The Isolation and Partial Characterization of the Major Glycoprotein (LGP-I) from the Articular Lubricating Fraction from Bovine Synovial Fluid

By DAVID A. SWANN, STUART SOTMAN, MARVIN DIXON and CAROLYN BROOKS

Departments of Biological Chemistry and Surgery, Harvard Medical School, Massachusetts General Hospital and Shriners Burns Institute, Boston, MA 02114, U.S.A.

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The articular lubricating fraction from bovine synovial fluid was prepared by repeated fractionation in three consecutive CsCl density gradients to remove completely traces of hyaluronic acid. The major glycoprotein constituent (LGP-I) was then isolated by repeated gel-permeation chromatography. The yield of the LGP-I component was about 20 mg/litre of synovial fluid. Sedimentation-equilibrium measurements showed that this glycoprotein was homogeneous and the mol.wt. was calculated to be 227 500. Amino acids represented 43% (w/w) and carbohydrate constituents 44% (w/w) of the molecule. Threonine, glutamic acid, proline and lysine (224, 127, 242 and 128 residues/1000 residues respectively) were the major amino acids. Galactosamine, galactose and *N*-acetylneuraminic acid (202, 162 and 114 residues/molecule of LGP-I component respectively) accounted for 98% of the total carbohydrate residues present. Small amounts of mannose and glucosamine (1 and 9 mol respectively/mol of LGP-I component) were also present. After treatment of LGP-I component with alkali and NaB^3H_4 radioactivity was incorporated into α -aminobutyric acid and alanine in a molar ratio of 4:1, and radioactive galactosaminitol was isolated by ion-exchange chromatography from a cleaved oligosaccharide fraction. These data demonstrate the presence of threonine and serine $-O\text{-GalNAc}$ linkages, but only 25% of the theoretical linkages involving threonine were cleaved by a β -elimination reaction. Digestion of LGP-I component with Pronase followed by chromatography on DEAE-cellulose yielded glycopeptide fractions with a similar amino acid and carbohydrate composition to the intact molecule. Treatment of desialylated and intact LGP-I component with galactose oxidase followed by reduction with NaB^3H_4 revealed the presence of 52 mol of terminal galactose in the intact molecule and 153 mol of galactose/mol of LGP-I component after treatment with neuraminidase. The data indicate that LGP-I component is composed of a single polypeptide chain containing more than 150 oligosaccharide side chains composed of *O*-GalNAc-Gal distributed over the length of the peptide chain and that terminal sialic acid residues are linked to galactose in two-thirds of these side chains.

The ability of bovine synovial fluid to lubricate the surface of articular cartilage in a test system *in vitro* was shown to be associated with a protein fraction which after reduction contained a mixture of peptide and glycopeptide constituents (Radin *et al.*, 1970; Swann & Radin, 1972). The isolation of this lubricating fraction was made possible because a method existed that allowed the lubricating ability of purified synovial-fluid fractions to be compared with that of whole-tissue fluid. This initial approach to the study of which molecules are involved in articular-cartilage lubrication led to the isolation of a fraction that lubricated and had fairly uniform composition with respect to both the number and amounts of constituents (Swann & Radin, 1972). The ability of fractions to lubricate articular cartilage in the test system used, however, is

dependent on a large number of variables, many of which, in the absence of detailed information about the chemical structure and mechanical properties of the articular-cartilage surface or the constituents in the fraction, cannot be controlled. Because of the complex nature of this function, and particularly the lack of a quantitative relationship between the lubricating activity and the concentration or structure of any one known constituent, it was decided to continue the study of articular lubrication by purifying and characterizing the individual constituents known to be present in the lubricating fraction. One of the major constituents in this fraction was a high-molecular-weight glycopeptide constituent (Swann & Nazimiec, 1973) which has been given the name 'Lubricating Glycopeptide-I' (LGP-I). In order to isolate sufficient quantities of

this component in a purified form it was necessary to modify the fractionation procedure used previously, and the present report is concerned with methods used and the partial characterization of the unique product obtained.

Materials and Methods

Preparation and treatment of synovial fluid

Pooled synovial fluid obtained from the metacarpal-phalangeal joints of adult cattle (immediately after death) was transported from the slaughterhouse to the laboratory, and within 2 h after death was clarified by ultracentrifugation (120000g, 30 min, 4°C) in batches of about 350 ml. The supernatant obtained was filtered (Millipore Corp., Bedford, MA, U.S.A.; 0.22 μ m pore size, 16 cm diameter; 4°C) by using vacuum to assist filtration, and the residue retained by the filter was then redissolved in 300 ml of sterile 0.15 M-NaCl before fractionation in a CsCl gradient.

Density-gradient fractionation

CsCl was added to the reconstituted filtration residue to give a density of 1.65 g/ml, and the resulting solution was then fractionated by sedimentation in a Beckman preparative ultracentrifuge with a 60 Ti rotor (Beckman Instruments, Palo Alto, CA, U.S.A.) at 220000g at 4°C for 64 h (gradient G-I, Fig. 1). The protein fraction from this first gradient was re-fractionated in a second gradient (G-II) under the same conditions as gradient I after adjustment of the density of the sample to 1.45 g/ml. The glycoprotein fraction from the second gradient was then re-fractionated in a third gradient (G-III) run under conditions identical with those for gradient G-II.

In a typical preparative procedure eight G-I gradients, corresponding to approx. 2800 ml of synovial fluid, were carried out. The pooled protein fractions obtained from the G-I gradients were then fractionated in four G-II gradients, and the glycoprotein-I fractions (Fig. 1) were then processed in a single G-III gradient.

Column chromatography

A descending-flow column (200 cm $\times$ 2.5 cm), packed with Bio-Gel A-5M (100–200 mesh; Bio-Rad Laboratories, Richmond, CA, U.S.A.) and equilibrated with 1 M-guanidine hydrochloride/0.01 M-Tris/HCl, pH 7.5, was used to separate the components present in the glycoprotein-II fraction obtained from gradient G-III. After reduction and alkylation the recycled B fraction was further fractionated on a Bio-Gel A-15M column (100 cm $\times$ 2.5 cm) eluted with 1 M-guanidine hydrochloride/0.01 M-Tris/HCl, pH 7.5. A column (90 cm $\times$

1.5 cm), packed with Sephadex G-25 (200–400 mesh; Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) and eluted with 0.1 M-acetic acid adjusted to pH 5.8 with pyridine, was used to separate the products obtained by treatment of LGP-I component with alkali or digestion with Pronase. Glycopeptides were also fractionated by chromatography on a DEAE-cellulose column (20 cm $\times$ 0.9 cm; Whatman Biochemicals, Maidstone, Kent, U.K.) eluted with a linear gradient of 0.002–0.3 M-acetic acid adjusted to pH 5.0 with pyridine (100 ml of each solution), followed by 30 ml of 0.4 M-acetic acid/pyridine, pH 5.0, then 30 ml of 0.5 M-acetic acid/pyridine, pH 5.0. All the columns were operated at 20°C and flow rates of 15–20 ml/h.

Reduction and alkylation

Samples (30 mg/ml) of recycled fraction B (Fig. 2b) were dissolved in 8 M-urea/0.5 M-Tris/HCl, pH 8.0, and treated with dithiothreitol (Calbiochem, San Diego, CA, U.S.A.; 3 mg/ml at 37°C for 19 h). Excess of sodium iodoacetate was then added (8 mg/ml) and the mixture incubated for a further 1 h at 37°C. The reaction mixture was then re-fractionated on the Bio-Gel A-15M column (Fig. 2c) to obtain LGP-I component.

Alkali treatment

LGP-I preparation 2 (Table 1) (50 mg) was treated with alkali under the following conditions: (a) 0.5 M-NaOH/0.02 M-NaBH₄ at 4°C for 91 h; or (b) 0.5 M-NaOH/0.02 M-NaBH₄ at 22°C for 18 h; or (c) 0.05 M-NaOH/1 M-NaBH₄ containing 20 mg of NaB³H₄ (New England Nuclear, Boston, MA, U.S.A.; specific radioactivity 185 mCi/mmol) at 45°C for 18 h. The final concentration of LGP-I component was 5 mg/ml of reaction mixture in each case. After these treatments the reaction mixtures were adjusted to pH 4.5 by addition of acetic acid, and concentrated almost to dryness on a rotary evaporator. After addition of water the samples were reconstituted to a volume of 3 ml and fractionated by chromatography on the Sephadex G-25 column.

Digestion with Pronase

LGP-I preparation 1 (Table 1) (40 mg) was dissolved in 5 ml of 0.15 M-NaCl/0.01 M-Tris/HCl (pH 7.8)/2 mM-CaCl₂, and after addition of Pronase (1 mg; Calbiochem; 45 proteolytic units/mg; Nomoto & Narahashi, 1959), the mixture was incubated at 37°C for 36 h, after which an additional 1 mg of Pronase was added. A further 2 mg of Pronase was added after a total of 72 h and the incubation continued for a further 48 h. Small quantities of toluene were added as a bacteriostatic agent. After

proteolysis the incubation mixture was applied directly to the Sephadex G-25 column.

Treatment with galactose oxidase and NaB^3H_4

The method described by Radin *et al.* (1969) and Suzuki & Suzuki (1972) was used to label terminal galactose and galactosamine constituents in both native and desialylated LGP-I component. A sample (5mg/ml) of LGP-I preparation 2 (Table 1) was incubated with neuraminidase (40 μg) (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) in 2ml of 0.1M-sodium acetate buffer, pH 5.0, at 37°C. After 16h a second 40 μg of enzyme was added and the incubation continued for another 24h. At the end of this time the mixture (total volume 2.25ml) was dialysed against 5 $\times$ 500ml of water and then 1 $\times$ 500ml of 0.1M-sodium phosphate buffer, pH 7.2. The desialylated and intact LGP-I samples (5mg in 2ml of 0.1M-sodium phosphate buffer, pH 7.2) were then treated with galactose oxidase [125 μg of enzyme (Worthington: 243 Worthington units/mg per 2.25ml of reaction mixture) for 4h at 37°C. A second 125 μg of enzyme was then added and the incubation continued for a further 20h. The samples (2.5ml) were dialysed against 4 $\times$ 500ml of water and reduced by reaction with NaB^3H_4 (New England Nuclear; specific radioactivity 200mCi/mmol, 0.1ml of solution containing 0.01mm- NaB^3H_4 in 1mm-NaOH) for 22h at room temperature (20°C). Unlabelled NaBH_4 (2mg) was then added, and, after incubation for 2h, the excess of borohydride was destroyed by the addition of 2M-acetic acid. Samples were dialysed against water and freeze-dried. The specific radioactivities of the LGP-I samples and the galactosamine component were calculated by determining the total quantities of radioactivity by using Biofluor (New England Nuclear), the content of galactosamine in the treated LGP-I samples and the quantity of radioactivity in galactosamine after analysis and fractionation on the amino acid analyser, as described below.

Analytical procedures

The presence of constituents which absorbed at 280nm in fractions obtained by the density-gradient and gel-permeation-chromatography fractionation procedures was determined by using a flow-through recording spectrophotometer (LKB Instruments, Rockwell, MD, U.S.A.). The pattern of peptide and glycoprotein constituents in fractions was determined by electrophoresis in 5% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulphate, after treatment with 4M-urea and before and after reduction with dithiothreitol (Furthmayr & Timpl, 1971). This gel-electrophoresis technique was also used to detect the presence of radioactive constituents

after the galactose oxidase treatment and NaB^3H_4 reduction of LGP-I component. After electrophoresis the gels were fixed, dehydrated and treated with 20% 2,5-diphenyloxazole in dimethyl sulphoxide by the method described by Bonner & Laskey (1974). The radioactive moieties were detected by exposure of the treated gels to X-ray film (Kodak RP Royal X-Omat, RP/R-54; Eastman Kodak, Rochester, NY, U.S.A.) at -70°C for 3 days. Hexuronic acid-containing constituents were determined by a carbazole method (Bitter & Muir, 1962), total carbohydrate by an anthrone procedure (Roe, 1955), and the sialic acid content of column effluents by the thiobarbituric acid method (Warren, 1959).

The presence of peptide and hexosamine constituents in column effluents was detected by hydrolysis and reaction with ninhydrin reagent. Portions (0.1ml) of the column fractions were mixed with 12M-HCl (0.1ml) and heated at 100°C for 3h. After this hydrolysis step the samples were dried under vacuum, dissolved in 0.1ml of 0.2M-sodium citrate buffer, pH 4.25, and ninhydrin reagent (0.1ml) was added. After heating at 100°C for 10min, 2ml of 50% (v/v) ethanol was added and the A_{570} measured. The radioactivity in column samples (0.1ml) was measured in a liquid-scintillation counter after mixing with NCS solubilizer (0.6ml; Amersham/Searle, Arlington Heights, IL, U.S.A.) and 10ml of counting fluid [6g of 2,5-diphenyloxazole and 75mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene per litre of toluene]. The counting efficiency was determined by using standards prepared under conditions identical with those used for the preparation of test samples.

Amino acid analyses were performed by using a two-column system after hydrolysis with 6M-HCl at 100°C for 24h in sealed tubes under vacuum. The 60cm $\times$ 0.9cm ion-exchange column, eluted with pH 3.25 and 4.24 buffers (Moore *et al.*, 1958), was temperature-programmed to allow separation of 4-hydroxyproline and aspartic acid. An extended column (20cm $\times$ 0.9cm), eluted with pH 5.28 buffer (Moore *et al.*, 1958), was used for the analysis of the basic amino acids and also glucosamine and galactosamine. For the hexosamine analyses, samples were hydrolysed with 6M-HCl at 100°C for 3h. Known quantities of glucosamine and galactosamine that had been submitted to the same hydrolysis and handling procedures as the samples were used as standards (Swann & Balazs, 1966). The mannose, galactose and *N*-acetylneuraminic acid contents of samples were determined by g.l.c. after methanolysis and preparation of the pertrimethylsilyl derivatives (Reinhold, 1972).

After treatment of LGP-I component with alkali or galactose oxidase and reduction with NaB^3H_4 , the quantities of radioactivity in amino acids, hexosaminitols and galactosamine were determined by applying portions of the hydrolysed samples to the

amino acid-analyser columns and operating the instrument with the ninhydrin-line disconnected. The column effluents were collected in a fraction collector. Radioactivity measurements were made on portions of the samples, and the elution positions were compared with those of standard amino acids detected by reaction with ninhydrin. With the 20cm×0.9cm column eluted with pH 5.28 buffer, glucosaminitol and galactosaminitol were co-eluted with galactosamine. Additional analyses by the method described by Donald (1968) (a 57cm×0.9cm column eluted with a borate/citrate buffer) were therefore used to separate glucosaminitol and galactosaminitol from the hexosamines. The types of labelled constituents present in LGP-I component after the treatments with galactose oxidase and NaB^3H_4 were also determined by t.l.c. after hydrolysis with either 1.5M-HCl at 100°C for 2h for the detection of neutral sugars, or 4M-HCl at 100°C for 3h for the hexosamines. After hydrolysis the samples were freeze-dried, dissolved in pyridine/acetic acid/water (5:1:10, by vol.), and samples containing 50000–100000 c.p.m. were applied to micro cellulose sheets (Brinkman MN Polygram Cell 400; Brinkman Instruments, Westbury, NY, U.S.A.) and fractionated by ascending chromato-

graphy with ethyl acetate/pyridine/acetic acid/water (36:36:7:21, by vol.) as solvent. The radioactive spots were located by dipping the chromatograms in a 7% (w/v) solution of 2,5-diphenyloxazole in ether (Randerath, 1970) and exposure to X-ray film for 6–21 days at -70°C .

Sedimentation-velocity and sedimentation-equilibrium measurements were made in a Beckman model E analytical ultracentrifuge. The sedimentation-equilibrium data were analysed by a computer procedure (Roark & Yphantis, 1969). Some of these sedimentation analyses were carried out by Dr. Don O'Hara and Dr. Judy Saide in the Laboratory of Physical Biochemistry at the Massachusetts General Hospital.

Results

Purification and properties of LGP-I component

The ultrafiltration procedure was a convenient pretreatment step before fractionation of the synovial fluid in a density gradient, because it decreased the quantities of non-lubricating proteins, and the filtration-residue samples could be frozen and stored at -20°C before fractionation

Fig. 1. *CsCl*-density-gradient fractionation of the ultrafiltration residue fraction of pooled bovine synovial fluid. The initial density was 1.65 g/ml for gradient G-I (a) and 1.45 g/ml for gradients G-II (b) and G-III (c). The sedimentation conditions (220000g at 4°C for 64h) were the same for each gradient procedure. Fractions (3 ml) were collected via a capillary tube inserted into the base of each tube. The density (●), hexuronic acid content (▲) and A_{280} (○) were then determined. The protein fraction from gradient G-I (obtained by pooling fractions 7–10) was re-fractionated by sedimentation in gradient G-II. The glycoprotein fraction I from gradient G-II (obtained by pooling fractions 4–8) was re-fractionated by sedimentation in gradient G-III. The gradient G-III fractions 3–7 were pooled to yield glycoprotein fraction II.

Fig. 2. Fractionation of glycoprotein fraction II (Fig. 1) by gel-permeation chromatography

(a) Chromatography on Bio-Gel A-5M (200cm $\times$ 2cm) to yield fractions A, B, C and D (obtained by pooling the fractions indicated). (b) Fraction B was recycled through the same Bio-Gel A-5M column. (c) The recycled fraction B after reduction and alkylation was re-fractionated by chromatography on a Bio-Gel A-15M column (100cm $\times$ 2.5cm). (1) and (2) indicate the elution positions of Blue Dextran 2000 (Pharmacia Fine Chemicals) and bovine serum albumin respectively. The columns were eluted with 1M-guanidine hydrochloride/0.01M-Tris/HCl, pH7.5, and the column fractions (18ml for the Bio-Gel A-5M column and 11ml for the Bio-Gel A-15M column) were monitored by measuring the A_{280} (protein, $\circ$) and by using the anthrone reaction (carbohydrate: A_{620} , $\bullet$).

Fig. 3. Sedimentation-velocity analysis of recycled fraction B (Fig. 2b)

Fraction B (3.4mg/ml) was analysed in 0.5M-guanidine hydrochloride/0.05M-Tris/HCl, pH8.0, before reduction (upper curve) and after reduction with 20mM-dithiothreitol (lower curve). The analysis was performed in double-sector cells with a 1° wedge window in the cell containing the unreduced sample. The centrifuge was operated at a speed of 42040 rev./min at 25°C, and the picture was taken at 54min after reaching top speed.

acid-containing constituents (hyaluronic acid) and protein, and so, to prevent any loss of glycoprotein constituents at this stage, all of the 280nm-absorbing fractions (nos. 7, 8, 9 and 10) were pooled for re-fractionation in gradient G-II. This second gradient step achieved a good separation of hyaluronic acid from the protein constituents, but analysis of the pooled glycoprotein fraction I revealed the presence of small quantities of hexuronic acid. A third gradient (G-III) was therefore performed to remove completely all traces of hyaluronic acid from the glycoprotein fraction. The principal constituents detected by gel electrophoresis in the protein fraction from gradient G-I were two peptides with mobilities similar to bovine serum albumin and a higher-molecular-weight component (mol.wt. about 150000), which was split by reduction to yield two peptides, one with a mobility similar to that of bovine serum albumin and a second one with a considerably greater mobility (gels 1 and 2, Plate 1). The glycoprotein fractions obtained from gradients G-II and G-III (gels 3 and 4, Plate 1) both showed bands with mobilities equivalent to those of the above components, as well as considerable quantities of high-molecular-weight constituents. Some of the latter components failed to enter the gels even after

in a CsCl gradient. After fractionation of reconstituted filtration residues in gradient G-I (Fig. 1), there was an incomplete separation of hexuronic

Table 1. *Chemical composition of fractions obtained by gel-permeation chromatography of glycoprotein fraction II (Fig. 1)*

The analytical values given for fractions A, C and D are averages of four separate preparations. The values for recycled fraction B indicate the range obtained with five separate preparations. The values for the LGP-I component are individual analyses performed on two preparations. Glucosamine and galactosamine were determined by ion-exchange chromatography and the remaining sugars by g.l.c. Fraction E is the void-volume fraction obtained by chromatography of the LGP-I component after treatment with alkaline NaB^3H_4 (Fig. 5).

Fraction ...	A	B	C	D	LGP-I		E
					1	2	
					Content (residues/1000 residues)		
Amino acid							
Aspartic acid	85	37-45	87	106	33	35	25
Threonine	124	196-206	97	65	229	218	204
Serine	83	57-68	108	71	56	50	32
Glutamic acid	121	120-129	127	132	130	124	127
Proline	127	228-257	87	55	244	241	300
Glycine	58	18-25	72	69	16	19	10
Alanine	74	81-85	65	73	88	84	70
Half-cystine	10	1-5	20	22	—	—	—
Valine	50	23-26	58	54	17	24	20
Methionine	12	3-5	12	10	2	4	—
Isoleucine	25	19-21	27	36	17	20	20
Leucine	50	17-22	66	83	14	18	13
Tyrosine	20	5-10	22	30	3	4	1
Phenylalanine	29	6-10	31	45	5	7	2
Lysine	79	106-127	69	82	129	128	132
Histidine	14	3-6	17	22	3	7	5
Arginine	39	15-21	35	45	14	17	7
α -Aminobutyric acid							32
Total (% , w/w)					41.5	44.3	41.1
Content (% , w/w)							
Carbohydrate							
Mannose	0.6	0.1-0.5	1.2	2.1	0.1	0.1	
Galactose	4.3	9.1-12.9	3.3	3.8	14.1	12.8	
Glucosamine	6.9	0.3-1.0	3.5	4.4	1.0	0.7	
Galactosamine	4.9	8.4-16.2	2.1	2.8	17.1	15.9	
N-Acetylneuraminic acid	7.1	15.0-25.1	5.1	6.0	23.0	15.5	

reduction. LGP-I component was observed as a single well-defined band which migrated with a mobility approx. 11 % of that of albumin and stained with both Coomassie Blue and periodic acid/Schiff reagent.

Fractionation of the glycoprotein fraction II obtained from gradient G-III by gel-permeation chromatography on a Bio-Gel A-5 column yielded four fractions (Fig. 2). A major portion of the void-volume fraction (A) did not enter the sodium dodecyl sulphate/polyacrylamide gels before reduction (gels 5 and 6, Plate 1), whereas fraction B (gels 7 and 8, Plate 1) was composed largely of LGP-I component. The mobility of this glycopeptide was not altered by reduction. Fraction C contained the 150000-mol.wt. constituent (gels 9 and 10, Plate 1) and fraction D the peptides with a mobility similar to albumin (gels 11 and 12, Plate 1). Also shown in Plate 1 is the gel-electrophoresis pattern obtained with plasma-protein fraction II (Nutritional Bio-

chemicals, Cleveland, OH, U.S.A.), which indicates that the major component in fraction C has similar properties to γ -globulin (gels 13 and 14, Plate 1). Rechromatography of the B fraction on the Bio-Gel column achieved a further separation of LGP-I component from the fraction A constituents, but analysis by gel electrophoresis showed that the recycled B fraction contained a high-molecular-weight constituent in addition to LGP-I component, and that the proportion of the LGP-I component was increased by reduction. It appeared therefore that the A fraction contained a constituent(s) that in the unreduced state could form stable aggregates with LGP-I component, with the result that these components were incompletely separated by the column fractionation procedure. Sedimentation-velocity measurements also indicated that the interaction between these constituents was decreased in the presence of reducing agent (Fig. 3). On the basis of these observations and the fact that the mobility

EXPLANATION OF PLATE I

Electrophoresis of protein fractions on 5% polyacrylamide gels in the presence of sodium dodecyl sulphate

Gels 1 and 2 are the protein fraction from gradient G-I, and gels 3 and 4 are the glycoprotein fraction II from gradient G-III (Fig. 1). Gels 5 and 6 are fraction A (Fig. 2a and Table 1), gels 7 and 8 are the recycled fraction B (Fig. 2b and Table 1), gels 9 and 10 are fraction C and gels 11 and 12 are fraction D (Fig. 2a and Table 1) obtained by gel-permeation chromatography of glycoprotein fraction II. The pattern of peptide constituents obtained with a γ -globulin fraction is shown on gels 13 and 14. Samples were analysed before reduction (odd-numbered gels) and after reduction with dithiothreitol (even-numbered gels). The arrows to the left of each set of gels indicate the position of bovine serum albumin. Peptide constituents were detected by staining with Coomassie Blue.

EXPLANATION OF PLATE 2

Radioautograms showing the distribution of radioactivity in the LGP-I component after treatment with galactose oxidase and reduction with NaB^3H_4 and detection by exposure to X-ray film

Picture no. 1 was obtained by electrophoresis of a treated intact LGP-I component on a polyacrylamide gel in the presence of sodium dodecyl sulphate and exposure to X-ray film for 3 days. Pictures 2 and 3 were obtained by t.l.c. of treated intact LGP-I component and treated desialo-LGP-I component respectively after hydrolysis with 1.5M-HCl at 100°C for 2h. The exposure time was 6 days for each sample. The sample shown in picture 4 was desialo-LGP-I component after hydrolysis with 4M-HCl for 2h at 100°C, and the exposure time was 3 weeks. The distances travelled by galactosamine and galactose on duplicate t.l.c. plates and of native LGP-I component on a duplicate polyacrylamide gel are indicated by the arrows. The dotted arrow indicates the direction of solvent flow with the t.l.c. radioautograms.

of the LGP-I component in the recycled B fraction was not altered by reduction (Plate 1), the recycled B fraction was reduced and alkylated and re-fractionated on a Bio-Gel A-15M column to obtain the LGP-I component essentially free of fraction A components. The yield of LGP-I component in six different preparations varied between 14 and 25 mg, with an average of 20 mg/litre of synovial fluid.

The analysis of fractions A, recycled B, C, D and two preparations of the LGP-I component obtained by gel chromatography are given in Table 1. The values given for fractions A, C and D are the averages of analyses obtained with four different preparations. The values shown for recycled fraction B indicate the range of values obtained with five different preparations. Threonine, glutamic acid, proline and lysine accounted for between 68 and 71% of the amino acid residues in the recycled B fraction. After reduction and alkylation and fractionation on the Bio-Gel A-15M column the content of threonine was increased slightly, and these four amino acids represented approx. 72% of the total. The values given in Table 1 were not corrected for losses that occurred during hydrolysis, and tryptophan was not determined. The average amino acid content of the two LGP-I preparations was 43%. The major carbohydrate constituents present in the recycled B fraction were galactose, galactosamine and *N*-acetylneuraminic acid. The mannose content of LGP-I component was always lower than that of recycled fraction B. Glucosamine was also present as a minor

component, but, in contrast with mannose, its concentration in LGP-I component was approximately the same as in the recycled B fraction. The average carbohydrate content of the LGP-I component was 44%. This glycoprotein thus contains approximately equal amounts of peptide and carbohydrate constituents.

Analysis of a solution of LGP-I component (5 mg/ml) in 0.5M-guanidine hydrochloride at 20°C in the analytical ultracentrifuge gave a single symmetrical boundary with an *s* value of 2.5S. Sedimentation-equilibrium measurements indicated that the LGP-I preparation was relatively monodisperse. A plot of the net fringe displacement as a function of radius is shown in Fig. 4. By using the formula

$$\text{mol.wt.} = \frac{RT}{(1-\bar{v}\rho)} \omega^2 \cdot \sigma$$

and a value of 0.69 for the partial specific volume ($\bar{v}$), the weight-average mol.wt. was calculated to be 227 500 (Roark & Yphantis, 1969). The effective reduced molecular weight (σ) was calculated from the net fringe-displacement data (Yphantis, 1964).

Alkali treatment of the LGP-I component

Treatment of LGP-I preparation 2 with 0.5M-NaOH and 0.02M-NaBH₄ at either 4°C for 91 h or 22°C for 18 h, followed by chromatography on Sephadex G-25, gave a single void-volume peak, and no low-molecular-weight oligosaccharide components were detected. Under the conditions suggested by Mayo & Carlson (1970) (0.05M-NaOH and 1M-NaBH₄ at 45°C for 18 h), two low-molecular-weight peaks were observed (Fig. 5). Most of the alkali-treated LGP-I component, however, was recovered in the void-volume fraction. The shaded areas shown in Fig. 5 were pooled and freeze-dried to yield fractions E (elution volume 46–65 ml), F (75–88 ml) and G (92–100 ml). Fraction E contained a greater proportion of proline and a lower content of threonine, serine and alanine than did the LGP-I component, and 32 residues of α -aminobutyric acid (Table 1). Two radioactive amino acids corresponding in position to alanine and α -aminobutyric acid were present in fraction E (Fig. 6). An initial peak of radioactivity corresponding in position to the acidic and neutral amino acids and a second peak corresponding in position to galactosaminitol were observed in fractions F and G (Fig. 7). Two other radioactive peaks were eluted in the same position as small ninhydrin-positive peaks, and the two remaining radioactive peaks did not correspond in position to ninhydrin-positive constituents. The carbohydrate compositions of fractions E, F and G and the amounts of radioactivity present in alanine and α -aminobutyric acid

Fig. 4. Sedimentation-equilibrium analysis of LGP-I preparation 2 (Fig. 2c) in the analytical ultracentrifuge

The sample was prepared for analysis by dissolving 0.5 mg of LGP-I preparation 2 in 0.5M-guanidine hydrochloride/0.05M-Tris/HCl, pH 8, and extensive dialysis against this solvent. The centrifuge was operated at 11036 rev./min at 20°C, and pictures were taken after sedimentation for 76 h.

Fig. 5. Fractionation of the LGP-I component on Sephadex G-25 after treatment with alkaline NaB^3H_4 .

LGP-I component was treated with 0.05 M-NaOH and 1 M- NaBH_4 containing 100 mCi of NaB^3H_4 , at 45°C for 18 h and then fractionated on a Sephadex G-25 column ($90\text{cm} \times 1.5\text{cm}$) eluted with 0.1 M-acetic acid adjusted to pH 5.8 with pyridine. The column fractions were analysed to determine the presence of radioactivity (—), carbohydrate (anthrone reaction, A_{620} ; ····) and ninhydrin-positive constituents (A_{570} , ----). V_0 and V_1 indicate the elution positions of Blue Dextran 2000 and CuSO_4 respectively. The shaded areas show the fractions pooled to give fractions E, F and G.

in fraction E and in galactosaminitol in fractions F and G are given in Table 2. The carbohydrate and amino acid compositions of fraction E were similar to that of untreated LGP-I component, which indicates that fraction E contained intact carbohydrate side chains.

The method of analysis used to determine the hexosamine content of fractions E and G did not separate galactosamine and galactosaminitol. Analysis of fraction F under conditions that resolve those hexosamines (Donald, 1968) showed that the galactosamine content of this fraction was approximately one-third that of the LGP-I component. A considerable portion of this galactosamine was probably derived from degraded LGP-I molecules, which contained uncleaved glycopeptide linkages. The amino acid content of fraction F (33%, Table 2) and the presence of radioactivity in the elution position of the acidic and neutral amino acids (Fig. 7) indicated that the alkali treatment of the LGP-I component had caused considerable degradation of

Fig. 6. Ion-exchange chromatography of radioactive acidic and neutral amino acids in fraction E (Fig. 5)

The distribution of radioactivity in the column effluent after hydrolysis of fraction E with 6 M-HCl, 24 h at 105°C , and fractionation of the hydrolysate on the $60\text{cm} \times 0.9\text{cm}$ column in an amino acid analyser. The arrows indicate the elution positions of alanine and α -aminobutyric acid (Abu).

Fig. 7. Ion-exchange chromatography of radioactive hexosamines in fraction F (Fig. 5)

The distribution of radioactivity (—) and ninhydrin-positive substances (----, A_{570}) in the column effluent after hydrolysis of fraction F with 6 M-HCl, 3 h at 100°C , and fractionation on the $20\text{cm} \times 0.9\text{cm}$ column in an amino acid analyser. The arrows indicate the elution positions of various known substances: (1) acidic and neutral amino acids; (2) galactosaminitol.

Table 2. *Chemical composition and incorporation of radioactivity into the Sephadex G-25 fractions after alkaline borohydride treatment of LGP-I preparation 2 (Fig. 7)*

The recovery is the freeze-dried weight of each fraction obtained after treatment of 50mg of LGP-I component. The method described by Donald (1968) was used to determine the galactosamine and galactosaminitol content of fraction F. The hexosamine contents of fractions E and G were determined by using an ion-exchange procedure that did not separate these constituents. The radioactivity values are the total quantities of counts present in each constituent in the various fractions. N.D., Not determined.

Fraction ...	Content (% w/w)		
	E	F	G
Recovery (mg)	18.14	4.89	2.44
Amino acids	41.1	33.0	N.D.
Glucosamine	1.6	0.16	0.05
Galactosamine	16.6	6.1	2.6
Galactosaminitol		2.0	
Galactose	12.9	14.9	9.7
N-Acetylneuraminic acid	22.8	29.4	1.5
	Radioactivity (c.p.m.)		
	E	F	G
Alanine	68841	N.D.	N.D.
α -Aminobutyric acid	274035	N.D.	N.D.
Galactosaminitol	N.D.	337590	167105

the peptide chain. This was confirmed when fraction E was rechromatographed on a Bio-Gel P-100 column. No radioactivity was observed in the void volume of the column, showing that none of the LGP-I molecules remained intact after this procedure. The low ratio of galactosaminitol/galactose in fraction F (Table 2), compared with the galactosamine/galactose ratio in the LGP-I component, indicated that considerable destruction of the galactosamine by chromagen production (Bray *et al.*, 1967) and peeling reactions occurred during the alkali treatment. These reactions would limit the formation of galactosaminitol (Mayo & Carlson, 1970). Fraction G contained only small amounts of N-acetylneuraminic acid.

Pronase digestion of the LGP-I component

The repeated digestion of the LGP-I component with Pronase over a 91h period yielded a major glycopeptide product (H), eluted at the void volume of a Sephadex G-25 column, together with a small amount of a second included glycopeptide fraction (J) (Fig. 8). The data obtained by analysis of these two fractions are shown in Table 3. After analysis, the remainder of glycopeptide fraction H was re-fractionated on a DEAE-cellulose column eluted

Fig. 8. *Fractionation of the LGP-I component on Sephadex G-25 after treatment with Pronase*

The enzyme-digest mixture was applied directly to the Sephadex G-25 column (90 cm $\times$ 1.5 cm), which was then eluted with 0.1M-acetic acid adjusted to pH 5.8 with pyridine. The presence of carbohydrate was determined by using the anthrone reaction ($\bullet$, A_{620}). The shaded areas indicate the fractions pooled to yield fractions H and J. V_0 and V_1 indicate the elution positions of Blue Dextran 2000 and $^3\text{H}_2\text{O}$ respectively.

with a pyridine/acetic acid gradient (Fig. 9). The fractions obtained by this latter chromatographic procedure were analysed to determine the quantities of amino acid and carbohydrate constituents present, and these data are also given in Table 3. Fractions H and J had lower lysine contents than did the LGP-I component, but were enriched in both threonine and proline. Re-fractionation of fraction H yielded six fractions, and of these fraction L, M, N and O, which represented more than 90% of the glycopeptides recovered from the DEAE-cellulose column, had a high content of threonine, proline, galactosamine, galactose and N-acetylneuraminic acid. Insufficient quantities of fraction K were available to obtain satisfactory analysis, but fraction P differed significantly in composition from both the LGP-I component and fractions L, M, N and O.

Galactose oxidase treatment of the LGP-I component

After treatment of both desialylated and intact LGP-I component with galactose oxidase and reduction with NaB^3H_4 , a single radioactive constituent with the same mobility as the LGP-I component was obtained (Plate 2). T.l.c. showed that the single major constituent oxidized by the galactose oxidase treatment was galactose and that small amounts of radioactivity were incorporated into galactosamine (Plate 2). The quantities of radioactivity incorporated into both intact and desialylated LGP-I component are given in Table 4. Fractionation on the amino acid analyser in a manner similar to that shown in Fig. 7 showed that approx.

Table 3. *Chemical composition of fractions obtained by Pronase digestion of LGP-I preparation 1*

Fractions H and J were obtained by chromatography on Sephadex G-25 (Fig. 8). Fractions L, M, N, O and P were obtained by re-fractionation of fraction H on DEAE-cellulose (Fig. 9). The recovery is the total amount of amino acids in the pooled column fractions indicated in Figs. 8 and 9 after dialysis and freeze-drying as outlined in the text. The (+) signs indicate that these constituents were present, but reliable estimates were not obtained.

Fraction	H	J	L	M	N	O	P
Content (residues/1000 residues)							
Amino acid							
Aspartic acid	20	20	21	11	9	10	57
Threonine	271	247	280	274	283	264	180
Serine	35	63	38	26	25	35	109
Glutamic acid	133	106	129	158	168	155	129
Proline	299	279	287	309	320	292	169
Glycine	14	20	13	5	6	10	133
Alanine	85	101	89	87	90	88	82
Valine	12	22	12	6	7	7	29
Methionine	1	1	1	1	—	1	—
Isoleucine	11	13	11	6	6	11	21
Leucine	13	9	10	6	9	16	32
Tyrosine	1	1	1	1	—	2	—
Phenylalanine	1	5	5	5	6	10	19
Lysine	100	101	97	102	71	98	41
Histidine	1	3	2	2	—	1	—
Arginine	5	9	6	3	—	1	—
Recovery of amino acids (mg)	8.0	1.7	0.8	0.6	0.4	0.3	0.05
Content ($\mu\text{M}/1000$ amino acid residues)							
Carbohydrates							
Mannose	3	6	(+)	(+)	(+)	(+)	(+)
Glucosamine	17	9	7	9	7	16	37
Galactosamine	271	213	242	299	228	228	149
Galactose	252	212	213	298	231	303	(+)
N-Acetylneuraminic acid	235	238	155	309	343	206	(+)

5% of the radioactivity was incorporated into galactosamine in intact LGP-I component and approx. 1.7% in desialylated LGP-I component (Table 4). These values indicate that in both samples terminal galactosamine was present in approximately equal quantities (approx. 3 residues/molecule of LGP-I component) for both samples. The results shown in Plate 2 indicate that the remainder of the radioactivity was incorporated into galactose. On the basis of this observation, the quantities of radioactivity apparently incorporated into galactose after the galactose oxidase treatment were calculated from the total radioactivity incorporated into the desialylated and intact LGP-I samples and the radioactivity determined in the galactosamine constituents. The values obtained, 5.72×10^{12} d.p.m./mol of LGP-I component and 1.68×10^{13} d.p.m./mol of desialylated LGP-I component, correspond to the occurrence of 52 and 153 molecules respectively of terminal galactose/molecule of LGP-I component.

Discussion

One of the major components of the lubricating fraction isolated previously (Swann & Radin, 1972)

was a high-molecular-weight glycoprotein (LGP-I), and preliminary studies showed that only synovial-fluid fractions containing this moiety possess positive lubricating ability (E. L. Radin & D. A. Swann, unpublished work). The major purpose of the present study was therefore to purify and characterize this molecule. The methods used previously were modified so that sufficient quantities of material would be made available for analysis by techniques that would completely separate LGP-I component from hyaluronate and which did not involve the use of enzymes. It was found that three successive CsCl-density-gradient procedures were necessary to satisfy this requirement. Further fractionation by using repeated gel-permeation chromatography and reduction and alkylation yielded preparations of LGP-I component essentially free of the other constituents previously detected in the lubricating fraction (Swann & Radin, 1972). Sedimentation experiments indicated that the molecule obtained was homogeneous in that there was no significant difference between the number-average and weight-average molecular weights over the whole range of solute concentration in the cell (Roark & Yphantis, 1969) and the mol.wt.

was calculated to be 227 500. Analyses of the LGP-I component showed that the molecule contained approximately equimolar amounts of galactosamine and threonine plus serine, and a high content of galactose and *N*-acetylneuraminic acid (Table 1), which indicated that this glycoprotein may contain many carbohydrate side chains composed of these

three sugars joined to the peptide chain by a threonine-*O*-GalNAc linkage (Spiro, 1970). The proportion of threonine residues present also suggested that these side chains might be fairly evenly distributed over the length of the peptide chain.

The results of the experiments involving the treatment of the LGP-I component with alkaline NaB^3H_4 provide strong evidence that at least some of the carbohydrate side chains in this glycoprotein are linked by threonine and serine *O*-GalNAc linkages. The presence of radioactivity in the elution positions of alanine, α -aminobutyric acid and galactosaminitol indicate that *O*-glycosidic linkages were cleaved by a β -elimination mechanism (Pigman & Moschera, 1973). The ratio of radioactivity in α -aminobutyric acid to that in alanine (Table 2) also indicates that 80% of the bonds cleaved involve threonine. Only a relatively small number of potentially available bonds, however, were cleaved by this reaction. It is difficult to estimate the actual number of threonine and serine residues involved in such linkages, because of the extensive degradation of the molecule that occurred during the reaction (Table 2). The high proline content (similar to that observed after Pronase digestion; Table 3) and the lower alanine content after reaction with alkali also indicate that parts of the molecule were lost by this treatment. By using the quantity of α -aminobutyric acid present after this treatment, a value of 14% can be calculated for the number of threonine residues involved in glycopeptide linkages. Tanaka & Pigman (1965) showed that when NaBH_4 alone was used in the absence of catalyst only 13% of the intermediate produced by β -elimination was reduced to α -aminobutyric acid. The ratio of threonine to proline before and after β -elimination, however, indicates that only 25% of the total threonine residues were destroyed by the alkali treatment. The apparently low conversion of threonine into α -aminobutyric acid for this glycoprotein, compared with others with a similar composition (Pigman & Moschera, 1973;

Fig. 9. Fractionation of fraction H (Fig. 8) on DEAE-cellulose

The glycopeptide constituents were fractionated on a DEAE-cellulose column (20cm $\times$ 0.9cm) in an acetic acid/pyridine, pH 5.0, gradient (for details, see the text). The fractions (2.5ml) were analysed to determine the presence of ninhydrin-positive constituents (A_{570} , ----) and sialic acid by using the thiobarbituric acid procedure (A_{549} , —). Fractions K, L, M, N, O and P were obtained by pooling the fractions indicated.

Table 4. Incorporation of radioactivity into galactosamine and galactose after treatment of LGP-I (preparation 2) and desialo-LGP-I (preparation 2) with galactose oxidase and reduction with NaB^3H_4

The data are expressed per mol of LGP-I component. Total incorporation is calculated from the determined values of radioactivity incorporated and the galactosamine content of the labelled sample by using a mol.wt. of 227 500 and a galactosamine content of 15.9% (w/w) (Table 1). The radioactivity in galactosamine was determined after isolation of galactosamine by ion-exchange chromatography. The radioactivity in galactose is the difference between the total radioactivity and the quantity detected in galactosamine. The number of terminal residues was calculated by using a value of 200 Ci/mol for the specific radioactivity of NaB^3H_4 and the fact that 1 mol of NaBH_4 reduces 4 mol of aldehyde.

Sample	Total incorporation (d.p.m.)	Galactosamine		Galactose	
		(d.p.m.)	Terminal residues	(d.p.m.)	Terminal residues
LGP-I	6.02×10^{12}	2.99×10^{11}	2.7	5.72×10^{12}	52
Desialo-LGP-I	1.71×10^{13}	2.97×10^{11}	2.7	1.68×10^{13}	153

Plantner & Carlson, 1975), appears to be the resistance of the threonine-*O*-GalNAc linkage to cleavage rather than failure of the NaBH_4 to reduce the unsaturated intermediate formed in the reaction. The extensive degradation of the peptide chain may have contributed to the stability of the threonine-*O*-glycosidic linkages, but this factor is insufficient to explain the limited cleavage that occurred. Gel-permeation chromatography of fraction E showed that most of the glycopeptides with intact carbohydrate side chains had apparent mol.wts. higher than 10000. In view of the composition of this fraction (Table 1), it appears unlikely that all the threonine residues involved in glycosidic linkages were in a terminal position and therefore resistant to cleavage (Derevitskaya *et al.*, 1967). Kieras (1974) also found a low conversion of destroyed threonine into α -amino-butyric acid in keratan sulphate glycopeptides, but the data presented suggested that this was caused by incomplete reduction of the unsaturated product formed by the β -elimination reaction.

The limited effect of alkali could be due to the presence of a glycopeptide linkage other than an *O*-glycosidic bond, or to the fact that this bond is stabilized, for example by specific amino acid sequences and the nature of the adjacent amino acids. The amino acid-composition data show that threonine and proline account for nearly 50% of the molecule and glutamic acid and lysine for a further 25%. If these amino acids were distributed evenly throughout the peptide chain it would be possible for each threonine residue to be adjacent to a proline and to a charged amino acid. The results obtained by Pronase digestion also indicate that the predominant amino acids and also the carbohydrate side chains are distributed throughout most of the molecule. All except one of the glycopeptide fractions obtained (Fig. 9; fraction P, Table 3) had a composition similar to that of the LGP-I component, except for the higher content of threonine and proline and the lower content of lysine. The LGP-I-like glycopeptides represented more than 90% of the material recovered, and in each case the molar proportions of galactosamine, galactose and *N*-acetylneuraminic acid provide evidence that these carbohydrate constituents are present in the same side chain.

Preliminary information about the structure of the carbohydrate side chains was obtained by galactose oxidase treatment of the desialylated and intact LGP-I molecule by using reduction with NaB^3H_4 to detect and determine the number of terminal galactose and galactosamine residues oxidized (Suzuki & Suzuki, 1972). Calculations based on the analytical values given in Table 1 and a mol.wt. of 227500 show that LGP-I preparation 2 contained 202 residues of galactosamine, 162 residues of galactose and 114 residues of *N*-acetylneuraminic acid per

molecule. Assuming that the completed carbohydrate side chains have the structure *O*-GalNAc-Gal-AcNeu,* the above molar compositions correspond to 114 completed side chains, 48 chains composed of *O*-GalNAc-Gal and 40 single galactosamine residues. The incorporation of radioactivity into galactosamine (Table 4) was clearly less than theoretical, but the presence of 52 terminal galactose residues in the intact molecule and 153 after neuraminidase treatment indicates that carbohydrate side chains with the above assumed structure are present in the LGP-I component. The apparently low incorporation of radioactivity into galactosamine if the above structure of the carbohydrate side chains is correct may have been caused by the proximity of this constituent to the peptide backbone, resulting in limited oxidation by galactose oxidase and/or reduction by the NaB^3H_4 . One of the glycopeptide fractions (fraction N, Table 3), however, had an AcNeu/Gal ratio of 1.5, which indicated that some of the oligosaccharide side chains have a more complicated structure than outlined above and this may also have contributed to the apparently low oxidation of galactosamine residues.

It is clear both from the analytical data and the galactose oxidase treatment that the LGP-I molecules contained a great many more carbohydrate side chains than could be demonstrated by the β -elimination reaction under alkaline conditions. It is concluded therefore, in view of the known variations in the degree of susceptibility of threonine and serine-*O*-glycosidic linkages to cleavage by alkali (Marshall & Neuberger, 1970), that specific structures in the LGP-I component were responsible for the limited cleavage of the threonine and serine residues linked *O*-glycosidically to *N*-acetylgalactosamine in this molecule.

Although the data discussed above strongly suggest that the LGP-I molecule contains many carbohydrate side chains composed of GalNAc-GalAcNeu linked to peptide by threonine and serine-*O*-GalNAc linkages, all of the fractions obtained both by alkali and Pronase treatments contained glucosamine and mannose. The quantities detected, however, were small compared with galactosamine, galactose and *N*-acetylneuraminic acid and the quantities of galactose oxidized by galactose oxidase. It is concluded therefore that either the LGP-I component contained a second type of carbohydrate side chain or it was contaminated by small amounts of a second glycoprotein which was not detected by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. In view of the difficulties experienced in separating the LGP-I component from the other glycoproteins present in the articular lubricating fraction, the latter possibility cannot be ruled out at the present time.

* Abbreviation: AcNeu, *N*-acetylneuraminic acid.

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