# Purification and Characterization of a Major Glycoprotein in Rat Liver Lysosomal Membrane

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**A major lysosomal membrane glycoprotein (LGP107) which has an apparent molecular weight ($M_r$) of 107 kilodaltons (kDa) was purified from rat liver by a simple method with a yield of 1 mg/87 g wet weight of liver. The purification procedures include; preparation of tritosomal membranes of triton-filled lysosomes (tritosomes), extraction of tritosomal membranes by Lubrol PX, wheat germ agglutinin (WGA)-Sepharose affinity chromatography, and monoclonal antibody-Sepharose affinity chromatography. The quantitative immunoblot analysis indicated that LGP107 represents 6.2% of the total protein of tritosomal membranes. The isoelectric point of the purified glycoprotein was 2.7, and it moved toward neutral pH after sialidase treatment, with its molecular weight decreased by about 10 kDa. LGP107 contained 52% carbohydrates, and the carbohydrate moiety was composed of Fuc, Man, Gal, GlcNAc and sialic acid in a molar ratio of 7.2 : 68.2 : 40.6 : 63.0 : 32.3, respectively, indicating that LGP107 was highly glycosylated with *N*-linked complex-type oligosaccharide chains. Out of the *N*-linked glycans released from the glycoprotein by hydrazinolysis/*N*-reacetylation, about 70% was sialylated. Anion exchange and reverse-phase high performance liquid chromatography analysis on the structure of *N*-glycans revealed that a disialyl biantennary form is a major component in the oligosaccharide chains of LGP107.**

**Keywords** glycoprotein; lysosomal membrane; monoclonal antibody; *N*-linked oligosaccharide chain; rat liver

## Introduction

There are two prominent glycoproteins with a molecular weight of 100—120 kilodaltons (kDa) on sodium dodecyl sulfate–polyacerylamide gel electrophoresis (SDS–PAGE) of lysosomal membranes.[1-5] In the last few years, biosynthesis and processing,[6-8] intracellular movement,[9-11] and complementary deoxyribonucleic acid (cDNA) determination[12-17] of these glycoproteins have been studied in various cells and species. According to the results on the cDNA sequence and biosynthesis, their extensive glycosylation is very unique. However, chemical analysis on the structure of the oligosaccharide chains of these proteins have not been well performed because it is difficult to obtain the glycoproteins in quantity owing to the low level content of lysosomes in the cell.

In the present study, we isolated a major glycoprotein, referred to as lysosomal membrane glycoprotein (LGP107), from rat liver tritosomes, and determined the carbohydrate composition of the glycoprotein. Furthermore, the anionic *N*-carbohydrate chains of LGP107 were characterized by anion exchange and reverse phase high performance liquid chromatography (HPLC).

### Materials and Methods

Male Wistar rats weighing 200—250 g and BALB/c mice were obtained from Seiwa, Experimental Animal Co. (Fukuoka, Japan). Wheat germ agglutinin (WGA)-Sepharose 6MB, Protein A-Sepharose CL 4B and Sepharose 4B, proteins for molecular weight standards and ampholine were purchased from Pharmacia & LKB (Uppsala, Sweden). Neuraminidase (*Clostridium perfringens*) was from Nakarai Co. (Kyoto, Japan). Various horseradish peroxidase-conjugated second antibodies were from Genzyme Co. (Boston, MA). Lubrol PX was from Sigma (St. Louis, MO). Other chemicals were of reagent grade.

**Preparation of an Immobilized Monoclonal Antibody against LGP107** The anti LGP107 monoclonal antibody was prepared according to the method of Köhler and Milstein.[18] Male mice (BALB/c) were immunized by partially purified tritosomal membrane glycoproteins (WGA bound fraction described below). Hybridoma secreting the specific antibody were developed by fusion of murine myeloma P3U1 and splenocytes obtained from the mice. A positive clone designated as AMS 156-30 was employed for large scale production of the specific antibody (mAb156; $IgG_1$) against LGP107 since the immunoprecipitation studies revealed that mAb 156 was bound specifically to the glycoprotein with apparent molecular weight ($M_r$) of 107 kDa. Pristane-primed male BALB/c mice were each inoculated intraperitonealy with 5 to $10 \times 10^6$ hybridoma cells from the midlog phase. Afterward, the ascitic fluid was repeatedly collected from each of the mice. Cells and debris were removed from the ascitic fluid by low speed centrifugation at $1000 \times g$ for 10 min.

Then additional fibrin and particulates were removed by centifugation at $50000 \times g$ for 60 min. Batches of ascitic fluid were tested for antigen binding ability by enzyme linked immunosorbent assay. The fluid was diluted 3 fold with phosphate buffered saline (20 mM sodium phosphate at pH 7.2 and 0.15 M NaCl). To about 20 ml of the diluted solution, 18 ml of saturated ammonium sulfate solution was added slowly with vigorous stirring at 0 °C. The suspension was kept in ice for 2 h and then centrifuged at $10000 \times g$ for 20 min. After the supernatant was well decantated and drained, the protein pellets were dissolved in 50 mM Tris–HCl (pH 8.9)/3 M NaCl and dialyzed against the same buffer. The dialyzed solution was centrifuged at $20000 \times g$ for 20 min to remove insoluble materials, then subjected to a column of protein A-Sepharose CL 4B (10 ml of packed gel) equilibrated with 50 mM Tris–HCl (pH 8.9)/3 M NaCl. After the column was washed with the equilibration buffer, the antibody was eluted with 0.1 M glycine–HCl (pH 3.5). The eluted antibody solution was immediately adjusted to pH 7.0 by adding Trizma base. Proteins were precipitated with 30% saturated ammonium sulfate and collected by centrifugation at $10000 \times g$ for 20 min. The protein pellets were dissolved in 0.1 M sodium borate (pH 8.0)/0.15 M NaCl and dialyzed extensively against the same buffer. The antibody was coupled to BrCN activated Sepharose 4B (10 mg of protein/ml of packed gel) according to the method of Cuatrecasas and Anfinsen.[19]

**Preparation of Membranes of Triton-Filled Lysosomes (Tritosomes)** Tritosomes were purified from rat liver according to the flotation method as described by Leighton *et al.*[20] To separate lysosomal membranes and contents, purified tritosomes were suspended in 20 mM Tris–HCl (pH 7.0) at 4 °C, then centrifuged at $105000 \times g$ for 60 min. The resultant pellets were washed with 1 M NaCl in 20 mM Tris–HCl (pH 7.0), followed by centrifugation at $105000 \times g$ for 60 min. The sedimented pellets were used as a tritosomal membrane fraction. The supernatants obtained through the two centrifugations were combined and used as a soluble fraction of tritosomes.

**Purification of LGP107 from Tritosomal Membrane** The tritosomal membranes were solubilized with 1% Lubrol PX in 20 mM Tris–HCl (pH 7.5) followed by centrifugation at $105000 \times g$ for 60 min. The resultant supernatant was applied to a column of WGA-Sepharose 6MB ($2 \times 5$ cm) previously equilibrated with the solubilization buffer. The column was extensively washed with 20 mM Tris–HCl (pH 7.5) containing 0.1% Lubrol PX and 1 M NaCl. Proteins were eluted with 0.5 M *N*-acetylglucosamine in 20 mM Tris–HCl (pH 7.5)/0.1% Lubrol PX. The eluates were concentrated with Amicon Dia Flow apparatus, then dialyzed against 20 mM Tris–HCl (pH 7.0) containing 0.1% Lubrol PX and 0.15 M NaCl (TBSL). The dialyzed materials were subjected to a column of monoclonal antibody (mAb

156)-Sepharose 4B (1.5 × 10 cm) equilibrated with TBSL. The column was washed with TBSL until absorbance at 280 nm dropped to a base line level (0.02—0.05 $A_{280}$). The bound proteins were eluted with 0.1 M sodium carbonate (pH 11)/0.2% Lubrol PX. The eluate was adjusted to a neutral pH by adding 0.5 M acetic acid and then concentrated with a Dia Flow apparatus.

**Gel Electrophoresis** SDS–PAGE was carried out on 12% slab gels according to the method of Laemmli.[21] Gel isoelectric focusing was performed in 4.5% disc gel containing 1% ampholine and 0.1% Lubrol PX by the method of Owen *et al.*[22] Proteins were stained with Coomassie Blue R-250, and carbohydrates by the periodate Schiff procedure.[23]

**Immunoblot Procedures** Anti-LGP107 polyclonal antibody was raised in rabbits by injections of purified LGP107, and used for immunoblotting. The quantitative immunoblot analysis was performed according to the method of Guengerich *et al.*[24] Stained bands on nitrocellulose sheets were scanned with a Shimadzu dual-wave length thin layer chromatography (TLC) scanner (model CS-910), utilizing fluorescent light in the reflectance mode. Areas were estimated either by triagulation or by use of an automated integrator. A standard curve based on purified LGP 107 run in the adjacent lanes was linear in up to 400 ng of the antigen.

**Protein Determination** Proteins were determined by the method of Lowry *et al.*[25] with bovine serum albumin as a standard.

**Determination of $NH_2$-Terminal Sequence** The $NH_2$-terminal sequence was determined with an Applied Biosystems, Model 470A gas phase protein sequencer. The phenylthiohydantoin (PTH) amino acid derivatives were automatically identified with an Applied Biosystems 120A PTH-analyzer used on-line with the sequencer.

**Amino Acid Analysis** The purified LGP107 was dialyzed against several changes of distilled water, lyophilized, and then hydrolyzed in 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole, under vacuum, for 24, 48, 72, and 96 h at 118 °C. Amino acids were analyzed on a Hitachi Model 835 automatic amino acid analyzer.

**Carbohydrate Composition Analysis** For quantitative carbohydrate analysis, the purified glycoprotein (100 μg) was mixed with mannitol and inositol solution (internal standard; 50 nmol each). After drying over $P_2O_5$ in a vacuum desiccator, the residue was dissolved in 2.5% methanolic HCl (0.2 ml) and methyl acetate (0.05 ml). After the solution was heated for 2 h at 90 °C, the methanolic HCl was evaporated to dryness under a $N_2$ stream at room temperature. To the residue was added acetic anhydride (20 μl) in methanol–pyridine (120 μl; 5:1, v/v) and the solution was left at room temperature for 12 h. Solvents and reagents were evaporated under $N_2$ at room temperature for re-*N*-acetylation. The sample was trimethylsilylated with 100 μl of silyl reagents (pyridine–HMDS–TMCS, 5:2:1) for 5 min at 60 °C. Analysis of trimethyl silylated methyl glycoside was carried out by gas liquid chromatography (GLC) on a CBP-1-W12-100 fused silica capillary column (0.53 mm × 12 m, Shimadzu) using a Shimadzu GC 9A gas chromatograph. The oven temperature was programmed first at 50 °C to 120 °C at 20 °C/min, and then from 170 °C to 250 °C at 2 °C/min.

Sialic acid was estimated by the periodate–resorcinol reaction method.[26]

**Release of *N*-Glycans and Labeling of *N*-Glycans with *p*-Aminobenzoic Acid Ethyl Ester (ABEE)** The release of *N*-linked oligosaccharides from LGP107 was performed by hydrazinolysis/*N*-reacetylation according to the procedure described by Takasaki *et al.*[27] and Bendiak.[28] The reaction mixture solution in saturated $NaHCO_3$ was applied to a column of Dowex-50($H^+$) and the column was washed with water. The combined washings and eluate were evaporated to dryness and the residues released from the glycoprotein were coupled with ABEE under the conditions as described previously.[29] The reaction mixture was diluted with water and extracted with ether. The aqueous layer was frozen and lyophilized, and the residue was dissolved in an appropriate volume of water. The aqueous solution of the reaction mixture was applied to a PRE-SEP $C_{18}$ cartridge (Senetek PLC, Mountain View, CA, U.S.A.) and the cartridge was eluted in a stepwise manner with water, water–acetonitrile mixtures, 95:5 and 9:1 by volume. Anionic and neutral oligosaccharide–ABEE were recovered in the water and 95:5 water–acetonitrile, and 9:1 water–acetonitrile fraction, respectively.

**Characterization of the Anionic Oligosaccharide–ABEE by HPLC** For further separation of the anionic oligosaccharide–ABEE, fractions eluted with water and water–acetonitrile (9:1) were pooled and evaporated to dryness. The residue was resolved in a small volume of water, then applied to HPLC with a TSK gel DEAE 5PW column (Tosoh Co., Tokyo). The column was eluted with 10 mM $NaH_2PO_4$ and subsequently with a linear gradient of 10—170 mM $NaH_2PO_4$ at a flow rate of 0.5 ml/min. Mono- to tetrasialyl oligosaccharide–ABEE were completely separated with HPLC under this condition when the authentic samples were used.

For analysis of the antennary structures, the anionic oligosaccharide–ABEE was treated with neuraminidase in 50 mM sodium acetate buffer (pH 5.4) for 20 h at 37 °C, and then subjected to reverse-phase HPLC on a Wakosil 5C18-200 column (Wako Co., Osaka). Elution was done using 9% acetonitrile in 50 mM $NaH_2PO_4$ at a flow rate of 1 ml/min. Oligosaccharide–ABEE was detected by a ultraviolet (UV) spectrophotometric detector at 304 nm.

## Results and Discussion

**Purification of LGP107 from Rat Liver Tritosomal Membranes** As shown in lanes 1 of Fig. 1, analytical SDS–PAGE of the tritosomal membranes shows conspicuous glycoprotein bands in the molecular weight range of 100 to 120 kDa. The presence of two glycoproteins in this region became distinct when stained with periodate-Schiff reaction rather than by Coomassie Blue dye. Most of the glycoproteins in the tritosomal membranes could be solubilized with a high percentage of recovery by 1% Lubrol PX (Fig. 1, lanes 2 and Table I). After application of the detergent lysate to affinity chromatography with a WGA-Sepharose column, major glycoproteins including LGP107 were concentrated in a WGA bound fraction (Fig. 1 lanes 3). Then, the WGA bound fraction was subjected to an anti-LGP107 monoclonal antibody-Sepharose column. About 8% of the protein applied was eluted from this immunoaffinity column by the high pH solution. As shown in the profile on SDS–PAGE (Fig. 2), LGP107 eluted in this fraction was free from other lysosomal membrane proteins. Through the procedures described above, we could

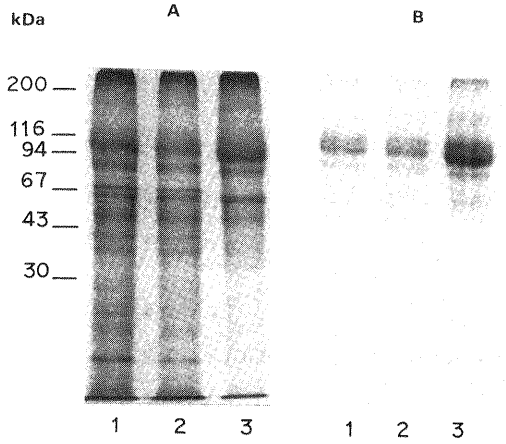


Fig. 1. SDS–PAGE of Rat Liver Tritosomal Membrane Glycoproteins

The tritosomal membranes were prepared as described under Materials and Methods. The membranes were solubilized with 1% Lubrol PX. After centrifugation at 105000 × *g*, for 60 min, the supernatants were applied to a column of WGA-Sepharose 6MB. The bound materials were eluted with 0.5 M *N*-acetylglucosamine. Lanes 1, 20 μg of the membranes; lanes 2, 20 μg of the membrane lysates; lanes 3, 20 μg of WGA bound fraction. The molecular weights (kDa) of marker proteins were shown in the left of gel (A): myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa). Gels were obtained after Coomassie Blue staining (A) or periodate-Schiff staining (B).

TABLE I. Purification of LGP107 from Rat Liver Tritosomal Membranes

| Step | Protein (mg) | Yield (%) |
|---|---|---|
| Lysosomal membranes | 28.0 | 100 |
| 1% Lubrol PX extracts | 20.5 | 73.2 |
| WGA-affinity chromatography | 9.3 | 33.2 |
| Immunoaffinity chromatography | 0.70 | 2.5 |

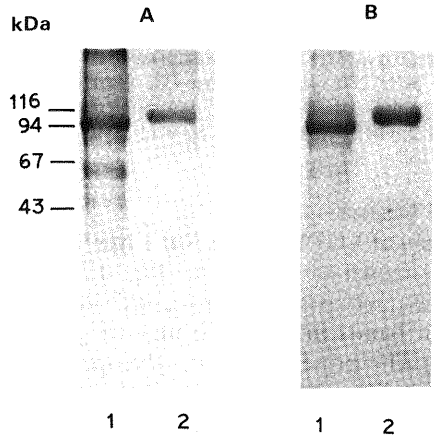


Fig. 2. SDS-PAGE of Fractions Obtained after an Immunoaffinity Chromatography of WGA Bound Fraction

The WGA bound fraction was subjected to immunoaffinity chromatography on a monoclonal antibody Sepharose 4B column as described under Materials and Methods. The unbound and bound fraction obtained were analyzed by SDS-PAGE. Lanes 1, 10 $\mu$g of the unbound fraction; lanes 2, 2 $\mu$g of the bound fraction. The molecular weights (kDa) of marker proteins were shown in the left of gel (A). Gels were obtained after Coomassie Blue staining (A) and the periodate-Schiff staining (B).

Table II. LGP107 Contents in Tritosomes, and Tritosomal Subfractions

| | Protein (mg/g wet weight liver) | LGP107 contents %[a)] | $\mu$g/g wet weight liver |
|---|---|---|---|
| Tritosomes | 0.46 | 2.5 | 11.5 |
| Membrane fraction | 0.12 | 6.2 | 7.4 |
| Soluble fraction | 0.34 | 0.98 | 3.3 |

a) Values listed are expressed as percentages of total protein in each fraction.

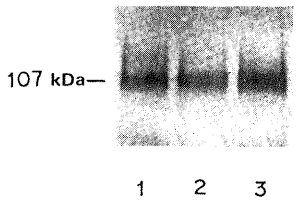


Fig. 3. Immunoblot Analysis of Rat Liver Tritosomes and Tritosomal Subfractions

The tritosomes and tritosomal subfractions were subjected to SDS-PAGE followed by immunoblotting using an anti-LGP107 rabbit antibody. Lane 1; tritosomes (10 $\mu$g), lane 2; tritosomal membranes (3 $\mu$g), lane 3; tritosomal contents (20 $\mu$g).

obtain 1 mg of the purified protein from about 87 g wet weight liver (Tables I and II).

**Determination of LGP107 Contents in Tritosomes** Specific contents of LGP107 in tritosomes and tritosomal subfractions (membrane and soluble fractions) were determined by using a quantitative immunoblot analysis. As shown in Fig. 3, a positive band with 107 kDa was detected in all the fractions tested. LGP107 accounts for 6.2% of the total protein in the tritosomal membranes (Table II). This figure indicates that LGP107 is a major protein in rat liver lysosomal membranes. About 30% of LGP107 was recovered in the soluble fraction of the tritosomes. LGP107 obtained in this fraction seems to be solubilized from the tritosomal membrane by a detergent of Triton WR1339 sequestered in the tritosomes.

Table III. Amino Acid Composition and *N*-Terminal Sequence of LGP107

| Amino acid | mol% A | mol% B |
|---|---|---|
| Asx | 11.14 | 11.14 |
| Thr | 7.30 | 9.07 |
| Ser | 10.31 | 10.62 |
| Glx | 8.45 | 7.51 |
| Gly | 6.98 | 5.70 |
| Ala | 6.58 | 7.77 |
| Cys | 1.78 | 2.07 |
| Val | 6.62 | 7.51 |
| Met | 2.10 | 2.33 |
| Ile | 3.65 | 4.40 |
| Leu | 13.84 | 8.81 |
| Tyr | 2.89 | 3.89 |
| Phe | 4.36 | 4.66 |
| Lys | 4.80 | 5.44 |
| His | 1.23 | 1.04 |
| Trp | 0.16 | 0.26 |
| Arg | 2.89 | 2.59 |
| Pro | 4.92 | 5.18 |

A: Determined by amino acid analysis. B: Calculated from a primary structure predicted by cDNA sequence.[15,16)] $NH_2$-Ala-Pro-Ala-Leu-Phe-Glu-Val-X-Asp-Asn-.

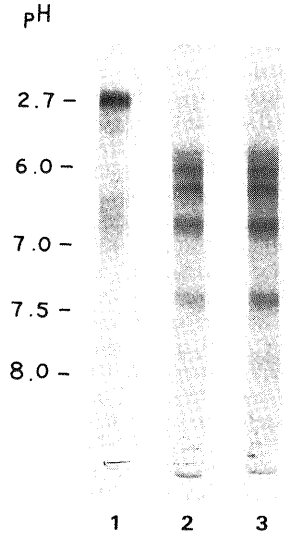


Fig. 4. Isoelectric Focusing of LGP107 before and after Neuraminidase Treatment

LGP107 before and after neuraminidase treatment were applied to gel isoelectric focusing. 20 $\mu$g of the purified LGP107 was incubated with 10 munits of neuraminidase at 37°C for 1 or 12 h in 50 mM acetate buffer (pH 5) containing 0.1% Lubrol PX. Lane 1; 20 $\mu$g of non-treated LGP107. Lanes 2 and 3; 20 $\mu$g of LGP107 were treated with neuraminidase for 1 and 12 h, respectively. All the gels were stained by the periodate-Schiff procedure.

**Amino Acid Composition and *N*-Terminal Sequence** The amino acid composition and *N*-terminal sequence of LGP107 were summarized in Table III. When the purified LGP107 protein was subjected to sequential Edman degradation for determining its $NH_2$-terminal sequence, the preparation showed only a single amino acid in each cycle of Edman degradation. This result indicates the high degree of purity of the glycoprotein obtained. The *N*-terminal amino acid sequence of LGP107 is identical to that predicted from the cDNA analysis of the glycoprotein.[15,16)] The amino acid composition of the purified LGP107 is also very similar to that deduced from the cDNA analysis.

**Isoelectric Point** In order to determine the isoelectric point (pI) of LGP107, the purified protein was subjected to gel isoelectric focusing. As shown in Fig. 4 (lane 1), LGP107 migrated as a single band at pH 2.7. After LGP107 was incubated with neuraminidase, the glycoprotein was transferred to the pH region from 6 to 8 with five heterogeneous bands (Fig. 4 lanes 2 and 3). These results indicated that the acidic pI of LGP107 was caused by the presence of a high level of negative charge from sialic acid. The high level of anionic charge on the protein should contribute to the maintenance of the protonated intralysosomal environment. The five isomorphic components of asialo LGP107 remained unchanged despite prolonged incubation with sialidase (compare lanes 2 and 3 in Fig. 4). Additionally, on SDS–PAGE, the sialidase-treated LGP107 was stained not as multiple bands but as a singlet with $M_r$ of 97 kDa (Fig. 5). The decrease in $M_r$ of LGP107 by sialidase-treatment was 10 kDa, which is in good agreement with the amounts of sialic acid in LGP107 determined by the chemical method as described later (Table IV). Taken together, the isoelectric heterogeneities of LGP107 are not caused by incomplete desialylation. Therefore, it is likely that some heterogeneous protein modification such as phosphorylation and sulfation occurs in LGP107, resulting in the isomorphic forms of asialo LGP107.

**Carbohydrate Composition** The result of quantitative analysis for the carbohydrates of LGP107 is shown in Table IV. The glycoprotein contained about 52% carbohydrates of which 22.6% was sialic acid. The cDNA sequence of LGP107 predicted that it contains 18—20 potential *N*-linked glycosylation sites per mole.[15,16] The presence of *N*-acetylglucosamine (GlcNAc), galactose and sialic acid (NeuAc) in high ratios in LGP107 indicated that *N*-oligosaccharide chains on LGP107 are mostly of the complex type.

According to Carlsson *et al.*,[5] a significant amount of *O*-linked oligosaccharide chains are contained in human lysosomal membrane glycoprotein (h-lamp 1) purified from myelogenous leukemia cells. However, Howe *et al.*[16] reported that lysosomal membrane glycoprotein (LGP120) from mouse macrophages was not glycosylated by the *O*-linked oligosaccharide chains. We could not detect the sugar component of the *O*-linked oligosaccharide chains, *N*-acetylgalactosamine (GalNAc), in the carbohydrates of LGP107. Occurrence of *O*-linked glycans in lysosomal membrane glycoproteins may be different among species and cell types.

**Analyses of *N*-Oligosaccharide Chains by HPLC** In order to characterize the structures of *N*-linked oligosaccharide of LGP107, *N*-glycans were released from the purified glycoprotein by hydrazinolysis/*N*-reacetylation. Then, they were labeled with ABEE through reductive amination. The ABEE-labeled glycans were separated into anionic and neutral fractions on a PRE-SEP $C_{18}$ cartridge. About 70% of the *N*-glycans were recovered in the anionic fraction. The anionic *N*-glycans from LGP107 were applied

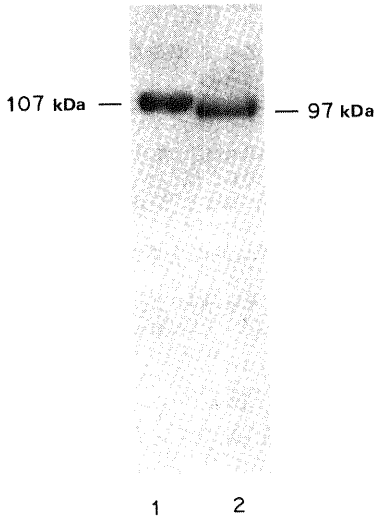


Fig. 5. SDS–PAGE of LGP107 before and after Neuraminidase Treatment

LGP107 (5 μg) was incubated with neuraminidase for 1 h under the same condition as in the legend of Fig. 4. Lane 1; non-treated LGP107, lane 2; neuraminidase-treated LGP107. The gels were stained by the periodate-Schiff procedure.

TABLE IV. Carbohydrate Composition of LGP107

| Carbohydrate | nmol/mol of glycoprotein |
|---|---|
| Fuc | 7.2 |
| Man | 68.2 |
| Gal | 40.6 |
| Glc | N.D. |
| GalNAc | N.D. |
| GlcNAc | 63.0 |
| NeuAc | 32.3 |

N.D.: not detectable.

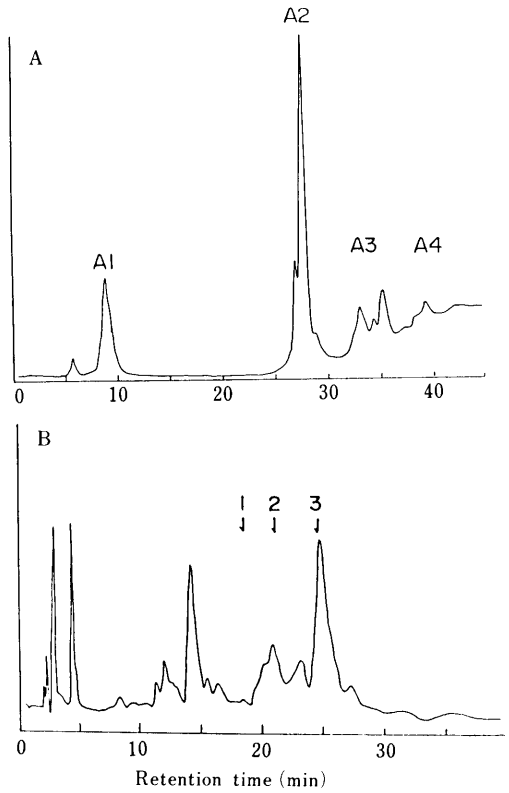


Fig. 6. HPLC of Oligosaccharide–ABEE from LGP107

A: Ion exchange HPLC of anionic oligosaccharide–ABEE on TSK gel DEAE 5PW. A1, A2, A3, and A4 indicate the elution position of mono-, di-, tri-, and tetrasialyl oligosaccharide ABEE, respectively. B: Reverse phase HPLC of asialo oligosaccharide ABEE on Wakosil 5C18-200. 1, 2, and 3 indicate the elution position of tetra-, tri-, and biantennary oligosaccharide–ABEE, respectively.

to anion exchange HPLC equipped with a TSK gel DEAE-5PW column. They were eluted at four positions consistent with authentic mono, di, tri, and tetrasialyl oligosaccharides from $\alpha_1$ acid glycoprotein (Fig. 6A). In addition, when the anionic *N*-glycans were treated with sialidase, all of them were recovered in the pass through fraction (data not shown). The elution profile shown in Fig. 6A evidently indicated that the anionic *N*-glycans were mostly composed of a disialylated complex type of oligosaccharides.

The ABEE labeled anionic *N*-glycans were desialylated, and then subjected to reverse-phase HPLC on a Wakosil 5C18-200 column. As shown in Fig. 6B, among bi-, tri-, and tetraantennary oligosaccharide chains, the biantennary oligosaccharide chain was a predominant component of the anionic carbohydrate chains in LGP107. Together with the results of anion exchange HPLC, we could conclude that a disialyl biantennary complex type oligosaccharide chain is a major component of the anionic *N*-glycans in LGP107.

There was another relatively large peak whose retention time was 14 min in the elution pattern on the reverse-phase HPLC (Fig. 6B). Considering its earlier retention time compared to that of the tetraantennary oligosaccharide, the *N*-glycans in this peak would have a much higher molecular weight than that of the tetraantennary. *N*-Glycans bearing lactosamine repeats found in h-lamp 1[5] might correspond to this unusual peak on the reverse-phase HPLC. However, the attachment of polylactosamine to the *N*-glycans of LGP107 is inconsistent with our results of the carbohydrate composition of the glycoprotein. Further studies are necessary for the identification of the sugar structure in this unknown peak.

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