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The Carbohydrate Moiety of IgM From Atlantic Salmon (*Salmo salar* L.)

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ABSTRACT. The carbohydrate moiety of salmon IgM was estimated to be about 12.5% of the total molecular weight of salmon IgM based on SDS-PAGE analysis and ≤8.0% based on FACE analysis. The carbohydrate moiety was restricted to the heavy chain and was all N-linked. Six different oligosaccharides were identified using the FACE oligosaccharide profiling technique. Monosaccharide composition analysis, as well as digestion with endoglycosidase H, suggested that the oligosaccharides were mainly of the complex type rather than high mannose type. Removal of about 80% of the carbohydrate affected the sensitivity of IgM to trypsin but had no effects on antigen binding or the complement fixation ability of anti s-RBC IgM. COMP BIOCHEM PHYSIOL 116B; 4:423–430, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. Endo H glycosidase, FACE, glycosylation, IgM, PNGase, F, O-Glycanase, *Salmo salar*, trypsin

INTRODUCTION

Glycosylation represents a major post-translational modification of proteins that can influence their structure and function. All immunoglobulins are glycosylated, and the amount of carbohydrate present and the nature of glycosylation are characteristic of each immunoglobulin class (13).

The two main types of oligosaccharide linkages to the protein are the N-linked and O-linked types (15). N-linked oligosaccharide is covalently bonded through nitrogen to asparagine (Asn) when it occurs in the sequence Asn-X-Ser/Thr, where X may be any amino acid other than proline. O-linked oligosaccharide is covalently bonded through oxygen to serine and threonine but the amino acid sequence where bonding occurs is not defined. The possible structural diversity of the oligosaccharide is enormous. There are, for example, several subgroups of N-linked oligosaccharides that share a common core structure but vary with respect to the composition and complexity of their outer chains (12,15,23,30).

Concurrent with the development of protein engineering, the importance of the normal glycosylation of the recombinant protein has become clearly apparent (35). It has also become apparent that the effects of glycosylation cannot be generalised but must be examined on a case-by-case basis (12,30,35). However, certain roles are commonly attributed to the oligosaccharide moiety of, for example, immunoglobulins: they may contribute to their structural stability and protect against proteases (33), they may be involved with effector functions like complement fixation and Fc receptor recognition (16,22,27,29,36), glycosylation can influence the avidity (5) and polymerization of IgM (11,26) and changes in the oligosaccharide moiety are sometimes associated with ageing, pregnancy or diseases (4,8). Most of these conclusions have been reached from studies of immunoglobulins from mammalian species that possess several Ig classes with different structure and function, including IgG, IgA and pentameric IgM.

The immune system of fish is in many ways similar to the mammalian system. There are, however, certain differences due to their position on the evolutionary ladder, their poikilothermic nature and adaptation to the aquatic environment (14). Most fish species have only one immunoglobulin class, tetrameric IgM. Exceptions are the chondrichthyan species, which possess pentameric IgM and a low-molecular-weight immunoglobulin of separate genetic origin (17,21). Very few reports exist in the literature about the glycosylation of fish IgM (1,10).

Information about the likely N-linked glycosylation of IgM from fish and higher vertebrates can be gained by examining the amino acid sequence analysis of IgM subunits, where this is available (2,3,7,17,19,37). Such an examination shows that normally only the heavy chain is glycosylated, commonly bearing five theoretical sites that are restricted to the constant regions. However, there are two noticeable differences in their distribution on IgM heavy chain of fish and higher vertebrates. First, fish μ chain does not seem to have a glycosylation site on the Cμ1 domain in the Fab region. This applies to both tetrameric IgM from

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teleosts and pentameric IgM from chondrichthyan species. One or two glycosylation sites are usually present in this domain of IgM from higher vertebrates. Second, the Cμ4 domain of fish IgM is glycosylated, whereas glycosylation is restricted to the C-terminal tailpiece of the μ chain of higher vertebrates (2,3,7,17,19,37).

In this article, the glycosylation of IgM isolated from the serum of Atlantic salmon (*Salmo salar* L.) was studied. Fluorophore-assisted carbohydrate electrophoresis (FACE) (20) was used to analyse the amount and nature of the oligosaccharide moiety, and some of its possible functional roles were also examined.

MATERIALS AND METHODS

The Fish

Atlantic salmon (0.5–1.0 kg) were kept in tanks with continuously flowing well water at approximately 10°C and fed commercial food pellets. Ten fish were used in this experiment.

IgM Purification

IgM was purified from serum pools of both immunised and control fish, using combined affinity and gel filtration chromatography using the fast protein liquid chromatography (FPLC) system from Pharmacia, Allerød, Denmark (24). Purity was checked by SDS-PAGE and Western blotting using polyclonal mouse antibodies (ascites) to salmon serum proteins and IgM. The protein concentration of purified IgM solution was measured at an optical density of 280 nm and using an extinction coefficient in E = 15 for 1% solution (24).

Salmon IgM can be separated into two subtypes by ion exchange chromatography (18,19). These are present in serum in roughly equal proportions. Isolation of the two types, when required, was carried out using MonoQ anion exchanger (HR5/5, Pharmacia). The equilibrium buffer was 20 mM Tris–HCl, pH 7.5, containing 0.15 M NaCl and elution was by NaCl gradient with an upper limit of 1M.

Unless specially noted, the analysis described below was carried out on purified IgM before the subtype separation.

SDS-PAGE and Western Blotting

Standard procedure was used for SDS-PAGE that was carried out in 14% separation gel and 4.5% stacking gel using the MiniProtean II system from BioRad (Hercules, CA, U.S.A.). Before electrophoresis, all samples were reduced for 2–5 min at 100°C with 5% mercaptoethanol in 125 mM Tris buffer, pH 6.8, containing 2% SDS. After electrophoresis the gels were stained for protein, using a silver staining kit from BioRad or transferred to a nitro-cellulose (NC) membrane (Hybond-ECL, Amersham, Buckinghamshire, England, U.K.). The transfer was carried out for 1 hr, using the semi-dry MilliBlot Graphite Electroblotter from Millipore (Bedford, MA, U.S.A.). A single buffer system of 25 mM Tris–glycine buffer, pH 8.8, containing 20% methanol, was used.

After the transfer, the NC membrane was blocked overnight at 4°C, in 2% BSA and 2% normal swine serum in 0.1M Tris–HCl buffered saline, pH 7.8, containing 0.1% Tween 20 (TBS-T). Primary antibody, as well as the peroxidase conjugated antibodies (Dako, Glostrup, Denmark), were diluted in TBS-T, containing 0.2% BSA and 0.2% swine serum. Each incubation was for 1 hr at room temperature. The blots were washed extensively in TBS-T between each step. The ECL system from Amersham was used for developing the blots. The primary antibody was polyclonal mouse anti salmon IgM primarily against the heavy (μ) chain.

The molecular weight estimations were extrapolated from standard graphs, using a range of molecular weight standards from two companies (Sigma, St. Louis, MO, U.S.A. and Bio-Rad) and were based on several SDS-PAGE separations.

PNGaseF Digestion

PNGase F, 500 units/μl (BioLabs, Beverly, MA, U.S.A.), removes N-linked oligosaccharides from glycoproteins. Purified IgM, unreduced or reduced, was digested with PNGaseF according to the manufacturers' directions. This analysis was also carried out on the two IgM sub-types separated by ion exchange. Under reducing conditions, 500 U of PNGase were used to digest 2 μg of reduced IgM in 20 μl for up to 1 hr at 37°C. The same amount of unreduced IgM was digested with 2500 U of PNGase for up to 3 hr at 37°C. If trypsin digestion was to follow the carbohydrate removal, the PNGase in the sample was first inactivated by heating at 100°C for 5 min. The results were analysed by SDS-PAGE after the reduction of the samples.

O-Glycanase Digestion

O-Glycanase, 1 milliunits/μl (Genzyme, Cambridge, MA, U.S.A.), removes O-linked oligosaccharides from glycoproteins. Purified reduced IgM was digested with O-Glycanase according to the manufacturers' directions. Approximately 2 milliunits of O-Glycanase were used to digest 2 μg IgM in 20 μl for 1 hr at 37°C. The results were analysed by SDS-PAGE.

Endo H Glycosidase Digestion

Endo H, 500 units/μl (BioLabs), cleaves the chitobiose core of high mannose oligosaccharides from glycoproteins. Purified reduced IgM was digested with Endo H according to the manufacturers' directions. Approximately 500 U of Endo H

were used to digest 2 μg IgM in 20 μl for 1 hr at 37°C. The results were analysed by SDS-PAGE.

Trypsin Digestion

The method for trypsin digestion of salmon IgM, using Trypsin-actigel-ald from Sterogene Biochemicals (San Gabriel, CA, U.S.A.), was a slight modification of a method described elsewhere (25). Trypsin-gel was washed and equilibrated in 0.1M Tris–HCl, 10 mM $CaCl_2$, pH 8.0 buffer on a glass filter and then suction dried. One milligram of suction dry Trypsin-gel (1.75 μg trypsin) was mixed with 2 μg salmon IgM in 20 μl reaction buffer (0.5 M sodium phosphate, pH 7.5) and incubated overnight ($\geq$16 hr) at 45°C. After the incubation period, the sample was centrifuged at 750 ***g*** for 10 min and the supernatant collected.

Because of the inhibitory effect of SDS and the reducing agent, mercaptoethanol, trypsin digestion was restricted to unreduced samples of IgM.

FACE Analysis

N-linked oligosaccharide profiling and analysis of the total monosaccharide composition of a purified sample of salmon IgM was performed by Glyko Inc. (Novaío, CA, U.S.A.) using the following procedures.

N-LINKED ANALYSIS. N-linked profiling was carried out on enzymatically released (PNGase) oligosaccharides. These were fluorescent-labelled and analysed in polyacrylamide gels. Location of oligosaccharides was determined relative to glucose polymers (4–12 glucose units [GU]) and quantity estimated by comparing band intensity to standards with the aid of a computer.

MONOSACCHARIDE ANALYSIS. Three separate hydrolysis reactions were performed on the sample to determine the maximum amine, neutral and sialic acid monosaccharides present. Fluorescent labelling and gel analysis followed and monosaccharides were identified and the quantity determined by comparing the bands to monosaccharide standards of defined amounts.

Immunization of Salmon with Sheep Red Blood Cells

Sheep blood was collected in Alsever's solution and red blood cells (s-RBC) washed three times by suspending in 0.85% saline followed by centrifuging at 750 ***g*** for 10 min. A 20% s-RBC suspension in 0.01 M phosphate-buffered saline, pH 7.4 (PBS) was mixed with an equal volume of Freund's complete adjuvant (FCA) and 0.5 ml injected intraperitoneally into seven fish. Three uninjected fish were kept as control. Five weeks later, the immunization was repeated with 10% suspension of s-RBC in PBS. At 4-week intervals, blood samples (3–5 ml) were collected from the caudal aorta, allowed to clot at room temperature for 1 hr and then overnight at 4°C. Serum was collected by centrifuging at 750 ***g*** for 10 min and stored at −80°C.

Haemolytic Assay

The haemolytic assay described by Nonaka *et al.* (28) was used, with some modifications, to determine the specific salmon antibody response to s-RBC and the complement fixing ability of purified IgM. The test makes use of the principle that when the specific antibody binds to the s-RBC it will fix complement, resulting in cell lysis, which can be visually or spectrophotometrically detected.

The test was carried out at room temperature; the diluent was complement fixation test (CFT) buffer, prepared from tablets (Oxoid Ltd., Hampshire, England, U.K.), containing 0.1% gelatine (CFT-G) and untreated serum from the control fish, stored in 100-μl aliquots at −80°C, was used as the complement source.

Fifty microliters, of 2-fold dilutions, of heat inactivated (45°C for 30 min) serum or purified IgM were made on a 96-well round bottom CFT microtray (Nunc, Roskilde, Denmark). A 0.5% suspension of washed s-RBC was prepared in CFT-G buffer and 50 μl added to the serum dilutions. The tray was sealed with adhesive tape and vigorously shaken for 30 min at room temperature. Fifty microliters of salmon complement, diluted 1:50, was added to each well. The tray was vigorously shaken for 30 min. Unlysed s-RBC were settled by centrifuging the microtray at 750 ***g*** for 10 min. Seventy-five microliters of the supernatant were transferred to a flat bottom 96-well microtray (Nunc) and the optical density (OD) read at 405 nm.

Under these conditions, complete lysis gave an OD reading of 0.8–1.0 and no lysis an OD reading of $<$0.1. Titre was defined as the reciprocal value of the serum dilution that gave OD reading of about three times the mean of the negative controls (0.2–0.3). Because of the interference of the reducing agent (mercaptoethanol or dithiothreitol), the haemolytic assay of purified IgM antibodies was restricted to unreduced samples.

RESULTS

Quantitative and Qualitative Analysis of the Carbohydrate Moiety

TREATMENT WITH GLYCOSIDASES. Untreated salmon IgM separated into its 70-kDa heavy chain and 25-kDa light chain components as shown in Fig. 1, lane 2. PNGase digestion, for 1 hr, of reduced IgM (Fig. 1, lane 3) reduced the μ chain to about 58 kDa, removing 12 kDa. This is about 12.5% of the total calculated molecular weight of tetrameric IgM (760 kDa). The light chain was not affected.

O-Glycanase had no detectable effect on the heavy or light chain, before or after PNGase treatment (Fig. 1, lanes 5 and 4, respectively) and Endo H cleaved 1–2 kDa from the heavy chain (Fig. 1, lane 6).

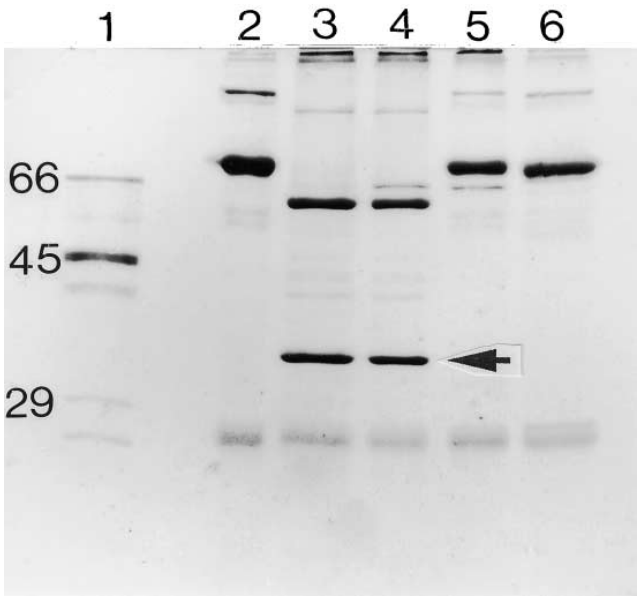

FIG. 1. Reduced salmon IgM digested with glycosidases for 1 hr followed by SDS-PAGE analysis of reduced samples. Molecular weight standards (kDa) (lane 1); untreated IgM (lane 2); IgM, deglycosylated with PNGase (lane 3); IgM, deglycosylated with PNGase, followed by digestion with *O*-Glycanase (lane 4); IgM, digested with *O*-Glycanase (lane 5); IgM, digested with Endo H glycosidase (lane 6). PNGase is indicated by an arrow.

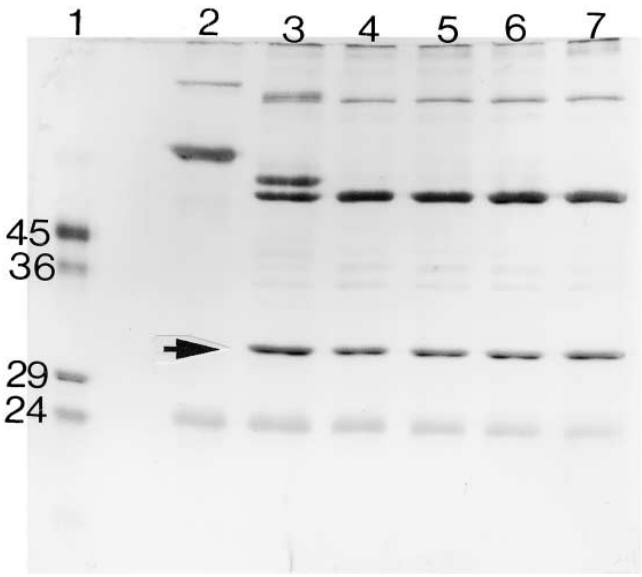

FIG. 2. Reduced salmon IgM digested with PNGase for different time intervals, followed by SDS-PAGE analysis of reduced samples. Molecular weight standards (kDa) (lane 1), untreated IgM (lane 2), PNGase digestion of IgM for ≤2 min (lane 3), for 10 min (lane 4), for 20 min (lane 5), for 40 min (lane 6) and for 60 min (lane 7). PNGase is indicated by an arrow.

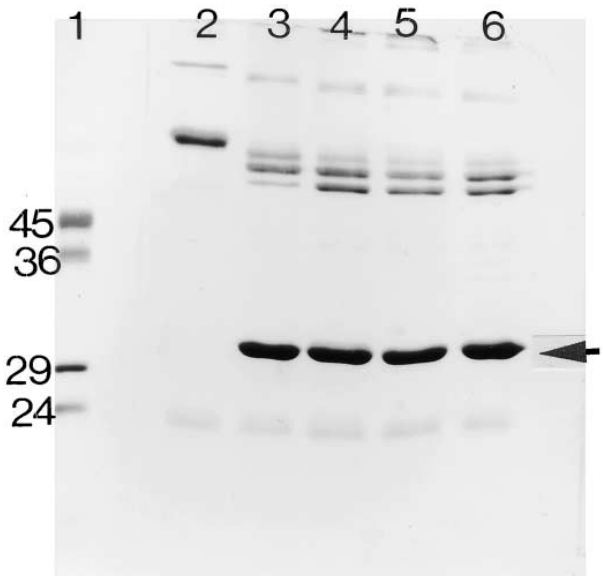

FIG. 3. Unreduced salmon IgM digested with PNGase for different time intervals followed by SDS-PAGE analysis of reduced samples. Molecular weight standards (kDa) (lane 1), untreated IgM (lane 2), PNGase digestion of IgM for 30 min (lane 3), for 60 min (lane 4), for 120 min (lane 5) and for 180 min (lane 6). PNGase is indicated by an arrow.

Fig. 2 shows the PNGase digestion of reduced IgM over a time span of 60 min. In the initial stages of digestion (≤2 min), an intermediate stage of a 62-kDa μ chain is observed, as well as the deglycosylated 58-kDa chain. Complete deglycosylation was obtained after 10-min incubation.

When unreduced IgM was treated with PNGase, using five times the amount of PNGase and an incubation time of up to 3 hr, the partially deglycosylated 62-kDa μ chain was observed after 30 min and persisted after 3 hr in roughly equal proportions to the completely deglycosylated 58-kDa μ chain (Fig. 3, lanes 3–6). The 66-kDa band also seen in these samples may be a partially deglycosylated heavy chain, although it was not always seen in repeated SDS-PAGE separations.

Because about half of the IgM sample was completely deglycosylated under unreducing conditions and the other half lost about two-thirds of its carbohydrate moiety (8 kDa), it was estimated that approximately 80% of the total carbohydrate was removed by the PNGase digestion of unreduced IgM.

Under the conditions used, the two IgM subtypes were separated by ion exchange chromatography with 3.53 and 3.91 M NaCl. Although the 62-kDa partially deglycosylated μ chain was slightly more prominent in the more charged subtype (eluted with 3.91 M NaCl), reduced and unreduced samples when treated with PNGase gave essentially the same digestion pattern as described above (not shown).

FACE ANALYSIS. A direct measure of fluorescence attached to the released oligosaccharides and monosaccha-

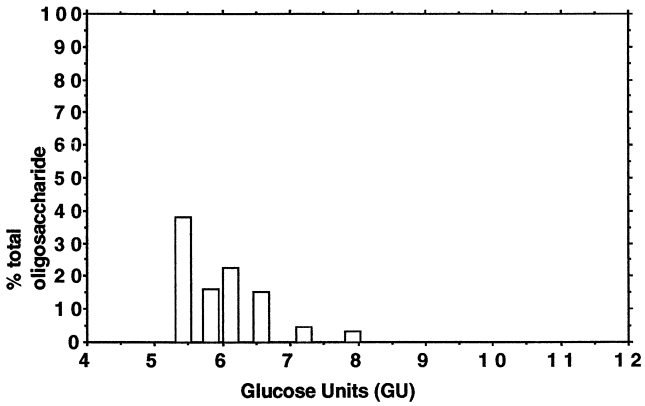

FIG. 4. FACE N-linked oligosaccharide profiling of the carbohydrate moiety of salmon IgM. Each column represents an individual oligosaccharide released and the relative amount (based on data from Glyko Inc. U.S.A.).

rides during the FACE analysis indicated that glycosylation of salmon IgM was 4–8% of the total molecular weight (Glyko).

Oligosaccharide profiling identified six different N-linked oligosaccharide structures with GU values ranging from 5.42 to 7.92 G (Fig. 4). The higher the GU value, the more complex the oligosaccharide and the more it may contain of *N*-acetylated amino sugars. The simplest form, corresponding to 5.42 G, was most abundant (38%), but the two more complex forms were under 5% of the oligosaccharide pool.

The results of the monosaccharide analysis are shown in Table 1. The neutral monosaccharides present were mannose, galactose and a small amount of fucose. *N*-acetylglucosamine was present and sialic acid of the *N*-acetylneuraminic form (NANA). A small amount of *N*-acetylgalactosamine was detected. Glucose, detected in the sample, was considered to be a contaminant by the analysers (Glyko).

Functional Role of the Carbohydrate Moiety

EFFECT ON THE ANTIGEN AND COMPLEMENT BINDING ABILITY OF SALMON IgM. Ten to 12 weeks after the initial immunization with s-RBC, all fish showed good antibody response to s-RBC. The titre reached a maximum (≥10,000) after about 15 weeks, but relatively high levels of antibody activity (>5000) were still detected when the experiment was terminated and the fish slaughtered at the end of 24 weeks. The control fish showed no antibody activity against s-RBC. Good haemolytic activity was obtained using purified immunoglobulin from the immunised fish in the concentration ≥6 μg IgM/ml in the haemolytic assay.

TABLE 1. Monosaccharide composition of the carbohydrate moiety of salmon IgM*

Monosaccharide	Molar ratio (%)
N-acetyl galactosamine (GalNAc)	1.7
Mannose	28.7
Fucose	3.8
Glucose	Contaminant
Galactose	17.2
N-acetyl glucosamine (GlcNAc)	31.7
N-acetylneuraminic acid (NANA)	17.0
N-glycolylneuraminic acid (NGNA)	0.0

*Based on data from Glyko Inc. U.S.A.

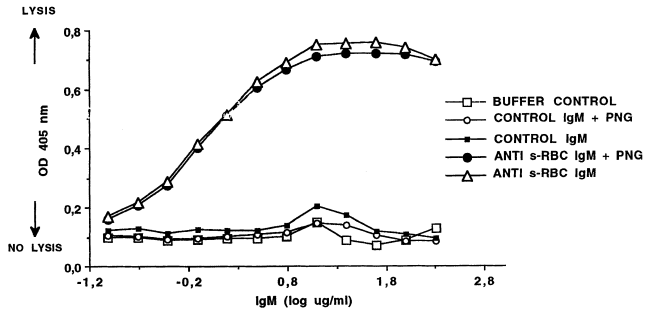

FIG. 5. Haemolytic assay of purified IgM from salmon immunised with s-RBC (anti-s-RBC IgM) before and after PNGase digestion of unreduced IgM. Control IgM was from unimmunized fish.

As can be seen in Fig. 5, PNGase digestion of the unreduced purified IgM, with anti s-RBC activity, did not have any effect on the lysis of the RBC. The slight reduction observed was not statistically significant.

EFFECT ON THE SENSITIVITY OF IgM TO PROTEASE DIGESTION. When untreated salmon IgM was digested with trypsin, the heavy chain partially broke down into 30.5- and 24-kDa pieces (Fig. 6, lane 3). Trypsin digestion of IgM after a partial removal of the carbohydrate moiety by PNGase digestion of unreduced IgM resulted in the complete breakdown of the heavy chain. Two bands crossing the lanes in the 50- to 65-kDa area were an artefact, sometimes observed after SDS-PAGE/Western blotting (34).

DISCUSSION

IgM is of interest both in phylogenetic and ontogenetic context and has been well conserved through evolution (32). One characteristic of IgM is a relatively high carbohydrate content compared with other immunoglobulin classes (13). In the present study the carbohydrate content of salmon IgM was only approximately defined due to the discrepancy between the two analytical methods used, SDS-PAGE (12.5%) and FACE (4–8%). This discrepancy was probably due to the inherent differences of the two methods.

The estimated molecular weight of the heavy and light chains, and hence the calculated molecular weight of tetrameric IgM, was slightly lower than that obtained by Håvarstein *et al.* (18) but without affecting the calculated %

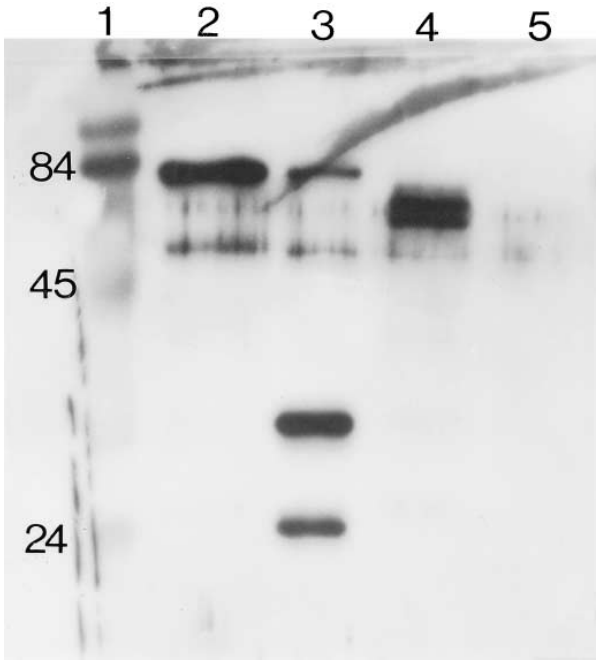

FIG. 6. Unreduced salmon IgM treated with trypsin before and after PNGase digestion followed by SDS-PAGE and Western blotting of reduced samples. Molecular weight standards (kDa) (lane 1), untreated IgM (lane 2), IgM digested with trypsin (lane 3), IgM treated with PNGase (lane 4), IgM treated with PNGase followed by trypsin digestion (lane 5). The primary antibody was polyclonal mouse anti salmon IgM with strong affinity for the heavy chain and its fragments.

glycosylation significantly (12%). However, estimation of glycosylation by SDS-PAGE is considered inaccurate due to the variable interference caused by the carbohydrate chains, for example by sialic acid (9). An accurate protein estimation, as well as successful deglycosylation and hydrolysis, are important factors in the FACE analysis. It is also possible that degradation of monosaccharides or inefficient labelling could result in lower values. The analyser (Glyko) consider 8% as the absolute maximum glycosylation of salmon IgM. Glycosylation of 7–15% has been reported for mammalian and fish IgM species (6,10,12,13) and lower carbohydrate content, 5–7%, has been reported in IgM of some bony fishes (1).

The carbohydrate moiety of salmon IgM was found to be restricted to the heavy chain and was primarily N-linked. This is the same arrangement as has been found in mammalian IgM (12,13). The detection of *N*-acetyl galactosamine (GalNAc, 1.7%) could indicate the presence of O-linked oligosaccharides. The analyser (Glyko) consider that this low value may be an artefact that is supported by the negative results of *O*-Glycanase digestion under the conditions used here. However, GalNAc (12.5%) has been described in IgM from another order of bony fishes (pike) (10) and O-linked oligosaccharide, as well as N-linked, has been described in murine and human IgA1 and IgD, although not in IgM (23).

The persistence of the partially deglycosylated 62-kDa μ chain after extensive PNGase digestion of unreduced IgM indicated that about one third of the carbohydrate moiety on about half of the IgM heavy chain pool was inaccessible to the glycosidase under these conditions. This reflects a heterogeneity in the glycosylation of salmon IgM that did not appear to be linked to the two IgM types isolated by ion exchange chromatography. Examination of the amino acid sequence of salmon IgM μ chain (19) indicated five possible sites for N-linked oligosaccharides and the two IgM types were identical in this respect. These sites were restricted to the Fc domains: Cμ2 (two sites), Cμ3 (one site) and Cμ4 (two sites). The oligosaccharide profiling analysis of salmon IgM revealed six different oligosaccharide types, present in variable amounts. This indicated that at least one of the available sites contained different oligosaccharide structures. The well-defined stages of deglycosylation, observed after the short PNGase digestion of reduced IgM, suggested that not all of the theoretical N-linked sites were occupied. This would further increase the site heterogeneity. Such a site heterogeneity is a well known characteristic of glycoproteins, including immunoglobulins, and produces so called glycoforms. These may have different physical and chemical properties and show functional diversity (30,38).

It was not attempted at this stage to analyse the composition of the individual oligosaccharide structures. However, the monosaccharide composition analysis gave an indication of their composition. N-linked oligosaccharides have been classified into several groups, the three most common ones being the complex type, the high mannose type and the hybrid type. All have a basic core structure of pentasaccharide Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-(Asn) but vary with respect to the structures attached to this core (15,30,35). The results of the monosaccharide composition analysis, and of the treatment with Endo H glycosidase, showed that the N-linked oligosaccharides were mainly of the complex type, with *N*-acetyl-glucosamine, galactose and sialic acid attached to the trimannosyl core and with core fucosylation present on at least some of the glycans. Available reports on the monosaccharide composition of the carbohydrate moiety of IgM from other bony fish similarly suggest primarily the complex type oligosaccharide, whereas high mannose type oligosaccharide is associated with human IgM (1,10,38).

The carbohydrate moiety did not seem to influence antigen binding, "staple" formation or complement fixation properties of salmon IgM, all three processes being involved if lysis was to take place in the haemolytic assay (13). However, the results of this test were not fully conclusive because, under the conditions used, IgM retained about 20% of the carbohydrate moiety and this could play a role in these functions.

Trypsin digestion of salmon IgM resulted in stable frag-

ments of heavy chain as previously described by Magnadóttir *et al.* (25). Salmon IgM was more sensitive to trypsin digestion after the removal of the carbohydrate. The complete breakdown of IgM with trypsin, after the 80% deglycosylation, suggests that the carbohydrate moiety gives the molecule a structural stability and protection against proteases. Increased susceptibility of deglycosylated human IgG to various proteases has been demonstrated (33) but varied depending on the type of glycosylation. This functional role could be important to the half-life of the immunoglobulin and in defence against certain bacterial infections (31).

It would be of considerable interest to see if mucosal or cellular IgM were differently glycosylated than the serum IgM, and work along these lines is now in progress at our laboratory. It would also be of interest to examine what effects the carbohydrate moiety of salmon IgM has on other functional roles that have been attributed to the oligosaccharide moiety of immunoglobulins of other species. These include, for example, its importance to IgM polymerization, especially because salmon IgM lacks a J-chain, and in the recognition of various receptor sites or participation in it signalling events of the immune system (11,26).

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APPENDIX

Unit definitions of the glycosidases used: PNGaseF, the amount of enzyme required to remove >95% of the carbohydrate from 10 μg of denatured RNase B at 37°C in 1 hr; O-Glycanase, the amount of enzyme required to catalyse the release of 1 μmol/min of Gal-GalNAc from native asialofetuin at 37°C; Endo H, the amount of enzyme required to remove >95% of the carbohydrate from 10 μg of denatured RNase B at 37°C in 1 hr.

References

1. Acton, R.T.; Weinheimer, P.F.; Hall, S.J.; Niedermeier, W.; Shelton, E.; Bennet, J.C. Tetrameric immune macroglobulins in three orders of bony fishes. Proc. Natl. Acad. Sci. USA 68: 107–111;1971.
2. Amemiya, C.T.; Litman, G.W. Complete nucleotide sequence of an immunoglobulin heavy chain gene and analysis of immunoglobulin gene organization in a primitive teleost species. Proc. Natl. Acad. Sci. USA 87:811–815;1990.
3. Andersson, E.; Matsunaga, T. Complete cDNA sequence of a rainbow trout IgM gene and evolution of vertebrate IgM constant domains. Immunogenetics 38:243–250;1993.
4. Axford, J.S. Second Jenner international glycoimmunology meeting. Immunol. Today 14:104–106;1993.
5. Bazin, R.; Darveau, A.; Martel, F.; Pelletier, A.; Piché, L.; St-Laurent, M.; Thibault, L.; Demers, A.; Boyer, L.; Lemieux, G.; Lemieux, R. Increased avidity of mutant IgM antibodies caused by the absence of COOH-terminal glycosylation of the μ H chain. J. Immunol. 149:3889–3893;1992.
6. Beale, D.; Buttress, N. Structural studies on bovine immunoglobulin M. Biochim. Biophys. Acta 257:372–383;1972.
7. Bengtén, E.; Leanderson, T.; Pilström, L. Immunoglobulin heavy chain cDNA from the teleost Atlantic cod (*Gadus morhua* L.): Nucleotide sequences of secretory and membrane form show an unusual splicing pattern. Eur. J. Immunol. 21: 3027–3033;1991.
8. Bond, A.; Jones, M.G.; Hay, F.C. Human IgG preparations isolated by ion-exchange or protein G affinity chromatography differ in their glycosylation profiles. J. Immunol. Methods 166:27–33;1993.
9. Carlsson, S.R. Isolation and characterization of glycoproteins. In: Fukuda, M.; Kobata, A. (eds). Glycobiology. A Practical Approach. Oxford: Oxford University Press; 1993:1–26.
10. Clerx, J.P.M.; Castel, A.; Bol, J.F.; Gerwig, G.J. Isolation and characterization of the immunoglobulin of pike (*Esox lucius* L.). Vet. Immunol. Immunopathol. 1:125–144;1980.
11. Davis, A.C.; Collins, C.; Shulman, M.J. Differential glycosylation of polymeric and monomeric IgM. Mol. Immunol. 26: 147–152;1989.
12. Davis, A.C.; Shulman, M.J. IgM-Molecular requirements for its assembly and function. Immunol. Today 10:118–128;1989.
13. Day, E. Advanced Immunochemistry. New York: Wiley & Sons, Inc.; 1990:107–181.
14. Du Pasquier, L. Antibody diversity in lower vertebrates—why is it so restricted? Nature 296:311–313;1982.
15. Dwek, R.A.; Edge, C.J.; Harvey, D.J.; Wormald, M.R.; Parekh, R.B. Analysis of glycoprotein-associated oligosaccharides. Annu. Rev. Biochem. 62:65–100;1993.
16. Furukawa, K.; Kobata, A. IgG galactosylation—its biological significance and pathology. Mol. Immunol. 28:1333–1340; 1991.
17. Harding, F.A.; Amemiya, C.T.; Litman, R.T.; Cohen, N.; Litman, G.W. Two distinct immunoglobulin heavy chain isotypes in a primitive cartilaginous fish. Nucleic Acids Res. 18: 6369–6376;1990.
18. Håvarstein, L.S.; Aasjord, P.M.; Ness, S.; Endresen, C. Purification and partial characterization of an IgM-like serum immunoglobulin from Atlantic salmon (*Salmo salar*). Dev. Comp. Immunol. 12:773–785;1988.
19. Hordvik, I.; Voie, A.M.; Glette, J.; Male, R.; Endresen, C. Cloning and sequence analysis of two isotypic IgM heavy chain genes from Atlantic salmon, *Salmo salar* L. Eur. J. Immunol. 22:2957–2962;1992.
20. Jackson, P. The use of polyacrylamide-gel electrophoresis for the high resolution separation of reducing saccharides labelled with the fluorophore 8-aminonaphthalene-1,3,6-trisulphonic acid. Biochem. J. 270:705–713;1990.
21. Kobayashi, K.; Tomonaga, S. The second immunoglobulin class is commonly present in cartilaginous fish belonging to the order Rajiformes. Mol. Immunol. 25:115–120;1988.
22. Leatherbarrow, R.J.; Rademacher, T.W.; Dwek, R.A.; Woof, J.M.; Clark, A.; Burton, D.R. Effector functions of a monoclonal aglycosylated mouse IgG2a: binding and activation of complement component C1 and interaction with human monocyte Fc receptor. Mol. Immunol. 22:407–415;1985.
23. Lipniunas, P.; Grönberg, G.; Krotkiewski, H.; Angel, A.-S.; Nilsson, B. Investigation of the structural heterogeneity in the carbohydrate portion of a mouse monoclonal immunoglobulin A antibody. Arch. Biochem. Biophys. 300:335–345; 1993.
24. Magnadóttir, B. Purification of immunoglobulin from the serum of Atlantic salmon (*Salmo salar* L.). Icel. Agr. Sci. 4:49–54;1990.

25. Magnadóttir, B.; Kristjánsdóttir, H.; Guðmundsdóttir, S. Characterisation of monoclonal antibodies to separate epitopes on salmon IgM heavy chain. Fish Shellfish Immunol. 6: 185–198;1996.
26. Muraoka, S.; Shulman, M.J. Structural requirements for IgM assembly and cytolytic activity. Effects of mutations in the oligosaccharide acceptor site at Asn 402. J. Immunol. 142: 695–701;1989.
27. Newkirk, M.M.; Rauch, J. Binding of human monoclonal IgG rheumatoid factors to Fc is influenced by carbohydrate. J. Rheumatol. 20:776–780;1993.
28. Nonaka, M.; Yamaguchi, N.; Natsuume-Sakai, S.; Takahashi, M. The complement system of rainbow trout (*Salmo gairdneri*). 1. Identification of the serum lytic system homologous to mammalian complement. J. Immunol. 126:1489–1494; 1981.
29. Perkins, S.J.; Nealis, A.S.; Sutton, B.J.; Feinstein, A. Solution structure of human and mouse immunoglobulin M by synchrotron X-ray scattering and molecular graphics modelling. J. Mol. Biol. 221:1345–1366;1991.
30. Rademacher, T.W.; Parekh, R.B.; Dwek, R.A. Glycobiology. Annu. Rev. Biochem. 57:785–838;1988.
31. Reinholdt, J.; Tomana, M.; Mortensen, S.B.; Kilian, M. Molecular aspects of immunoglobulin A1 degradation by oral streptococci. Infect. Immun. 58:1186–1194;1990.
32. Rosenshein, I.L.; Schluter, S.F.; Vasta, G.R.; Marchalonis; J.J. Phylogenetic conservation of heavy chain determinants of vertebrates and protochordates. Dev. Comp. Immunol. 9: 783–795;1985.
33. Tao, M.-H.; Morrison, S.L. Studies of aglycosylated chimeric mouse-human IgG. Role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. J. Immunol. 143:2595–2601;1989.
34. Tasheva, B.; Dessev, G. Artefacts in sodium dodecyl sulfate-polyacrylamide gel electrophoresis due to 2-mercaptoethanol. Anal. Biochem. 129:98–102;1983.
35. Varki, A. Biological roles of oligosaccharides: All of the theories are correct. Glycobiology 3:97–130;1993.
36. Walker, M.R.; Lund, J.; Thompson, K.M.; Jefferis, R. Aglycosylation of human IgG1 and IgG3 monoclonal antibodies can eliminate recognition by human cells expressing FcγRI and/or FcγRII receptors. Biochem. J. 259:347–353;1989.
37. Wilson, M.R.; Marcus, A.; Van Ginkel, F.; Miller, N.W.; Clem, L.W.; Middleton, D.; Warr, G.W. The immunoglobulin M heavy chain constant region gene of the channel catfish, *Ictalurus punctatus*: An unusual mRNA splice pattern produces the membrane form of the molecule. Nucleic Acids Res. 18: 5227–5233;1990.
38. Wormald, M.R.; Wooten, E.W.; Bazzo, R.; Edge, C.J.; Feinstein, A.; Rademacher, T.W.; Dwek, R.A. The conformational effects of *N*-glycosylation on the tailpiece from serum IgM. Eur. J. Biochem. 198:131–139;1991.