerning the exact positions of the 1.2-, 1.3- and 1.5- linkages and the anomeric configuration of this fraction could be evaluated.

Table 7. Alkaline hydrolysis of myrrh gum: linkage analysis of the fractions B/a.h.1, B/a.h.2C, B/a.h.3 and B/a.h.4 (mol%)

Sugar	Linkage	B/a.h.1	B/a.h.2C	B/a.h.3	B/a.h.4
Ara(f)	1-	—	—	—	33.2
	1.2-	—	—	—	37.3
	1.3-	—	—	—	15.0
	1.5-	—	—	—	8.4
	Σ	—	—	—	93.9
4-*O*-Me-Glc[a]	1-	7.5	4.6	8.3	—
	1.2-	29.8	41.2	34.5	—
	Σ	37.3	45.8	42.8	—
Gal	1.3-	15.7	6.5	6.9	1.9
	1.4-	28.2	47.7	42.9	4.1
	1.3.6-	18.8	—	7.4	—
	Σ	62.7	54.2	57.2	6.1

[a]Deutero reduced 4-*O*-Me-GlcA.

Fraction B/5

The purified fraction B/5 was reduced with $NaBD_4$ (Taylor & Conrad, 1972) and hydrolysed by alkali. GPC separation showed a similar elution profile as for the crude gum B. Besides two fractions with a higher M_r of $\sim$ 6000 (B/5d.r.A) and $\sim$ 4000 (B/5d.r.B), a small amount was obtained showing a M_r of about 600 (B/5d.r.C). This fraction consisted of 23% Hyp and 67% Ara. The linkage analysis yielded equal parts of terminal, 2-linked and 3-linked Ara. Thus an arabinose consisting oligosaccharide related to the one mentioned for fraction B/a.h.4 reflects these data. This type of oligosaccharide is linked via Hyp to the protein core of fraction B/5.

The fractions B/5d.r. A und B showed the linkage pattern given in Table 8.

The two fractions contained 4-Me-Glc and Gal only. The Hyp content was 2.9% for fraction B/5d.r.A and 3.3% for B/5d.r.B.

Thus, both fractions, apart from their different M_r values, seem to exhibit similar structural features: a chain comprising of 4-*O*-Me-GlcA and Gal shows branches formed by 1.3.6-Gal with 4-*O*-Me-GlcA as the only type of terminal sugar. These structures are linked via Hyp to the protein core of the glycoprotein molecule of fraction B5 of the crude gum.

In conclusion, treatment with hot alkali revealed a reproducible degradation of myrrh gum B and the main

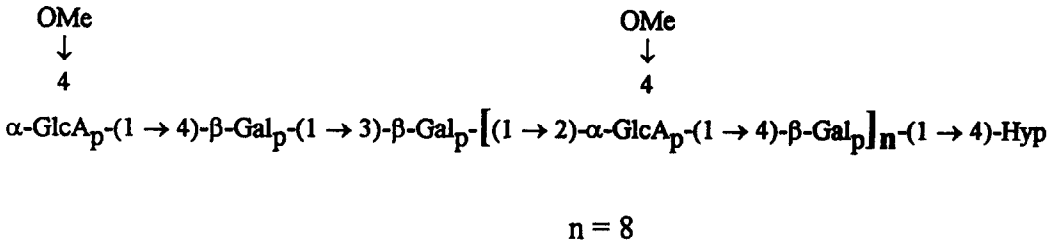

Fig. 5. Proposed structure of fraction B/a.h.2C of alkaline hydrolysed myrrh gum.

acidic fraction B/5 to shorter compounds. The M_r of the samples was reduced from 120 000–200 000 to 400–7000. Treatment of non reduced samples showed a loss of uronic acid, probably due to β-elimination.

In all fractions Hyp was present as the *cis* and *trans* epimers. This amino acid is known as a linkage partner between sugar moiety and protein core in extensins and glycoproteins (Lamport, 1980; Fincher *et al.*, 1983). Other covalent linkages between amino acids and sugar can be excluded on the basis of the data gathered.

The above results are in good agreement with the data of the partial hydrolysis, where the main acidic fraction B/5 showed no linkage of arabinose to other sugars. The existence of arabinose monomers/oligomers linked to the protein moiety was concluded from the data of the partial hydrolysis experiments with fractions B/2–3.

CONCLUSIONS

Myrrh gum was obtained by aqueous extraction and further purification by IEC. Five distinct fractions could be established, all of them contained protein (10–49%) and carbohydrate moieties. Due to the coelution of protein- and carbohydrate moieties in every chromatography applied and due to the failure of every attempt to remove the protein, either by enzyme digest, by SDS–gel electrophoresis, or by precipitation techniques, the covalent binding of protein and carbohydrate moiety is most probable. Additionally, the finding of amino acids typical for plant glycoproteins, especially of Hyp, and the susceptibility of the investigated fractions to alkaline hydrolysis clearly demonstrated the presence of proteoglycans. The only uronic acid present was identified as 4-*O*-methyl glucuronic acid.

In the crude gum the two aldobiuronic acids 4-*O*-(4-*O*-methyl-D-glucuronosyl)-D-galactose and 6-*O*-(4-*O*-methyl-D-glucuronosyl)-D-galactose were identified and quantified in a ratio of 6:1.

The main fractions B/4 and B/5 were proteoglycans with a M_r of 200 000, an approximate protein content of 10% with Hyp as the main amino acid (40%). After reduction and labelling of the uronic acid, the ratio of the comprising sugars Ara:Gal:4-*O*-methyl-GlcA was approximately 1:4:4. Structural investigations using mild acid hydrolysis, Smith degradation and alkaline hydrolysis demonstrated the following results: two different types of sugar chains are connected to the protein core: (i) short chains of arabinose (2- and 3-linked); and (ii) slightly branched chains of Gal (mostly 4-linked) and 4-*O*-methyl-GlcA (terminal and 2-linked). Both polymers are linked to the protein moiety via Hyp of the protein chain. A structure of alternating Gal and UA, linked via C2 and C4, respectively, could be made possible. This pattern resembles a different type of polymer, i.e. rhamnoglacturonan 1, a cell wall polysaccharide mainly composed of rhamnose and galacturonic acid, which is widely distributed in higher plants, but not found in exudate gums (Stephen *et al.*, 1990).

The less acidic fractions B/2 and B/3, having an average M_r of $\sim$ 67 000 showed the properties of arabino-3.6-galactans. The main chain is composed of 3-linked Gal partially branched via C6, bearing side chains containing Ara, 4-*O*-methyl glucuronic acid and 4-linked Gal.

These hypothetical arabinogalactans as well as oligoarabinosides are covalently linked to the protein chain. Showing a protein amount of 31 and 16%, respectively, these two fractions could be classified as arabinogalactan proteins.

The complexity of the structures is reflected in very complex NMR-spectra. Nevertheless, spectral data showed the presence of a methoxy group due to the occurrence of 4-*O*-methyl-GlcA and and also a signal for its unesterified carboxyl group.

In conclusion it can be stated that the gum fraction

Table 8. Alkaline hydrolysis of the reduced fraction B/5: linkage analysis of the fractions B/5d.r.A and B/5d.r.B (mol%)

Sugar-	Linkage-	B/5d.r.A	B/5d.r.B
4-*O*-Me-Glc[a]	1-	5.9	9.0
	1.2-	37	38.4
	Σ	43	47.4
Gal	1.3-	4.5	5.3
	1.4-	40.2	39.7
	1.16-	11.9	7.9
	Σ	56.6	52.9

[a]Deutero reduced.

from myrrh is composed of a heterodisperse mixture of proteoglycans with a dominating amount of uronic acid rich compounds. The prevailing estimation as an AGP-type gum is not correct, instead a 4-*O*-methyl-glucuronogalactan protein type is proposed. So far, these structures have not been described in gums from other plants.

It remaines questionable whether the different proteoglycans represent different polymers resulting from the biosynthesis sequence or whether they derive from a partial degradation during isolation or strorage of myrrh.

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