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THE STRUCTURE OF THE *O*-GLYCOSYLICALLY-LINKED OLIGOSACCHARIDE CHAINS OF LPG-I, A GLYCOPROTEIN PRESENT IN ARTICULAR LUBRICATING FRACTION OF BOVINE SYNOVIAL FLUID*

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ABSTRACT

Periodate oxidation of LPG-1 established that *N*-acetylneuraminic acid residues are linked preponderantly α-(2→3) to D-galactose residues. The resistance of 2-acetamido-2-deoxy-D-galactose residues to periodate oxidation suggests that they are linked at either O-3 or O-4 to D-galactose residues. After treatment of LPG-I with alkaline sulfite, ~80% of 2-acetamido-2-deoxygalactose was recovered as the sulfonic acid derivative. The Gal→GalNAc disaccharide released from sialic-acid-free LPG-I by digestion with *endo*-2-acetamido-2-deoxy-α-D-galactosidase (which suggests an α-D-GalNAc→L-Ser or -L-Thr linkage) gave a high color-yield in the Morgan–Elson reaction, indicating that 2-acetamido-2-deoxy-D-galactose residues are linked at C-3 to D-galactose residues. The migration of the released Gal-GalNAc disaccharide was the same as that of a standard sample of *O*-β-D-galactosyl-(1→3)-2-acetamido-2-deoxy-D-galactose. Treatment of sialic acid-free LPG-I with *Streptococcus pneumoniae* β-D-galactosidase, which hydrolyzes only galactosides linked β-D-(1→4) gave no free D-galactose, whereas treatment of LPG-I with bovine testes β-D-galactosidase released >90% of D-galactose. These results provide evidence for β-D-Gal*p*-(1→3)-α-D-GalNAc*p*-(1→3)-L-Ser or -L-Thr and α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-α-D-GalNAc*p*-(1→3)-L-Ser or -L-Thr structures. The sensitivity of the methods used and the recovery of constituents following treatment of LPG-I do not rule out the occurrence of small amounts of other tri- or tetra-saccharide chains.

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INTRODUCTION

Previous studies[2] showed that the synovial fluid fraction responsible for the boundary lubrication of articular cartilage contains two glycoprotein constituents. The major constituent (LPG-I) has a mol. wt. of 227 500, and is composed of ~equal proportions of amino acid and carbohydrate. LPG-I contains a single polypeptide chain and more than 150 oligosaccharide side-chains consisting of *N*-acetylneuraminic acid, D-galactose, and 2-acetamido-2-deoxy-D-galactose residues linked to the hydroxyl group of L-threonine or L-serine. The second glycoprotein component (LPG-II) has a mol. wt. of ~70 000 based on its electrophoretic mobility on dodecyl sodium sulfate gels.

Lubrication tests carried out with synovial fluid fractions containing mixtures of these glycoproteins[3,4] indicated that the ability to lubricate articular cartilage was dependant on the presence of LPG-I. In order to better understand the molecular basis for the boundary-lubricating properties of synovial fluid, we have studied the chemical structure of this glycoprotein and we report herein information on the structure of the oligosaccharide chains.

EXPERIMENTAL

Materials. — LPG-I was isolated from pooled bovine synovial fluid obtained from the metacarpal-phalangeal joints of adult cattle by cesium chloride density-gradient fractionation and purification by column chromatography[2]. *Streptococcus pneumoniae* β-D-galactosidase[5], *S. pneumoniae* neuraminidase[5], and bovine testes β-D-galactosidase[6] were prepared as previously described. Standard 2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-D-galactose was prepared by extensive hydrolysis of antifreeze glycoprotein[7] with *endo*-2-acetamido-2-deoxy-α-D-galactosidase[5].

Removal of N-*acetylneuraminic acid.* — *N*-Acetylneuraminic acid-free LGP-I was prepared by treating the glycoprotein (1 mg) in 0.1M acetate buffer (pH 5.0, 1 mL) with neuraminidase (50 units, from *Vibrio cholerae*, 500 units/ml, Behring Diagnostics, Somerville, N.J. 08876) for 18 h at 37°. The solution was dialyzed against water and lyophilized.

Analytical procedures. — *Paper chromatography.* Descending chromatography was performed on Whatman No. 1 paper in 4:1:5 (v/v) upper phase, 1-butanol–acetic acid–water. Spots were detected by the silver nitrate–sodium hydroxide procedure[8,9].

Determination of N-*acetylneuraminic acid and neutral sugars.* These compounds were determined by g.l.c. after methanolysis as pertrimethylsilyl derivatives, as described by Reinhold[10].

Determination of hexosamines. Samples were hydrolyzed with 6M hydrochloric acid for 3 h at 100°. The analyses were performed with an amino acid analyzer equipped with an ion-exchange column eluted with pH 5.28 buffer[11]. The 2-acetamido-2-deoxygalactose content of the disaccharide obtained by treating desialosylated

LGP-I with *endo*-α-*N*-acetylgalactosaminidase[5] was measured colorimetrically by a modified Morgan–Elson procedure for 2-acetamido-2-deoxysugars[12].

Determination of sulfonyl derivatives of 2-amino-2-deoxygalactose and amino acids. The content of 2-deoxy-2-sulfonylaminogalactose and sulfonylamino acids present in LGP-I following alkaline sulfite treatment was determined with an amino acid analyzer: The acid hydrolyzate (6M HCl, 100° for 24 h) was fractionated on an ion-exchange column eluted with pH 3.25 buffer[11].

Determination of amino acids. The samples were hydrolyzed with 6M hydrochloric acid at 100° for 24 h, and the amino acid content was determined with a two-column system[11]. The basic amino acids were eluted with pH 5.28 buffer, and the acid and neutral amino acids with pH 3.25 and 4.25 buffers. The amino acids and hexosamines were detected by their reaction with ninhydrin, and the absorbance at 570 nm and 440 nm was measured by a micro flow through a spectrophotometer connected to a two-channel integrating recorder. The amino acid analyzer was a manually operated system, laboratory-constructed with Durrum parts and using Durrum DC-4A ion-exchange resin.

Periodate oxidation. — Two samples of LGP-I (1 mg) in 0.1M acetate buffer pH 5.0 (1 mL) were treated with *Vibrio cholerae* neuraminidase (50 units) as described earlier, and two control samples of LGP-I were treated in parallel but without addition of neuraminidase. After incubation for 18 h at 37°, an enzyme-treated and a control sample were treated with sodium metaperiodate (1 mg) in water (1 mL), and the solutions were kept in the dark for 24 h at 22°. The reaction was terminated by the addition of 1,2-ethanediol (0.2 mL). After 3 h at 22°, the solutions were dialyzed against water and lyophilized. The residues were analyzed for galactose and *N*-acetylneuraminic acid by g.l.c., and for aminodeoxy sugars with the amino acid analyzer.

Alkaline sulfite treatment. — LGP-I (1 mg) was treated[13] with 0.1M sodium hydroxide containing 0.5M sodium sulfite for 72 h at 37°. The solution was then acidified with hydrochloric acid and lyophilized. The 2-deoxy-2-sulfonylaminogalactose, 2-amino-3-sulfonylbutyric acid, and cysteic acid contents of the acid hydrolyzate were analyzed with the amino acid analyzer.

Digestion with endo-*2-acetamido-2-deoxy*-α-D-*galactosidase.* — *N*-Acetylneuraminic acid-free LGP-I (1.67 mg in 500 μL of 50mM sodium cacodylate, pH 6.0) was treated with *S. pneumoniae endo*-2-acetamido-2-deoxy-α-D-galactosidase (0.05 unit) for 90 h at 37°, and then lyophilized. The released disaccharide was determined colorimetrically[12]. A part of the enzyme-treated LGP-I was applied to Whatman No. 1 paper to compare the mobility of the products with a standard of 2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-D-galactose[7]. The enzyme-treated LGP-I was hydrolyzed with 6M hydrochloric acid for 3 h at 100°, and the hydrolyzate was analyzed by paper chromatography.

Digestion with bovine testes and S. pneumoniae β-D-*galactosidases.* — Incubations were performed at 37° under the conditions specified in Table V.

TABLE I

CARBOHYDRATE COMPONENTS BEFORE AND AFTER PERIODATE OXIDATION OF NATIVE AND DESIALOSYLATED LGP-I

Compound	*Components*							
	Gal[a]		*GalNAc*[b]		*GlcNAc*[b]		*NeuAc*[a]	
	Mol[c]	%	Mol[c]	%	Mol[c]	%	Mol[c]	%
LGP-I	146	11.6	150	11.8	5	0.4	119	16.2
Periodate-treated[d]								
Recovered	89	7.0	145	11.4	5	0.39	0	0
Oxidized		39		(29)[e]		0		100
Sialic acid-free LGP-I	125	9.9	135	10.6	4	0.32	7	0.94
Periodate-treated[d]	0	0	129	10.1	4	0.35	0	0
Oxidized		100		(36)[e]		0		100

[a]Components analyzed by g.l.c. after methanolysis. [b]Components analyzed by amino acid analyzer after hydrolysis. [c]Mol per mol of glycoprotein. [d]Periodate oxidation carried out for 24 h at 22°. [e]Based upon a theoretical recovery of LGP-I of 100% (ref. 2).

RESULTS AND DISCUSSION

The results of the periodate oxidation of intact and desialosylated LGP-I are given in Table I. The complete oxidation of *N*-acetylneuraminic acid is consistent with the occurrence of this constituent as an end group[2]. The recovery of galactose after oxidation of intact LGP-I and from a sample treated with neuraminidase were 61 and 0%, respectively. This indicates that *N*-acetylneuraminosyl groups are primarily linked to O-3 of the D-galactose residues in the trisaccharide chains, NeuAc→D-Gal→D-GalNAc. The oxidation of a part of the D-galactose residues in intact LGP-I suggests the presence of free D-Gal→D-GalNAc disaccharide units. The almost complete release of *N*-acetylneuraminic acid (94%) by *V. cholerae* neuraminidase indicates that the *N*-acetylneuraminosyl groups are linked in α configuration[14]. In addition, the resistance to periodate oxidation of ~145 of 2-acetamido-2-deoxy-D-galactose residues suggests either a (1→3)- or a (1→4)-linkage between the D-galactose and 2-acetamido-2-deoxy-D-galactose residues. The lower recovery of 2-acetamido-2-deoxy-D-galactose residues after periodate treatment of LGP-I is probably due to the occurrence of 2-acetamido-2-deoxygalactose residues (~40) linked to L-threonine or L-serine residues[2]. A similar oxidation of 2-acetamido-2-deoxy-D-galactose residues has been reported previously for a glycoprotein containing several hundred chains having 1 to 5 carbohydrate components[15].

Treatment of oligosaccharides having the sequences hexosyl-(1→6)-2-acetamido-2-deoxyhexose, hexosyl-(1→4)-2-acetamido-2-deoxyhexose, and hexosyl-(1→3)-2-acetamido-2-deoxyhexose with alkaline sulfite was reported[16] to give the sulfonyl derivative of hexosamine in 15, 2, and >90% yields, respectively. This procedure was

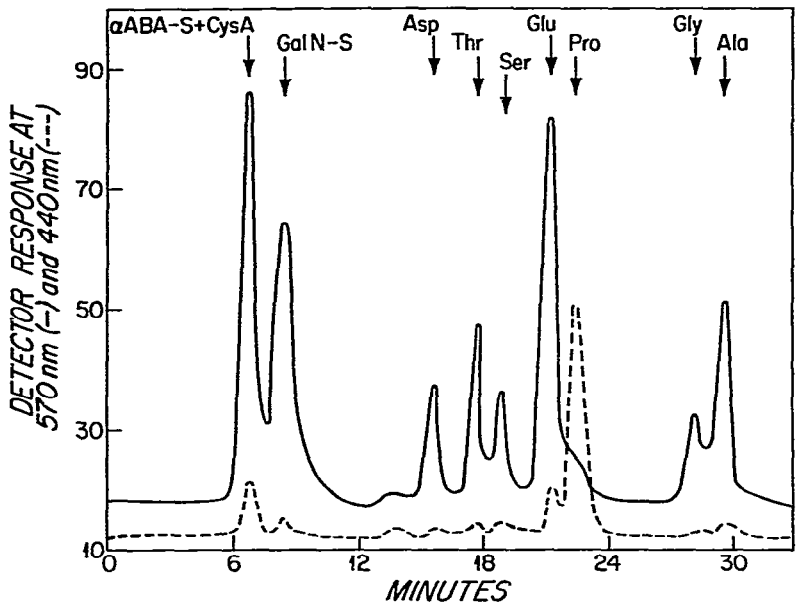


Fig. 1. Elution profile of ninhydrin-positive substances present in the acid hydrolyzate of LGP-I after alkaline sulfite treatment. Abbreviations: αABA-S, 2-amino-3-sulfonylbutyric acid; CysA, cysteic acid; and GalN-S, 2-deoxy-2-sulfonylamino-D-galactose.

TABLE II

2-AMINO-2-DEOXYHEXOSE CONTENT OF LGP-I TREATED WITH ALKALINE SULFITE

Component[a]	*LGP-I*		*Net change*
	Untreated	*Treated*	
2-Amino-2-deoxy-D-glucose	9	10	+1
2-Amino-2-deoxy-D-galactose	202	39	−163
2-Deoxy-2-sulfonylamino-D-galactose	0	158	+158

[a]Mol/mol of LGP-I.

used to determine the structure of the glycosidically linked carbohydrate units of fetuin[17]. In order to determine whether O-3 or O-4 of the 2-acetamido-2-deoxy-D-galactose residues were linked to D-galactosyl residues, LGP-I was treated with alkaline sodium sulfite. The profile of the ninhydrin-reactive peaks eluted from the ion-exchange column with pH 3.25 buffer is shown in Fig. 1. Two major, new ninhydrin-positive peaks were detected. The first peak was co-eluted with cysteic acid and 2-amino-3-sulfonylbutyric acid, which cannot be separated with the amino acid-analyzer system used[16,17]. The second peak was eluted at the same position as 2-deoxy-2-sulfonylamino-D-galactose[17]. The recovery of 2-deoxy-2-sulfonylamino-

TABLE III

AMINO ACID COMPOSITION OF LGP-I BEFORE AND AFTER ALKALINE SULFITE TREATMENT[a]

Amino acid	*LGP-I*		*Net change*
	Untreated	*Treated*	
Lysine	123	121	−2
Histidine	2	2	0
Arginine	16	16	0
2-Amino-3-sulfonylbutyric acid + cysteic acid[b]	0	144	+144
Aspartic acid	35	40	+5
Thronine	220	63	−157
Serine	63	35	−28
Glutamic acid	146	142	−4
Proline	229	262	+33
Glycine	18	29	+11
Alanine	89	86	−3
Valine	18	15	−3
Methionine	12	12	0
Isoleucine	16	14	−2
Leucine	14	15	+1
Tyrosine	2	2	0
Phenylalanine	7	7	0

[a]Residues per 1000 residues. [b]Eluted as a single, ninhydrin-reactive peak at the same position as cysteic acid[16,17].

TABLE IV

RELATIVE COLOR INTENSITIES OF DISACCHARIDES IN THE MORGAN–ELSON REACTION

Compound	*Relative color intensity (%)*[a]	*Ref.*
β-D-Gal*p*-(1→3)-D-GalNAc	110	5
β-D-GalA*p*-(1→3)-D-GlcNAc	130	20
β-D-GalA*p*-(1→4)-D-GlcNAc	2	20
Isolated disaccharide, Gal→GalNAc, from LGP-I	110	This work

[a]Compared to an equimolar amount of 2-acetamido-2-deoxyhexose.

hexose (based on the color value of standard 2-deoxy-2-sulfonylamino-D-galactose[17]) and the loss of aminodeoxysugar are reported in Table II. The recovery of cysteic acid and 2-amino-3-sulfonylbutyric acid (based on the color value of cysteic acid), and the amino acid composition of the alkaline, sodium sulfite-treated LGP-I are shown in Table III. The amounts of sulfonated derivatives of 2-amino-2-deoxy-D-galactose and amino acids, and the almost equivalent loss of 2-acetamido-2-deoxy-D-galactose, and L-threonine and L-serine suggest a (1→3)-linkage between D-galactosyl and 2-acetamido-2-deoxy-D-galactose residues.

The color yield of substituted 2-acetamido-2-deoxyhexoses in the Morgan–Elson reaction depends upon the position of substitution[18]. Substitution at O-3 increases the color yield to ~130% of the unsubstituted constituent, whereas substitution at C-4, which prevents chromogen formation[19], decreases the color yield to about 2%[20]. *endo*-2-Acetamido-2-deoxy-D-galactosidase has been shown to hydrolyze the glycoside bonds between a 2-acetamido-2-deoxy-D-galactose residue and the hydroxyl group of L-threonine or L-serine residues in glycoproteins and glycopeptides containing β-D-Gal*p*-(1→3)-D-GalNAc*p* side-chains[21,22]. Incubation of desialosylated LGP-I with this enzyme released a Morgan–Elson reactive disaccharide. Its color yield based on the 2-acetamido-2-deoxy-D-galactose content of intact LGP-I was 110% (Table IV), suggesting the presence of a (1→3)-linkage between the D-galactosyl and 2-acetamido-2-deoxy-D-galactose residues.

The yield of disaccharide residues released from sialic acid-free LGP-I by treatment with *endo*-2-acetamido-2-deoxy-D-galactosidase was >95%, based on

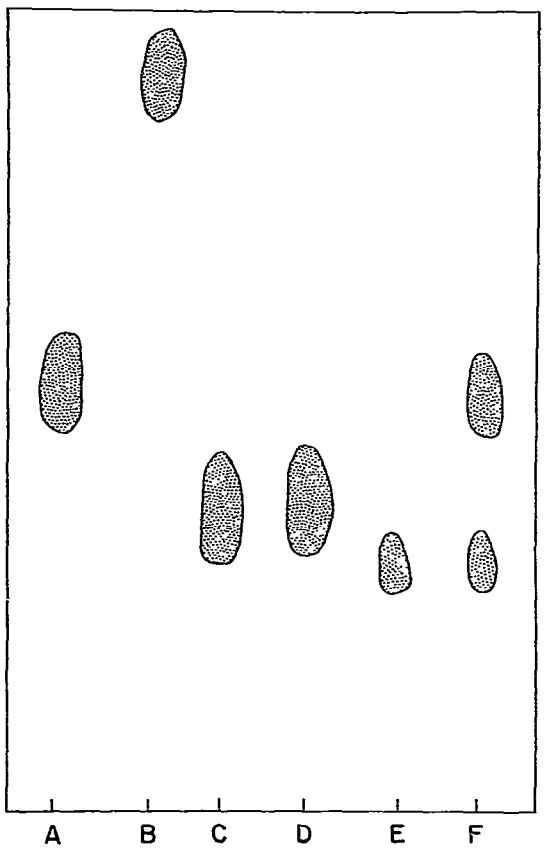


Fig. 2. Comparison by paper chromatography of desialosylated LGP-I treated with *endo*-α-*N*-acetylgalactosaminidase (C), the acid hydrolyzate following enzyme treatment (F), and standards of β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactopyranose (D), D-galactose (A), 2-acetamido-2-deoxy-D-galactose (B), and 2-amino-2-deoxy-D-galactose hydrochloride (E).

TABLE V

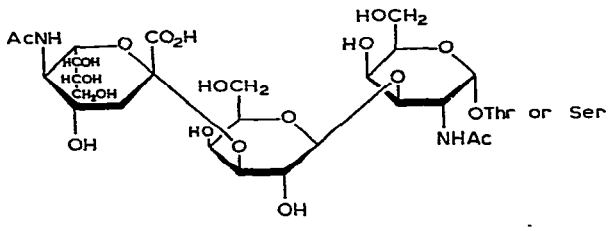
ENZYMIC RELEASE OF D-GALACTOSE FROM LGP-I[a]

Sequential enzyme treatment[b]				*D-Galactose released*[c]	
(*1*) S. pneumoniae *neuraminidase*	(*2*) S. pneumoniae *β-D-galactosidase*	(*3*) endo-2-*Acetamido*-2-*deoxy*-α-D-*galactosidase*	(*4*) *Bovine testes* *β-D-galactosidase*	*nmol*	%[d]
5	1			1	1
			10	8.8	15
			30	16	27
5			5	19	32
5			10	34	58
5			30	40	68
5		5	5	39	66
5		5	10	51	86
5		5	30	54	92

[a]Samples (50μL) of LGP-I (1.5 mg dissolved in 1 mL of 10 mM sodium cacodylate 1 mL), pH 6.0, were treated sequentially at 37°, as follows: with *S. pneumoniae* neuraminidase (1.0 unit/mL for 4 h); *S. pneumoniae* β-D-galactosidase (0.4 unit/mL for 40 h); bovine testes β-D-galactosidase (0.72 unit/mL for 40 h); and *endo*-2-acetamido-2-deoxy-D-galactosidase (2.3 units/mL for 4 h). Sodium citrate 0.5M buffer (pH 4.0, 10 μL) was added before bovine testis β-D-galactosidase treatment. [b]μL of enzyme added. [c]The release of D-galactose was determined by the D-galactose dehydrogenase assay[23]. [d]The percentage of D-galactose released by the enzyme treatment is expressed as a percentage of the total D-galactose released (59 nmol) from native LGP-I by hydrolysis with 2M hydrochloric acid for 2 h at 100°.

substituted 2-amino-2-deoxy-*O*-galactose residues. These results show that the 2-acetamido-2-deoxygalactosyl residues are α-D-linked with L-threonine (or L-serine) residues. Identification by paper chromatography (Fig. 2) of the carbohydrate compounds liberated by enzyme treatment gave a single spot, which corresponded to a standard sample of *O*-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactose[7]. In addition, hydrolysis with 6M hydrochloric acid for 3 h at 100° of the released carbohydrate compounds, followed by paper chromatography (Fig. 2), showed two spots reacting with the silver nitrate–sodium hydroxide reagent[8,9] and migrating to the same position as D-galactose (R_{Gal} 1.0) and 2-amino-2-deoxy-D-galactose hydrochloride (R_{Gal} 0.69).

Additional proof for a β-D-(1→3) linkage between the galactosyl and 2-acetamido-2-deoxygalactose residues was obtained by enzymic hydrolysis of the galactosyl residues of LGP-I (see Table V). Treatment of desialosylated LGP-I with *S. pneumoniae* β-D-galactosidase [which was shown to hydrolyze only β-D-(1→4)-linked galactoside[24]] gave trace amounts of free D-galactose, whereas bovine testes β-D-galactosidase[6], which hydrolyzes galactosides in β-D-(1→3) linkages, released 27% of the total galactose from intact LGP-I, 68% of the galactose from desialosylated LGP-I, and 92% of the galactose from desialosylated LGP-I treated with *endo*-2-



Scheme 1

acetamido-2-deoxy-D-galactosidase. The yields of galactose released by the enzyme are probably dependent on the nature of the substrate, because it is known[5] that oligosaccharides are cleaved more readily than glycoproteins by bovine testes β-D-galactosidase, whereas glycoproteins are better substrates than oligosaccharides for the *S. pneumoniae* β-D-galactosidase.

These results are consistent with a previous report[2] that LGP-I contains ~110 oligosaccharide side-chains composed of NeuAc→Gal→GalNAc linked primarily to L-threonine, and indicate that these chains have the structure *O*-*N*-acetylneuraminosyl-(2→3)-*O*-β-D-galactopyranosyl-(1→3)-*O*-2-acetamido-2-deoxy-D-galactopyranosyl-(1→3)-L-threonine or (L-serine) (Scheme 1). Cleavage of *N*-acetylneuraminic acid with neuraminidase and treatment with *endo*-2-acetamido-2-deoxy-α-D-galactosidase gave a single disaccharide, suggesting that the ~40 disaccharide chains of LGP-I also contain D-galactopyranosyl residues linked β-D-(1→3) to 2-acetamido-2-deoxy-D-galactopyranose residues. This disaccharide unit has been reported in anti-freeze glycoprotein[7], submaxillary gland mucin[25], and in many other glycoconjugates[26].

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