Purification and Characterization of Glycoprotein from the Skin Mucus of the Rainbow Trout, Salmo gairdneri

Toshihisa SUMI, Yoichiro HAMA, Daisuke MARUYAMA, Makio ASAKAWA,* and Hiroki NAKAGAWA†

Department of Applied Biological Sciences, Faculty of Agriculture, Saga University, Honjo-yo-machi, Saga 840, Japan
*Laboratory of Food Science, Faculty of Education, Kumamoto University, 3-Kurokami, Kumamoto 860, Japan
Received October 21, 1996

Mucus glycoprotein (RGP) was purified and characterized from the skin mucus of rainbow trout, Salmo gairdneri. RGP was found to contain 30.1% NeuAc, 26.0% GlcNAc, 5.0% Gal, and 26.0% amino acids. The protein moiety of RGP is very rich in Thr (32.4 mol%). Neither NeuGc nor KDN (2-keto-3-deoxy-d-glycero-d-galacto-nononic acid) was found in RGP. Alkaline borohydride treatment of RGP yielded a major disaccharide alditol, NeuAc2→6GlcNAc-ol and more than 4 minor oligosaccharide aldits including NeuAc-(GlcNAc1→)GlcNAc-ol. It was evident that an average RGP molecule has approximately 500 NeuAc-containing oligosaccharide chains, which are attached to the Thr and Ser residues of the protein moiety and spaced at an average of 3 amino acids apart.

Key words: sialoglycoprotein; mucus glycoprotein; mucus; rainbow trout; mucin

The mucus that covers the body surface of fishes contains a variety of secretions from epidermal goblet cells and epithelial cells. In addition to these secretions, the mucus coat contains cellular macromolecules, electrolytes, and remnants of epithelial cells. It has been recognized that the mucus coat of fish has a variety of important physiological functions such as a mechanical and chemical protective barrier against parasites and microorganisms, a lubricant to facilitate swimming, and a sensor and regulator for osmolality. The most important constituent of the mucus coat is thought to be skin mucus glycoprotein. Although several important functions have been ascribed to mucus glycoproteins, the knowledge of their chemical compositions and properties is still fragmentary. Our previous studies on the mucus glycoproteins of loach, eel, and several species of fishes found that different species of fishes secrete their characteristic mucus glycoproteins, which are diverse in their carbohydrate moieties.

This paper describes the purification and characterization of glycoprotein from rainbow trout skin mucus.

Materials and Methods
Materials. Live cultured rainbow trout (Salmo gairdneri) were obtained directly from a culturist. The following were purchased from commercial sources: Sephadryl S-200 HR, Sepharose CL-4B, Sephadex G-25 (superfine), and DEAE-Sephadex A-25, Pharmacia Biotech; DE52 (DEAE-cellulose), Whatman; Dowex20-X8 (200–400 mesh), Dow Chemical; silica gel-60 TLC plates, Merck; NeuAc and nucleos P., Seikagaku Kogyo; hexamethyldisilazane and trimethylchlorosilane, Wako Pure Chemical Industries; pyridine, Pierce; pullulan molecular weight markers, Showa Denko; Actinase E, Kaken Seiyaku; neuraminidase (acylneuraminyl hydrolase, EC 3.2.1.18) from Arthrobacter ureafaciens, Nakarai Chemicals. α-N-Acetylglactosaminidase (2-acetamido-2-deoxy-α-N-galactosaminidase, EC 3.2.1.49) was isolated from skipjack liver. NeuAc2→6GlcNAc-ol was isolated from ovine submaxillary mucin, which was prepared from ovine submaxillary glands essentially according to the method described in Materials and Methods for Extraction and purification of glycoprotein (RGP) from rainbow trout skin mucus.

Sialic acid determination. Sialic acid was determined by the modified thiobarbituric acid method using NeuAc as the standard after the samples were hydrolyzed with 0.1 N H2SO4 at 80°C for 1 h.

Analysis of oligosaccharide alditos by TLC. Small amounts (about 7 µg with respect to NeuAc in 15 µL of distilled water) of oligosaccharide alditols were put on a precoated silica gel-60 TLC plate. The plate was developed twice with 1-butanol-acetic acid-water (2:1:1, v/v/v), sprayed with diphenylamine reagent, and heated at 115°C for 15 min to reveal oligosaccharide alditos.

Carbohydrate analysis by GLC. Carbohydrate components were determined as trimethylsilyl derivatives by GLC after methanolation under strong or mild conditions. Briefly, the sample (200 µg) was mixed with 6 µg each of manninitol and myo-inositol (internal standards), lyophilized, and dried in a vacuum desiccator over P2O5. Methanolysis of the dried sample was carried out in 1 ml of 1 N methanolic HCl at 90°C for 3 h (strong methanolation). After methanolysis, the reaction mixture was evaporated to dryness with a stream of nitrogen. The methyl glycosides of amino sugars after methanolysis were recylated with 20 µL of acetic anhydride in 200 µL of 10% pyridine-methanol at room temperature for 15 min. After evaporation to dryness, the methyl glycosides were reacted with 100 µL of pyridine-hexamethydisilazane-trimethylchlorosilane (5:1:1, v/v/v) at room temperature for 30 min. After the silylating reagents were removed with a stream of nitrogen, the residue was extracted with 500 µL of hexane. The extract was concentrated to 50 µL and a 1-µL aliquot of the concentrate was analyzed by a Shimadzu GC-14A gas chromatograph using a fused silica capillary column (CBP-1, 0.25 mm x 25 m, Shimadzu). After the column temperature was maintained at 40°C for 3 min, it was increased to 180°C at 20°C/min and then to 240°C at 1.5°C/min. The amounts of carbohydrate components were calculated using manninitol as the internal standard for hexose and hexosamine, and myo-inositol for sialic acid. Sialic acid was also determined by GLC as the trimethylsilyl derivative after methanolysis in 0.05 N methanolic HCl at 80°C for 1 h (mild methanolysis) as described by Yu and Ledeen. In this case, the acetylation step was omitted from the above procedure.

Methylation analysis. An oligosaccharide alditol preparation (100 µg) was permethylated as described by Ciucanu and Kerek. The permethylated oligosaccharide alditol was treated with 1 ml of 0.1 N methanolic HCl at 80°C for 1 h, evaporated to dryness with a stream of nitrogen, and acetylated with 100 µL each of pyridine and acetic anhydride at 80°C for 30 min. The mixture was evaporated to dryness, dissolved in

* To whom correspondence should be addressed.
† Abbreviations: RGP, glycoprotein from skin mucus of rainbow trout; KDN, deaminated neuraminic acid (2-keto-3-deoxy-d-glycero-d-galacto-nononic acid).
50 μl of hexane, and analyzed with a Shimadzu QP-5000 gas chromatograph-mass spectrometer using a fused silica capillary column (DB-1, 0.25 mm × 30 m, J&W Scientific). After the column temperature was maintained at 50°C for 3 min, it was increased to 180°C at 40°C/min and then to 260°C at 4°C/min. The mass spectrometer was operated in the electron ionization mode under the following conditions: ionization energy, 70 eV; ion source temperature, 220°C; scan interval, 0.5 s.

Amino acid analysis. Protein samples were hydrolyzed in 6 N HCl at 110°C for 24 h under a vacuum. The amino acid composition was determined using a JEOI-300 automatic amino acid analyzer. Corrections were made for destruction of threonine and serine during hydrolysis.\(^{16}\) Tryptophan was measured by the method of Sartin et al.\(^{17}\)

Exoglycosidase digestion. Neuraminidase digestion and analysis for free NeuAc; A glycoprotein preparation (200 μg) was digested with 0.1 unit of neuraminidase at 37°C for 24 h in 0.5 ml of 0.05 M sodium acetate buffer, pH 5.0, in the presence of toluene. The toluene mixture was added to the small (0.5 ml) of Dowex-50X-8 (H+). The effluent and washings were combined and lyophilized with 6.5 μg of myo-inositol (internal standard). The dried sample was treated with 0.05 M methanolic HCl at 80°C for 1 h, trimethylsilylated, and analyzed by GLC for free NeuAc, α-N-Acetylaminosaminidase digestion and analysis for free GalNAc; A glycoprotein preparation (300 μg) was digested with 0.3 unit of α-N-acetylglucosaminidase at 37°C for 24 h in 1 ml of 0.05 M sodium acetate buffer, pH 4.2, in the presence of toluene. The toluene mixture was added to 5 ml of ethanol and the ethanol mixture was treated as described above. The dried sample containing 6.5 μg of mannitol as the internal standard was trimethylsilylated and analyzed by GLC for free GalNAc.

Preparation of rainbow trout skin mucus. Fifteen live rainbow trout (average weight 320 g) were placed in a plastic bag and kept frozen at −35°C overnight. The frozen fishes were immersed in 3 liters of ethanol for 5 min at room temperature. This treatment caused the skin mucus to coagulate into a jelly that could be easily removed from the skin by scraping. The jelly-like mucus was collected by filtration using a Buchner funnel. The mixture was stored at 4°C until used and was reduced pressure to obtain 3.5 g of rainbow trout skin mucus powder.

Extraction and purification of glycoprotein (RGP) from rainbow trout skin mucus. The rainbow trout skin mucus powder (3.5 g) was suspended in 350 ml of 0.05 M Tris–HCl buffer, pH 7.8, and sonicated using an ultrasonic disruptor (Tomy Seiko model UR-200P) for 5 min. The temperature was maintained at 4°C by adding ice water. The mixture was centrifuged at 20,000 × g for 30 min. This extraction was repeated once more. The combined supernatant (700 ml) was adjusted to pH 5.0 with acetic acid and centrifuged to remove the resulting precipitates. The clear supernatant was concentrated to 100 ml in an Amicon ultrafiltration stirred cell using a PM-10 membrane. The concentrate was digested with 200 units of nuclease P1 at 37°C for 24 h in the presence of toluene. After incubation, the incubation mixture was adjusted to pH 7.0 with 1 N NaOH, heated at 100°C for 5 min, and centrifuged to remove the precipitates. The supernatant was concentrated and equilibrated with 0.05 M Tris-HCl buffer, pH 7.8, by ultrafiltration with a PM-10 membrane to give a final volume of 40 ml. The concentrate was applied to a DE52 column (2.5 × 27 cm) which had been equilibrated with 0.05 M Tris-HCl buffer, pH 7.8. After the column was washed with this buffer, it was eluted with a 0–0.5 M NaCl gradient in the buffer at 70 ml/h and 10.6-ml fractions were collected (Fig. 1a). The fractions which contained sialic acid were pooled and concentrated to 10 ml by ultrafiltration with a PM-10 membrane. The concentrate was applied to a Sepharose CL-4B column (4 × 77 cm) which had been equilibrated with 0.05 M Tris–HCl buffer, pH 7.8, containing 0.1 M NaCl. The column was eluted with the same buffer at 22 ml/h and 8.3-ml fractions were collected (Fig. 1b). The fractions that contained sialic acid were pooled, dialyzed against water, and lyophilized to obtain 9.5 g of glycoprotein (RGP).

Actinase digestion of RGP and purification of the resulting glycopeptide (Actinase-digested RGP). RGP (45 mg) dissolved in 5 ml of 0.05 M Tris–HCl buffer, pH 7.8, containing 0.02 M calcium acetate was digested with 6 mg of total of Actinase at 37°C for 72 h in the presence of toluene. Actinase (2 mg × 3) was added at 24-hour intervals while the pH of the mixture was maintained at 7.2 to 7.8 by the addition of 1 N NaOH. After digestion, the mixture was heated at 100°C for 3 min and centrifuged to remove the precipitates. The supernatant was applied to a DEAE-Sephadex A-25 column (0.2 × 19 cm) which had been equilibrated with 0.05 M Tris-HCl buffer, pH 7.8. After the column was washed with this buffer, it was eluted with a 0–0.5 M NaCl gradient in the buffer at 80 ml/h and 10.0-ml fractions were collected (Fig. 4a). The fractions which contained sialic acid were pooled, concentrated and equilibrated with 0.1 M NH₄HCO₃ by ultrafiltration with a YM-3 membrane to give a final volume of 3 ml. The concentrate was applied to a Sephacryl S-200 HR column (2.2 × 89 cm) which had been equilibrated with 0.1 M NH₄HCO₃. The column was eluted with the same solution at 12 ml/h and 3.5-ml fractions were collected (Fig. 4b). The fractions that contained sialic acid were pooled and lyophilized.

Liberation of carbohydrate chains from RGP. RGP (25 mg) was dissolved in 20 ml of 0.05 M KOH containing 1 M NaOH and incubated at 37°C for 48 h.\(^{18}\) The reaction mixture was neutralized with acetic acid, diluted with 100 ml of distilled water, and passed through a Dowex-50X-8 column (2 × 10 cm, H⁺) at 5°C. The effluent was lyophilized. The resulting boric acid was removed by repeated codistillation with methanol in a rotary evaporator to obtain carbohydrate chains (oligosaccharide alditois). The residue was dissolved in 1 ml of distilled water.

Fractionation of carbohydrate chains. The carbohydrate chains (oligosaccharide alditois) dissolved in 1 ml of distilled water was applied to a Sephacryl G-25 column (1.7 × 147 cm, superfine) which had been equilibrated with 0.1 M NH₄HCO₃. The column was eluted with the same solution at 12 ml/h and 2.3-ml fractions were collected. Sialic acid in the fractions was monitored for oligosaccharide alditois. The fractions containing the major carbohydrate chain were pooled and lyophilized (Fig. 5a).

Results

Extraction and Purification of RGP

Ultrasound disruption of the mucus suspension in a large volume of 0.05 M Tris–HCl buffer, pH 7.8, facilitated quantitative extraction of RGP from the rainbow trout skin mucus powder. Acid treatment of the extract at pH 5.0 was very effective to remove nucleic acid and protein impurities as precipitates. After centrifugation of the acidified extract, a clear and less viscous supernatant was obtained. From the thiobarbituric acid assay of the precipitates, a considerable amount of RGP-bound sialic acid appeared to precipitate concomitantly with these impurities. However, the absorption spectrum of the thiobarbituric acid-positive substance in the precipitates showed its maximum at 530 nm, suggesting that most of the thiobarbituric acid-positive substance was deoxyribose from DNA.\(^{19}\) Since RGP is stable on heating at neutral pH, heat treatment of the extract at 100°C for 3 min also reduced protein impurities. Digestion of the extract with nuclease P₁ before chromatographic purification was imperative for the complete removal from degradation products of nucleic acids. The concentrated solution obtained after heat treatment following nuclease P₁ digestion was subjected to DE52 chromatography. Figure 1a shows the elution profile of RGP from the DE52 column. RGP was eluted as a single peak with 0.2 M NaCl. The fractions that contained RGP were pooled, concentrated, and subjected to Sephacryl CL-4B gel filtration. Figure 1b shows the elution profile of RGP from the Sephacryl CL-4B column. RGP emerged immediately after the void volume of the column. The purified RGP did not show substantial absorbance at 280 nm. Recoveries of RGP in the purification steps were summarized in Table I. Its yield was 72 mg with a recovery of 62% from 3.5 g of the starting mucus material. A flow
Glycoprotein from Rainbow Trout Skin Mucus

Fig. 1. Purification of RGP from Rainbow Trout Skin Mucus by DE52 Chromatography (a) and Sepharose CL-4B Gel Filtration (b), and Estimation of the Apparent Average Molecular Weight of RGP by the Gel Filtration (c).

a: The concentrate of the supernatant obtained after heat treatment following nuclease P1 digestion was subjected to DE52 chromatography. b: The concentrate of the pooled fractions indicated by the bar in a was subjected to Sepharose CL-4B gel filtration. c: Estimation of the apparent average molecular weight of RGP by Sepharose CL-4B gel filtration using pullulan molecular weight markers. Sialic acid in fractions was monitored for RGP. Sialic acid was determined by the thioarbitruric acid method after the samples were hydrolyzed with 0.1N H2SO4 at 80°C for 1 h. ʿ·········· sialic acid; ·········· absorbance at 280 nm. The numbered arrows shown in b indicate the elution volumes (the tops of the elution peaks) of pullulan markers which were subjected to the Sepharose CL-4B gel filtration under the same conditions as used for RGP. 1, pullulan M1, 12,200; 2, pullulan M2, 48,000; 3, pullulan M3, 380,000; 4, pullulan M4, 853,000.

Table 1. Recoveries of RGP from 3.5 g of Rainbow Trout Skin Mucus Powder

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Sialic acid (mg)</th>
<th>RGP (mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucus powder</td>
<td>35.0</td>
<td>116</td>
<td>100</td>
</tr>
<tr>
<td>Extraction</td>
<td>33.3</td>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td>Acid treatment at pH 5.0</td>
<td>25.6</td>
<td>84</td>
<td>73</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>24.9</td>
<td>82</td>
<td>71</td>
</tr>
<tr>
<td>DE52 chromatography</td>
<td>23.1</td>
<td>76</td>
<td>66</td>
</tr>
<tr>
<td>Sepharose CL-4B gel filtration</td>
<td>21.7</td>
<td>72</td>
<td>62</td>
</tr>
</tbody>
</table>

The amount of RGP was obtained by multiplying the amount of sialic acid by 3.3 since the purified RGP contained 30% sialic acid (Table II).

Fig. 2. A Flow Chart for Purification of RGP from Rainbow Trout Skin Mucus.

chart for the purification of RGP is shown in Fig. 2.

Characterization of RGP

Carbohydrate composition. The carbohydrate components of RGP were analyzed by GLC. The gas chromatogram is shown in Fig. 3 and the carbohydrate composition of RGP is given in Table II (left). Sialic acid is only NeuAc. No other species of sialic acids such as NeuGc and KDN (2-keto-3-deoxy-D-glycero-D-galacto-nononic acid) were detected. NeuAc and GalNAc are major carbohydrate components. A considerable amount of Gal was also found. No other carbohydrates were detected. The total of these three carbohydrate components occupied 61% of the glycoprotein by weight.

Amino acid composition. Table III (left) shows the amino acid composition of RGP before and after alkaline treatment. The protein moiety of RGP accounts for only 26% of the dried weight. RGP is unusually rich in Thr (32 residues/100 residues) and 84 out of 100 residues of amino acids are Thr, Ser, Glu (or Gln), Pro, Gly, Ala, and Val. This glycoprotein is poor in Cys, Met, Tyr, and Phe. Trp was not detected in the glycoprotein.
Table II. Carbohydrate Compositions and Amino Acid Contents of RGP and Actinase-digested RGP

<table>
<thead>
<tr>
<th>Components</th>
<th>RGP (%)</th>
<th>Actinase-digested RGP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NeuAc</td>
<td>30.1</td>
<td>37.3</td>
</tr>
<tr>
<td>GalNAc</td>
<td>26.0</td>
<td>31.8</td>
</tr>
<tr>
<td>Gal</td>
<td>5.0</td>
<td>6.1</td>
</tr>
<tr>
<td>Amino acid</td>
<td>26.0</td>
<td>22.6</td>
</tr>
<tr>
<td>Total</td>
<td>87.1</td>
<td>97.8</td>
</tr>
</tbody>
</table>

Actinase-digested RGP was purified by DEAE-Sephadex A-25 chromatography and Sephacryl S-200HR gel filtration following Actinase digestion of RGP (see Actinase digestion).

Treatment RGP with 0.3 N NaOH at 30°C for 48 h destroyed 75% (24 out of 32 residues) of the Thr and 50% (6 out of 12 residues) of the Ser. This indicates that the Thr and Ser residues destroyed by acid hydrolysis following alkaline treatment (β-elimination) are involved in the linkage of the carbohydrate chains to the protein moiety through GalNAc residues.

Average molecular weight. The apparent average molecular weight of RGP was estimated to be approximately 500,000 by Sepharose CL-4B gel filtration using standard pullulan preparations as the molecular weight markers (Figs. 1b and 1c).

Exoglycosidase digestion. Liberation of the two major carbohydrate components, NeuAc and GalNAc, by neuraminidase and α-N-acetylgalactosaminidase digestions was examined. The digestion of RGP by neuraminidase liberated more than 95% of the NeuAc originally found in this glycoprotein, indicating that the NeuAc occupies the nonreducing ends of the carbohydrate chains. The extensive digestion of RGP with a sufficient amount of α-N-acetylgalactosaminidase liberated approximately 7% of the GalNAc. This suggests that at least 7% of the GalNAc residues in this glycoprotein occurs as nonreducing ends in the carbohydrate chains and the rest of the GalNAc residues are involved in the linkage of the carbohydrate chains to the protein moiety.

Actinase digestion. Extensive digestion of RGP by Actinase gave nondialyzable glycopeptide fragments (Actinase-digested RGP) in which virtually all of the carbohydrate components originally present in RGP were recovered. Actinase-digested RGP was purified by DEAE-Sephadex A-25 chromatography followed by Sephacryl S-200 HR gel filtration (Figs. 4a and 4b). The apparent average molecular weight of Actinase-digested RGP was approximately 50,000 (Fig. 4c). The carbohydrate content of the glycopeptide was considerably higher than that of original RGP (Table II), and its amino acid composition became devoid of Cys, Met, Tyr, and Phe, suggesting that Actinase digested the polypeptide region containing these amino acids in RGP (Table III). It is evident that Actinase-digested RGP is a polypeptide bearing a number of sialic acid-containing carbohydrate chains that protected the polypeptide against the protease.

Liberation and fractionation of carbohydrate chains. The carbohydrate chains were liberated as oligosaccharide alditols from RGP by alkaline borohydride treatment. The liberated oligosaccharide alditols were analyzed for GalNAc and GalNAc-ol by GLC (strong methanolysis). Approximately 90% of the GalNAc originally present in RGP was converted to GalNAc-ol. The oligosaccharide alditols were fractionated by Sephacryl G-25 gel filtration. Figure 5 shows the elution profile of oligosaccharide alditols and TLC analysis of these alditols in fractions. On the TLC analysis, NeuAc2→6GalNAc-ol from ovine submaxillary mucin was run at the same time as a reference compound. Since NeuAc-containing oligosaccharides are stained violet, several violet bands of oligosaccharide alditols were detected on the chromatogram. This TLC analysis revealed that a
### Table III. Amino Acid Compositions of RGP before and after Alkaline Treatment and Amino Acid Composition of Actinase-digested RGP

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Before (µg/mg)</th>
<th>(res/100 res)</th>
<th>After (µg/mg)</th>
<th>(res/100 res)</th>
<th>Actinase-digested RGP (µg/mg)</th>
<th>(res/100 res)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>7.4</td>
<td>2.5</td>
<td>7.3</td>
<td>3.8</td>
<td>1.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Thr</td>
<td>84.8</td>
<td>32.4</td>
<td>14.1</td>
<td>8.4</td>
<td>83.5</td>
<td>36.4</td>
</tr>
<tr>
<td>Ser</td>
<td>26.8</td>
<td>11.9</td>
<td>9.1</td>
<td>6.3</td>
<td>27.1</td>
<td>13.4</td>
</tr>
<tr>
<td>Glx</td>
<td>34.1</td>
<td>10.2</td>
<td>34.0</td>
<td>15.8</td>
<td>28.6</td>
<td>10.0</td>
</tr>
<tr>
<td>Pro</td>
<td>23.6</td>
<td>9.4</td>
<td>23.3</td>
<td>14.4</td>
<td>22.5</td>
<td>10.1</td>
</tr>
<tr>
<td>Gly</td>
<td>10.3</td>
<td>7.0</td>
<td>10.3</td>
<td>10.8</td>
<td>9.8</td>
<td>7.4</td>
</tr>
<tr>
<td>Ala</td>
<td>14.4</td>
<td>7.8</td>
<td>14.3</td>
<td>12.1</td>
<td>14.2</td>
<td>8.6</td>
</tr>
<tr>
<td>Cys</td>
<td>1.7</td>
<td>0.6</td>
<td>1.7</td>
<td>1.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Val</td>
<td>14.6</td>
<td>5.7</td>
<td>14.3</td>
<td>8.7</td>
<td>14.3</td>
<td>6.2</td>
</tr>
<tr>
<td>Met</td>
<td>1.4</td>
<td>0.4</td>
<td>1.5</td>
<td>0.7</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Ile</td>
<td>8.7</td>
<td>3.0</td>
<td>8.6</td>
<td>4.5</td>
<td>6.0</td>
<td>2.3</td>
</tr>
<tr>
<td>Leu</td>
<td>5.1</td>
<td>1.8</td>
<td>5.0</td>
<td>2.6</td>
<td>2.4</td>
<td>0.9</td>
</tr>
<tr>
<td>Tyr</td>
<td>2.1</td>
<td>0.5</td>
<td>1.9</td>
<td>0.7</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Phe</td>
<td>2.0</td>
<td>0.5</td>
<td>2.0</td>
<td>0.8</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>His</td>
<td>7.5</td>
<td>2.1</td>
<td>7.4</td>
<td>3.2</td>
<td>5.1</td>
<td>1.6</td>
</tr>
<tr>
<td>Lys</td>
<td>8.3</td>
<td>2.5</td>
<td>8.0</td>
<td>3.7</td>
<td>4.8</td>
<td>1.6</td>
</tr>
<tr>
<td>Arg</td>
<td>7.2</td>
<td>1.8</td>
<td>7.1</td>
<td>2.7</td>
<td>4.6</td>
<td>1.3</td>
</tr>
<tr>
<td>Trp</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Total</td>
<td>260.0</td>
<td>100</td>
<td>197.9</td>
<td>100</td>
<td>226.3</td>
<td>100</td>
</tr>
</tbody>
</table>

Alkaline treatment: 100 µg of RGP was treated with 0.2 ml of 0.3 N NaOH at 30°C for 48 h.

The values of Thr and Ser were corrected with factors of 1.027 and 1.10, respectively, for their destruction during hydrolysis.16)

### major carbohydrate chain and more than 4 minor ones occur in RGP. The major oligosaccharide alditol had the same mobility as that of NeuAcz2→6GalNAc-ol. Minor oligosaccharide alditols with lower mobilities than NeuAcz2→6GalNAc-ol are presumed to be NeuAc-containing oligosaccharide alditols with larger molecular weights. GLC analysis of an oligosaccharide alditol in fraction 90 found that it is composed of equimolar amounts of NeuAc, GalNAc, and GalNAc-ol (data not shown). The fractions 92–100 were pooled and evaporated to dryness to obtain the major oligosaccharide alditol for structural studies. This oligosaccharide alditol gave a homogeneous band on TLC.

### Characterization of the major carbohydrate chain. The major oligosaccharide alditol was analyzed for carbohydrate composition by GLC (mild methanolysis). Figure 6 shows the gas chromatogram obtained. The oligosaccharide alditol was composed of equimolar amounts of NeuAc and GalNAc-ol. Approximately 80% of the GalNAc-ol liberated from RGP by alkaline borohydride treatment was found in the major oligosaccharide alditol preparation.

To determine substitutions in the linkage between the NeuAc and GalNAc-ol, the oligosaccharide alditol was subjected to methylation analysis. The resulting permethylated methyl glycoside methyl ester of NeuAc and partially permethylated GalNAc-ol-monoacetate were separated by GLC. As shown in Fig. 7a, several peaks were detected. Based on the mass spectrum of each peak, Peaks 2 and 5 were identified to be 1, 3, 4, 5-tetra-O-methyl-6-O-acetyl-2-deoxy-2-N-methylacetamido-galactitol (M = 335) and the permethylated methyl glycoside methyl ester of NeuAc, respectively, by the following interpretation. The electron ionization mass spectrum of Peak 2 and the fragmentation scheme for 1, 3, 4, 5-tetra-O-methyl-6-O-acetyl-2-deoxy-2-N-methylacetamido-galactitol are shown in Figs. 7b and 7d, respectively. The largest ion m/z 290 (M – 45) is derived from the loss of the fragment CH3OCH3 from the molecular ion due to the cleavage between C-1 and C-2. The cleavage between C-2 and C-3 produces the ion pair m/z 130 and m/z 205. Similarly, the ion pair m/z 174 and m/z 161 are the products of the cleavage between C-3 and C-4, and the ion pair m/z 218 and m/z 117 are the products of the cleavage between C-4 and C-5. The detection of the characteristic ion m/z 218 clearly indicates the presence of an acetyl group at C-6 since C-5 of hexose and hexosamine can not be involved in glycosidic linkage due to the pyranose ring structure. Thus, it is evident that C-6 of the GalNAc-ol of the oligosaccharide alditol is involved in the glycosidic linkage. Other ions such as m/z 88, 101, 142, 230, and 258 are secondary ions derived from the loss of ketene, acetic acid, or methanol from the ions mentioned above.

The electron ionization mass spectrum of Peak 5 and the fragmentation scheme for permethylated methyl glycoside methyl ester of NeuAc are shown in Figs. 7c and 7e, respectively. The largest ion m/z 376 (M – 31) must be due to the loss of OCH3 from the molecular ion. The characteristic ion m/z 348 (M – 59) is derived from the loss of COOCH3. Thus, the molecular ion of Peak 5 is estimated to be m/z 407, which corresponds to the permethylated methyl glycoside methyl ester of NeuAc. Based on these results and the fact that practically all of the NeuAc of RGP were hydrolyzed by neuraminidase from *Arthrobacter ureafaciens*, the structure of the major oligosaccharide alditol is concluded to be NeuAcz2→6GalNAc-ol.

### Discussion
Fish skin that is in constant contact with the external aqueous environment is coated with mucus consisting of
mucus cell secretions, cellular macromolecules, and remnants of epithelial cells. The mucus coat of fish has been implicated in many important physiological functions. In spite of its biological importance, very little is known about the chemical nature of fish skin mucus. Mucous glycoproteins that are mucin-type glycoproteins are considered to be the most important constituents of the mucus coat. In our previous studies we found that cel skin mucus glycoprotein has many NeuAc-containing disaccharide chains and in contrast loach skin mucus glycoprotein has many KDN-containing oligosaccharide chains. This was the first evidence that KDN occurs also in fish skin mucus glycoprotein. KDN, a deaminated neuraminic acid analogue, was first isolated from the polysialoglycoprotein of rainbow trout eggs. Subsequently, KDN-containing glycoconjugates have been isolated from the vitelline envelope, ovarian fluid, and sperm of rainbow trout. These findings prompted us to study rainbow trout skin mucus glycoprotein.

Similar to mammalian mucus, fish skin mucus is a gel composed of complex chemical constituents and is practically insoluble in water. In order to isolate glycoprotein from the mucus, it is necessary to solubilize the mucus glycoprotein, which is entangled through noncovalent bonds with proteins and nucleic acids in the mucus gel. We found that ultrasonic disruption of the rainbow trout mucous suspension in a large volume of 0.05 M Tris–HCl buffer, pH 7.8, could solubilize practically all of the mucus glycoprotein. Gel filtration on Sepharose CL-4B was very effective to remove protein impurities since the mucus glycoprotein is a large molecule. For complete removal from degradation products of nucleic acids,
however, treatment of the mucus extract by nuclease was imperative. Based on Sepharose CL-4B gel filtration, the apparent average molecular weight of RGP was estimated to be approximately 500,000.

To elucidate the nature and various functions of fish skin mucus glycoproteins, characterization of their carbohydrate chains is important since the characteristics of glycoproteins depend on their carbohydrate chains. Although a large proportion of glycoproteins from eggs, vitelline envelope and ovarian fluid of rainbow trout contain NeuGc and KDN as sialic acid components, RGP did not contain these sialic acids. Major carbohydrate components of RGP are NeuAc (30.1%) and GalNAc (26.0%) in a molar ratio of 1.0:1.2. The exhaustive digestion of RGP by neuraminidase and α-N-acetylgalactosaminidase liberated practically all of the NeuAc and only 7% of the GalNAc. This result strongly suggests that most of the carbohydrate chains of RGP are disaccharide NeuAc–GalNAc. Alkaline borohydride treatment (β-elimination under reductive conditions) of RGP liberated its carbohydrate chains in the form of oligosaccharide aldolts. By this treatment, approximately 90% of the GalNAc initially present in RGP was found as GalNAc-ol in the oligosaccharide aldolts. Considering the incomplete reaction of β-elimination and the fact that exhaustive digestion of RGP by α-N-acetylgalactosaminidase liberated 7% of the GalNAc, it is estimated that approximately 93% of the GalNAc is involved in the glycosidic linkage to the protein moiety. From the average molecular weight of RGP and the amount of the GalNAc involved in the carbohydrate–protein linkage, an average RGP molecule is estimated to have approximately 500 carbohydrate chains. Fractionation of oligosaccharide aldolts by Sephadex G-25 gel filtration and their TLC analysis revealed that a major carbohydrate chain and more than 4 minor ones occur in RGP. Using methylation analysis, together with the result of neuraminidase digestion of RGP, the major oligosaccharide aldol was identified to be NeuAc2→6GalNAc-ol. Its original disaccharide NeuAc2→6GalNAc is identical to the carbohydrate chain occurring in ovine submaxillary glycoprotein and cel skin mucus glycoprotein. In the case of these glycoproteins, no other carbohydrate chains have reported. In RGP, a considerable amount of an oligosaccharide aldol (fractions 88–90 in Fig. 5b) and small amounts of at least 3 oligosaccharide aldolts were also detected as minor carbohydrate chains. The former was found to be composed of equimolar amounts of NeuAc, GalNAc and GalNAc-ol. This trisaccharide aldol is presumed to be NeuAc2→6(GalNAc1→3) GalNAc-ol since we have found a novel trisaccharide chain, KDNz2→6(GalNAc1→3) GalNAc as a minor carbohydrate chain from loach skin mucus glycoprotein. Minor oligosaccharide aldolts were not further characterized due to the limited amounts of the samples available.

As in the case of mammalian epithelial mucus glycoproteins, RGP is very poor in Cys, Met, Thr, and Phe. The extremely low UV absorbance of RGP agrees with the fact that this glycoprotein is extremely poor in aromatic amino acids (Table III). RGP is unusually rich in Thr. The sum of the Thr and Ser residues to which the carbohydrate chains can be O-glycosidically attached accounts for 44 residues/100 residues of the total amino acids. During acid hydrolysis following alkaline treatment (β-elimination), 30 residues out of the 44 residues were destroyed, suggesting that more than 70% of the hydroxy amino acids were involved in the O-glycosidic linkage of carbohydrate chains. There is little doubt that all carbohydrate chains are O-glycosidically attached to the hydroxyl groups of Thr and Ser residues in the protein moiety through GalNAc residues. From the average molecular weight and the amino acid content (26%, Table III) of RGP, its protein moiety is estimated to have a molecular weight of 130,000.
and thus to be composed of approximately 1200 amino acid residues. More than 400 of the Thr and Ser residues out of the 1200 amino acid residues are presumed to be involved in the carbohydrate-protein linkage. This estimate agrees with the number (approximately 500) of carbohydrate chains calculated from the amount of liberated GalNAc-ol. The presence of a small amount (0.6 residue/100 residues) of Cys (half-cystine) in RGP is noteworthy. The lower Cys value may have been obtained due to oxidation of cysteine and cystine during acid hydrolysis. It can be estimated...
from the Cys content that the protein moieties contains more than 10 Cys residues, which take part in disulfide bonds between several highly glycosylated subunits. Exhaustive digestion of RGP by Actinase resulted in obtaining a highly glycosylated polypeptide preparation with an apparent average molecular weight of approximately 50,000 (Fig. 4c). The carbohydrate content in this preparation is considerably higher than that of the original glycoprotein (Table II) and the amino acid composition became devoid of Cys, Met, Tyr, and Phe. Our these results are essentially in agreement with mucin oligomer structures proposed for human cervical mucin and porcine submaxillary mucin, in which several highly glycosylated polypeptides (protease resistant and Ser- and Thr-rich) and nonglycosylated polypeptides (protease susceptible and Cys-rich) are attached tandemly to form a mucin oligomer.

Based on these results, the following overall structure can be proposed for RGP. An average RGP molecule contains approximately 500 oligosaccharide chains such as the major disaccharide NeuAcα2→6GalNAc and several minor NeuAc-containing oligosaccharides that are linked to threonine and serine residues of the protein moiety through GalNAc residues. These oligosaccharide chains are spaced at an average of 3 amino acids apart. The multiple, mutually repelling and highly hydrated negative charges of the oligosaccharide chains give the glycoprotein an extended rod-like conformation, which is largely responsible for the characteristic viscoelastic property in aqueous solution. The fish skin mucus glycoprotein involves through non-covalent bonds mucus cell secretions, cellular macromolecules, electrolytes, and remnants of epithelial cells to form a mucus-gel layer on fish skin. One of the important roles of the fish skin mucus is considered to be a biochemical barrier against microorganisms and parasites in the water.

References