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Comparison of immunoglobulin (IgM) from four fish species

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SUMMARY

Immunoglobulin (IgM) was isolated from the serum of four fish species, Atlantic salmon (*Salmo salar* L.), halibut (*Hippoglossus hippoglossus* L.), haddock *(Melanogrammus aeglefinus* L.) and cod (*Gadus morhua* L.) and a comparison made of some physical and biochemical properties. The molecular weight of IgM varied between the different species and between the different analytical methods used. IgM from all four species was tetrameric in serum although a proportion of the molecule was held together by non-covalent forces. Salmon and haddock IgM were composed of two IgM types as regards the overall charge whereas halibut and cod IgM were homogeneous in this respect. The molecular weight of the heavy and light chains was similar for all four species. The oligosaccharide moiety, which was N-linked and associated with the heavy chain varied from 7.8 to 11.4% of the total molecular weight. Lectin analysis indicated variable composition of the carbohydrate moiety between species. The sensitivity to PNGase and trypsin varied between the four species.

Key words: Atlantic salmon, cod, halibut, haddock, IgM, immunoglobulin, oligosaccharide, trypsin.

YFIRLIT

Samanburður á mótefni (IgM) fjögurra fisktegunda

Mótefni (IgM) var einangrað úr blóðvatni (sermi) fjögurra fisktegunda, laxs *(Salmo salar* L.), lúðu (*Hippoglossus hippoglossus* L.), ýsu (*Melanogrammus aeglefinus* L.) og þorsks (*Gadus morhua* L.), og nokkrir lífeðlis- og lífefnafræðilegir eiginleikar þess bornir saman. Mólþungi IgM var breytilegur á milli tegunda og milli greiningaraðferða. Öll mótefnin voru fjórgild í sermi, enda þótt hluti undireininganna væri ekki tengdur með samgildum tengjum. Laxa- og ýsu-IgM greindust í tvær gerðir eftir heildarhleðslu, en lúðu- og þorska-IgM voru einsleit að þessu leyti. Mólþungi þungra og léttra keðja allra IgM-tegundanna var svipaður. Sykruþátturinn, sem var af N-tengdri gerð og staðsettur á þungu keðjunni, var 7,8–11,4% af heildarmólþunga IgM. Lektín-greining gaf til kynna breytilega samsetningu sykruþáttarins á milli tegunda. Næmni fyrir PNGasa og trypsíni var breytileg á milli tegunda.

INTRODUCTION

IgM is an important immunoglobulin class. It is important in phylogenetic research being the first immunoglobulin to appear in evolution and commonly the only immunoglobulin class described in fish. It is also important in an ontogenetic context, being the first formed antibody in a new-born mammal and the major antibody of primary response in higher vertebrates. A better understanding of the structure and function of fish IgM has become all the more important in recent years due to the need of the fish farming industry for effective prevention and control of various fish diseases.

Amino acid sequence analysis of IgM has demonstrated a significant homology between the IgM heavy chain of diverse species of ver-

tebrates, indicating an evolutionary well-conserved molecule (Rosenshein *et al.*, 1985; Bengtén *et al.*, 1991; Fellah *et al.*, 1992; Andersson and Matsunaga, 1993; Lee *et al.*, 1993).

In spite of this homology, IgM displays considerable structural and biochemical heterogeneity. There are, for example, variations in the polymeric arrangement between different vertebrates. IgM is pentameric in higher vertebrates and cartilaginous fish (Kobayashi *et al.*, 1984), tetrameric in teleosts (Acton *et al.*, 1971) and hexameric form has been demonstrated in some amphibia (Hsu and Du Pasquier, 1984). Hexameric, as well as pentameric form has also been found in mice (Hughey *et al.*, 1998). Monomeric form has similarly been described in some species (Clem and McLean, 1975) and separate isotypes have, for example, been identified in salmon (Hordvik *et al.*, 1992). The so-called J-chain, which is associated with the polymerisation of mammalian IgM, is commonly absent from fish IgM. However, its presence has been indicated in a few fish species like catfish and rainbow trout (Weinheimer *et al.*, 1971; Sanchez *et al.*, 1989). Heterogeneity with respect to the number and position of disulphide bonds has been demonstrated (Kobayashi *et al.*, 1982; Partula and Charlemagne, 1993; Whittington, 1993) as well as variations in the number, position and nature of the carbohydrate moiety (Acton *et al.*, 1971; Lee *et al.*, 1993; Magnadóttir *et al.*, 1997). Possible functional specificity has in some cases been attributed to these variants of fish IgM (Lobb and Clem, 1981; Rombout *et al.*, 1993).

There are also indications that another class of immunoglobulin may be found in some fish species. An unusual IgM type has been described in eggs of Chum salmon (Fuda *et al.*, 1992) and a second class of immunoglobulin has been described in cartilaginous fishes (Kobayashi *et al.*, 1984; Tomonaga and Kobayashi, 1985). Recently Wilson *et al.* (1997) described a new chimeric Ig heavy chain in Channel catfish (*Ictalurus punctatus*). This molecule showed similarities to mammalian IgD but also shared the C termini and the first constant domain of IgM heavy chain.

The aim of the present work was to compare some physical and biochemical properties of IgM isolated from four teleost species. The fish species were Atlantic salmon (*Salmo salar* L.), and three marine species: halibut (*Hippoglossus hippoglossus* L.), haddock (*Melanogrammus aeglefinus* L.) and cod (*Gadus morhua* L.).

MATERIALS AND METHODS

The fish

The fish came from experimental fish farms in Iceland. Salmon brood fish was of ocean ranched origin; halibut, haddock and cod were of wild stock and had been reared for several months. Salmon was 2.5–4 kg, halibut 4–5 kg, haddock 2–4 kg and cod 8–10 kg, in weight.

From 5–10 ml blood was collected from the caudal aorta, except in the case of halibut where blood was collected from an aorta in the gills. The blood was allowed to clot at room temperature for 2 hours and then overnight at 4°C and serum collected after centrifuging at 2000 rpm for 10 minutes. A pool of serum from 2–4 fish was used for the IgM isolation. Serum was stored at –50°C.

IgM purification

IgM was purified as described by Magnadóttir (1990). Briefly, it involved chromatography of serum on a CM Affi Gel Blue column (Bio-Rad, USA) using the FPLC system from Pharmacia (Denmark) followed by ammonium sulphate precipitation and gel filtration on Sepharose 6 (Pharmacia) as described below. Purity was checked by Western blotting using mouse antibodies to the serum proteins of each fish species as well as mouse antibodies to each IgM species.

Polyclonal antibody production

Polyclonal antibodies to serum proteins and IgM of each fish species was prepared in mouse ascitic fluid according to the method of Overkamp *et al.* (1988). The anti-serum protein an-

tibodies reacted in Western blotting with several proteins including IgM. The anti-IgM antibodies showed strong affinity for IgM and did not react with other serum proteins, i.e. were monospecific.

Protein measurements

Two methods were used: (1) Bradford's method, using a protein assay kit from BioRad (Bradford, 1976). Bovine serum albumin and human IgG were used as standards (BioRad) for the analysis of serum and crude IgM preparations respectively. (2) To estimate the protein concentration of purified IgM, the optical density at 280 nm was measured and the concentration worked out, using an extinction coefficient of E=13.7, for 1% solution and 1 cm cuvette (Williams and Chase, 1968). Lower E values have been quoted for e.g. cod (Pilström and Petersson, 1991).

Gel filtration

For analysis of serum proteins by gel filtration 250 µl of serum, diluted to contain about 0.5 mg protein, were put on a Superose 6 column (HR10/30, Pharmacia) using the FPLC system from Pharmacia. The diluting and elution buffer was 0.1 M Tris-HCl, pH 8.0 containing 0.15 M NaCl and 0.01% sodium azide. Fractions were tested for IgM by Western blotting.

For size estimation by gel filtration approx. 250 µg of purified IgM was put on the Superose 6 column as above. A calibration kit for high molecular weight proteins, from Pharmacia, was used, as well as bovine IgM (Sigma, USA) to plot the standard graph.

Ion exchange chromatography

For isotype analysis purified IgM was equilibrated by dialysis in 20 mM Tris-HCl, pH 7.5 containing 0.15 M NaCl. Then 250 µg in 250 µl were put on a MonoQ anion exchanger (HR5/5, Pharmacia), equilibrated in the same buffer, using the FPLC system from Pharmacia. Elution was carried out using a NaCl gradient which upper limit was 1 M.

Electron microscopy (EM)

Freshly purified halibut IgM was diluted to give 25 µg protein ml^{-1} in 0.2 M NH_4HCO_3, pH 8.2 and sonicated on ice for 30 sec. For negative staining the following procedure was used: A drop of the IgM solution was put on formvar/carbon coated copper grid, drained after 1–2 min and fixed for 1 min with 1% gluteraldehyde in 0.02 M potassium phosphate buffer at pH 7.2. Stained with 1% uranyl acetate for 1–2 min, drained and allowed to dry. Examined and photographed in JEM-100SX (JEOL) electron microscope.

SDS-PAGE analysis

SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) analysis was carried out using the Mini-Protean II system from BioRad according to the manufacturer's instructions.

Analysis of IgM, after reduction with 5% mercaptoethanol and 2% SDS, was carried out in a 4.5% acrylamide stacking gel and a 14% acrylamide resolving gel.

Analysis of unreduced IgM, was carried out in a homogeneous 3.5% acrylamide gel containing 0.6% agarose according to the method of Avtalion and Mor (1992).

After the SDS-PAGE separation the gels were analysed for protein by silver staining (BioRad silver staining kit).

Western blotting and immunostaining

Following SDS-PAGE separation, proteins were transferred to nitro-cellulose paper (Hybond ECL, Amersham, UK) using a semi-dry MilliBlot Graphite Electroblotter (Millipore, USA). The manufacturer's instructions were used with the exception that a single buffer system of 25 mM Tris-glycine buffer, pH 8.8, containing 20% methanol, was used instead of the triple buffer systems described by Millipore. The procedure used for immunostaining, after transfer, has been described before (Magnadóttir *et al.*, 1995). Incubation with polyclonal mouse anti-IgM (or anti-serum pro-

tein) antibody was followed by peroxidase conjugated rabbit anti-mouse Ig antibody (Dako, Denmark). The blots were developed using the ECL (enhanced chemiluminescence) system from Amersham. Prestained (BioRad) or biotinylated (Sigma) molecular weight markers were used in immunoblotting. Streptavidin labelled peroxidase (Dako) was used to develop the biotinylated markers.

Competitive ELISA (C-ELISA)

C-ELISA was used to measure the IgM concentration in serum. The method has been described before (Magnadóttir and Guðmundsdóttir, 1992) and only a brief outline will be given here, estimation of salmon IgM in serum being used as an example.

Salmon serum, diluted 1/100, 1/500 and 1/1000, was incubated with polyclonal mouse anti-salmon IgM antibodies on salmon IgM coated ELISA tray. The dilution of the mouse anti-salmon IgM antibody was determined from the titration curve of the antibody, the criteria being that the curve was descending (i.e. the antibody was not in excess) and the OD (optical density) at 492 nm was ≥1.0. Following the incubation and washing, bound antibody was detected with peroxidase labelled rabbit antibody to mouse immunoglobulin (Dako) and colour developed with OPD (1,2-phenylendiamin dihydroclorid) substrate. For plotting of a standard graph purified salmon IgM, in two fold serial dilutions from 40 µg/ml to 0.625 µg/ml, in place of the serum dilutions, was incubated with the mouse anti-salmon IgM antibodies and the IgM concentration vs OD at 492 nm plotted. The serum IgM concentration of each dilution was interpolated from this and the mean calculated.

Sensitivity to trypsin

A protocol had previously been devised to obtain stable breakdown fragments of salmon IgM heavy chain by a partial digestion with trypsin coupled to actigel from Sterogen Biochemicals (USA) (Magnadóttir *et al.*, 1996). This basic protocol was used for comparing the susceptibility of the four IgM species to trypsin digestion. Briefly, Trypsin-actigel was equilibrated with 0.1 M Tris-HCl buffer, pH 8.0, containing 10 mM $CaCl_2$ (TB-Ca). A suspension of 10 mg suction dry trypsin gel in 100 µl TB-Ca was then prepared. A 50 µg ml^{-1} solution of IgM was prepared in TB-Ca and 20 µl mixed with 15 µl of the trypsin-actigel suspension (trypsin:IgM ratio approx. 2:1). Incubation was for 17–18 hours at 22°, 37° and 45°C and samples were analysed by SDS-PAGE after reduction.

Carbohydrate analysis

Enzyme cleavage by PNGaseF. PNGaseF from BioLabs (USA), which removes N-linked oligosaccharides, was used. The reaction conditions used were as suggested by the manufacturer. Digestion was carried out before and after reduction of IgM with mercaptoethanol. Approximately 50 units (U) of the glycosidase was used to cleave the N-linked oligosaccharides from 10 to 15 µg IgM, at 37°C for 1 hour. The samples were then reduced and analysed by SDS-PAGE. The successful removal of the oligosaccharides was checked by using GlycoTrack detection kit from Oxford GlycoSystems (UK).

Lectin analysis. After SDS-PAGE and Western blotting of reduced samples of IgM, a panel of biotinylated lectins (Genzyme Corp., USA) was used to characterise the oligosaccharide moiety of IgM. Table 1 lists the lectins used and their specificity according to the manufacturer's information. The procedure was according to the manufacturer's instructions with the modification that streptavidin labelled peroxidase (Dako) was used instead of streptavidin labelled alkaline phosphatase and the blots developed using the ECL system from Amersham. The results were analysed using gel documentation and analysis system, GSD 8000, from UVP Ltd, UK.

RESULTS

IgM concentration in serum

The gel filtration profile of serum from the

Table 1. The main specificity of five lectins for mono- and oligosaccharides.
1. tafla. Aðal sértækni fimm lektína við einsykrur og fjölsykrur.

Lectin	Specificity[a)]—*Sértækni*
ConA	β-Man, α-Glc, α-GlcNAc, branched mannoses
RCA	Terminal Gal, GalNAc, (terminal NeuNAc can block the lectin)
SNA	β-Gal, terminal NeuNAc α2–6 Gal(NAc)
DSA	β-GlcNAc, GlcNAc β1–4 GlcNAc oligomers, Gal β1–4 GlcNAc
WGA	β-GlcNAc, GlcNAc β1–4 GlcNAc (dimers/trimers)

a) Man: mannose, Glc: glucose, GlcNAc: N-acetylglucosamine, Gal: galactose, GalNAc: N-acetylgalactosamine, NeuNAc: sialic acid.

four fish species differed considerably with respect to the relative size of the IgM peak (Figure 1). IgM constituted a small peak in salmon serum, both relative to the other serum proteins and to the other fish sera. The IgM peak of cod on the other hand appeared to be about 50% of the total serum protein.

Using the competitive ELISA, salmon IgM serum concentration was found to be ≤1 mg ml^{-1}, halibut serum contained about 4 mg IgM ml^{-1}, haddock serum 7 mg IgM ml^{-1} and cod serum 11.5 mg IgM ml^{-1}. The protein concentration of each serum pool was: salmon 44 mg ml^{-1}, halibut 50 mg ml^{-1}, haddock and cod 54 mg ml^{-1}, hence the IgM concentration was respectively about 2%, 8%, 13% and 20% of the serum proteins.

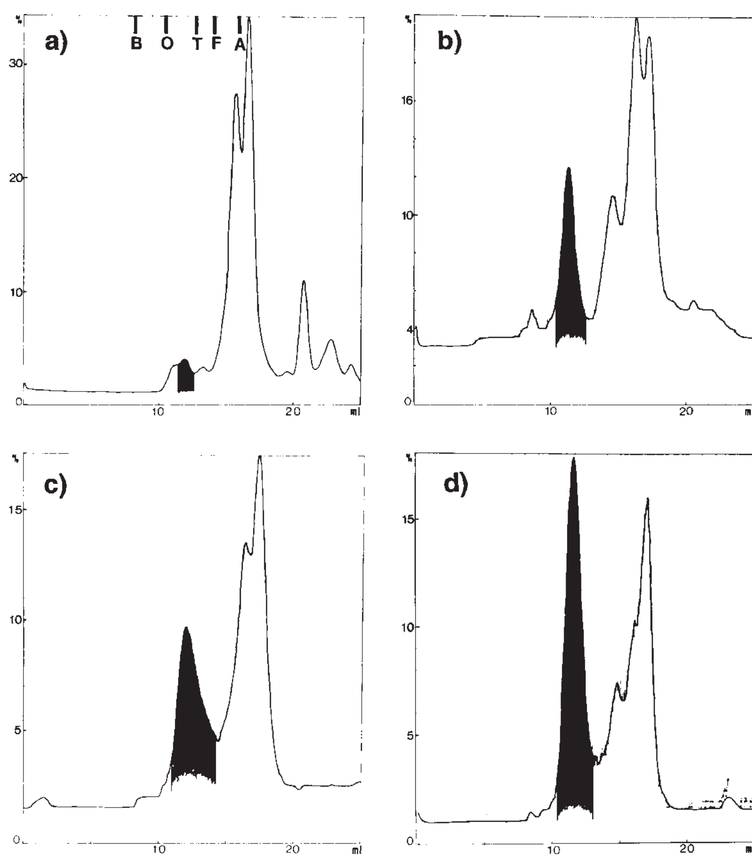

Figure 1. Serum proteins analysed by gel filtration; (a) salmon, (b) halibut, (c) haddock, and (d) cod. The shaded areas contained IgM detected by Western blotting with specific antibody to the relevant IgM species. Elution of molecular size markers is indicated in Figure (a); B: blue dextran, 2×10^3 kDa; O: bovine IgM, 975 kDa; T: thyroglobulin, 669 kDa; F: ferritin, 440 kDa; A: aldolase, 158 kDa.
1. mynd. Serum (blóðvatn) greint með gelsíu-súluskiljun: (a) lax, (b) lúða, (c) ýsa og (d) þorskur. Skyggði hlutinn sýnir IgM greint með ónæmiþrykki með hjálp sérvirkra mótefna gegn IgM hverrar tegundar. Staðsetning staðalpróteina er sýnd á mynd (a); B: blátt dextran, 2×10^3 kDa; O: kúa-IgM, 975 kDa; T: thyroglobulin, 669 kDa; F: ferritin, 440 kDa; A: aldolase, 158 kDa.

Table 2. The molecular weight of fish IgM and its sub-units using different analytical methods.
2. tafla. Mólþungi fiska-IgM og undireininga þess greindur með mismunandi aðferðum.

Fish IgM	Molecular weight, kDa—*Mólþungi*				
	Whole IgM (tetrameric)			H-chain	L-chain
Fiska-IgM	g.f.[a)]	SDS-PAGE [b)]	Calculated	SDS-PAGE	SDS-PAGE
Salmon—*Lax*	870	850 [c)]	784	71.5 (60.3) [d)]	26.5
Halibut—*Lúða*	933	830	788	72.0 (64.4)	26.5
Haddock—*Ýsa*	840	700	788	72.0 (63.0)	26.5
Cod—*Þorskur*	832	700	792	72.5 (61.7)	26.5

a) Gel filtration—*Gelsíun*.
b) In thin gel—*Í þunnu geli*.
c) Average of 5–6 bands—*Meðaltal 5–6 banda*.
d) Deglycosylated in brackets—*Afsykrað innan sviga*.

IgM molecular weight, polymeric arrangement and isotypes

The molecular weight of tetrameric IgM was obtained by gel filtration (not shown), by SDS-PAGE analysis of unreduced IgM in thin gel (Figure 2) and by calculation based on the individual heavy and light chain sizes (8×(H+L)). Considerable discrepancy was observed between the three methods (Table 2). However, due to the lack of good molecular standards for plotting a standard graph the values obtained by SDS-PAGE analysis in thin gel must be viewed with caution.

The gel filtration analysis gave higher values than the other two methods (Table 2). This was especially noticeable for halibut IgM which showed molecular size approaching that of pentameric IgM (933 kDa). It was therefore decided to examine the structure of halibut

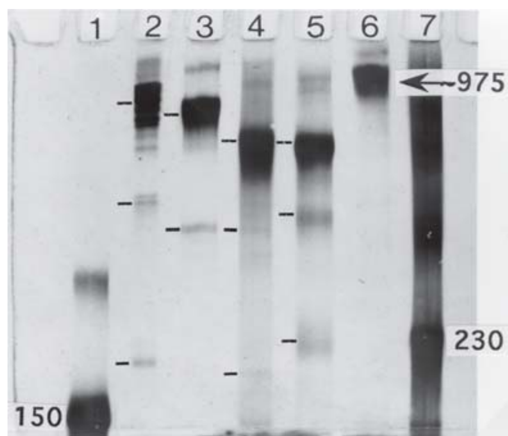

Figure 2. SDS-PAGE analysis in a thin gel (3.5% acrylamide) of non-reduced IgM. Lanes 1 and 7: molecular weight standards as shown; lane 2: salmon IgM; lane 3: halibut IgM; lane 4: haddock IgM; lane 5: cod IgM; lane 6: bovine IgM (975 kDa). Tetrameric, dimeric and monomeric forms are indicated by hyphens.
2. mynd. SDS-PAGE rafdráttur í þunnu geli á óafoxuðu IgM. Brautir 1 og 7: mólþyngdarstaðlar eins og sýnt er; braut 2: laxa-IgM; braut 3: lúðu-IgM; braut 4: ýsu-IgM; braut 5: þorska-IgM; braut 6: kúa-IgM (975 kDa). Fjórgild, tvígild og eingild form eru sýnd með bandstriki.

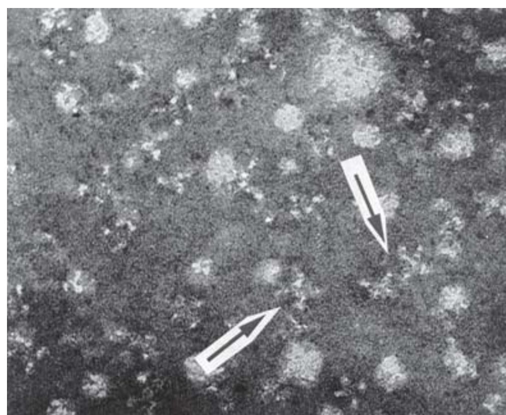

Figure 3. Electron micrograph of halibut IgM, ×240.000. Arrows point to tetrameric shapes.
3. mynd. Rafeindasmásjármynd af lúðu-IgM, ×240.000. Örvar sýna fjórgild form.

IgM by electron microscopy. Only star shaped, four armed structures could be detected (Figure 3) indicating a true tetrameric form.

Analysis of unreduced IgM by SDS-PAGE in a thin gel showed that tetrameric salmon IgM, unlike the other three IgM species, was heterogeneous in size (i.e. the banded appearance in Figure 2). The analyses by SDS-PAGE in thin gel also showed dimeric (about 400 kDa) and monomeric (about 200 kDa) subunit components as well as the typical tetrameric configuration (Figure 2). Salmon and cod IgM contained both dimers and monomers whereas only dimers were detected in halibut IgM and subunits were negligible in haddock IgM (Figure 2). Subunit configurations were not detected in the control bovine IgM.

Analysis of the molecular weight of heavy and light chains by SDS-PAGE of reduced IgM, showed a little or no variation in the heavy and light chain sizes of the four species (Table 2, Figure 4 and 5). No J chain (10–15 kDa) was discerned.

When analysed by anion exchange chroma-

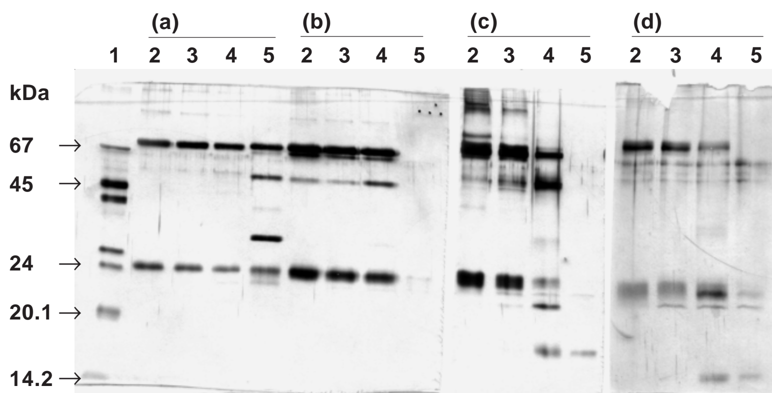

Figure 4. Trypsin digestion of unreduced IgM at different temperatures, the results analysed by SDS-PAGE after reduction; a) salmon IgM, b) halibut IgM, c) haddock IgM and d) cod IgM. Lane 1: molecular weight standards as indicated; lane 2: untreated IgM; lane 3: trypsin digested IgM at 22°C; lane 4: trypsin digested IgM at 37°C; lane 5: trypsin digested IgM at 45°C.

4. mynd. Trypsín-melting óafoxaðs IgM við mismunandi hitastig, útkoman greind í SDS-PAGE eftir afoxun; a) laxa-IgM, b) lúðu-IgM, c) ýsu-IgM og d) þorska-IgM. Braut 1: mólþyngdarstaðlar eins og sýnt er; braut 2: ómeðhöndlað IgM; braut 3: trypsín-melting við 22°C; braut 4: trypsín-melting við 37°C; braut 5: trypsín-melting við 45°C.

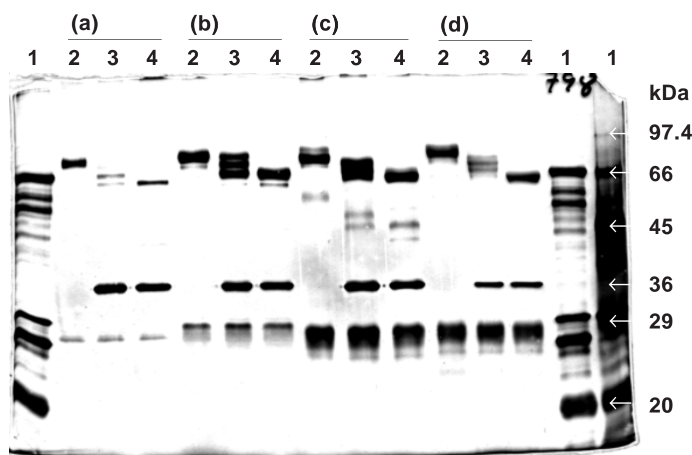

Figure 5. PNGase digestion of unreduced and reduced IgM, the results analysed by SDS-PAGE after reduction; a) salmon IgM, b) halibut IgM, c) haddock IgM and d) cod IgM. Lanes 1: molecular weight standards as indicated; lane 2: untreated IgM; lane 3: unreduced IgM; PNGase digested; lane 4: reduced IgM, PNGase digested.

5. mynd. PNGase-melting á óafoxuðu og afoxuðu IgM, útkoman greind í SDS-PAGE eftir afoxun; a) laxa-IgM, b) lúðu-IgM, c) ýsu-IgM og d) þorska-IgM. Brautir 1: mólþyngdarstaðlar eins og sýnt er; braut 2: ómeðhöndlað IgM; braut 3: óafoxað IgM, PNGase melt; braut 4: afoxað IgM, PNGase melt.

Table 3. Isotypes of fish IgM separated by ion exchange chromatography. The table shows the M NaCl of isotype elution.
3. tafla. Mismunandi IgM-gerðir greindar með jónaskiptasúlu. Taflan sýnir mólstyrk NaCl sem mismunandi IgM losna við.

Fish IgM	Isotype I (M NaCl)	Isotype II (M NaCl)
Salmon	0.44	0.48
Halibut	0.43	None
Haddock	0.36	0.15
Cod	0.34	None

tography, purified salmon and haddock IgM separated into two types of roughly equal proportions whereas halibut and cod IgM appeared to be homogeneous with respect to the overall charge (Table 3). The two types of salmon IgM showed similar overall charge and similar to that of halibut and cod IgM and one type of haddock IgM. The second type of haddock on the other hand showed a relatively neutral overall charge.

The sensitivity to trypsin

The four IgM species were all stable in the absence of trypsin at 22–45°C for 18 hours (data not shown).

Salmon IgM was relatively resistant to trypsin digestion under the conditions used. No digestion was detected after incubation at 22°C or 37°C and only a partial breakdown of the heavy chains was observed after overnight incubation at 45°C, 50 and 31 kD breakdown pieces being detected by silver staining and probably a 25 kDa band superimposed on the light chain (Figure 4a).

Halibut IgM was resistant to trypsin at 22 and 37°C but a complete breakdown of both heavy and light chains took place at 45°C. A slight increase in the 50 kDa band, also detected in the control, can be seen after incubation at 37°C (Figure 4b).

Haddock IgM was resistant to trypsin at 22°C whereas partial digestion of heavy and light chains occurred when haddock IgM was treated with trypsin at 37°C, resulting in several breakdown fragments, i.e. ca 48, 32, 22 and 18 kDa. After incubation at 45°C the breakdown was complete, except for faint remains of the 18 kDa fragment (Figure 4c).

Cod IgM behaved somewhat similar to haddock IgM, being resistant to trypsin at 22°C, partial digestion taking place after incubation at 37°C and more or less complete breakdown of both heavy and light chain taking place at 45°C. However, the fragments obtained after incubation at 37°C were different in size. There was no major 48 kDa band as seen in haddock IgM, the main fragments being 18 kDa and possibly a 25 kDa band superimposed on the light chain band and a very faint ca 35 kDa band (Figure 4d).

The bands seen extending across the gel in the 50–67 kDa area are artifact (Tasheva and Dessev, 1983).

Carbohydrate analysis

Digestion with PNGase. The carbohydrate moiety of the four IgM species was in all instances associated with the heavy chain, the light chain not being affected by treatment with PNGase (Figure 5) and showing no reaction with oligosaccharide detection kit (results not shown).

Under non-reducing conditions the four IgM species showed incomplete removal of the carbohydrate moiety with PNGase. Salmon, halibut and haddock IgM gave 1–2 intermediate sizes as well as the completely deglycosylated heavy chain. On the other hand, only intermediate sizes were observed when cod IgM was treated with PNGase under non-reducing conditions.

Complete deglycosylation was obtained when reduced IgM was treated with PNGase. Under these conditions PNGase removed approx. 11.2, 7.6, 9.0 and 10.8 kDa from the heavy chain of salmon, halibut, haddock and cod IgM respectively (Table 2). This constituted about 11.4%, 7.8%, 9.1% and 10.9% of the molecular weight of tetrameric salmon, halibut, haddock and cod IgM respectively.

Table 4. Oligosaccharide analysis of fish IgM heavy chain using lectin binding.
4. tafla. Greining fjölsykra á þungu keðju fiska-IgM með lektín-bindingu.

Fish-IgM/ *Lectin*	The relative density of reaction [a] *Hlutfallsleg þéttni svars* ConA	RCA	SNA	DSA	WGA
Salmon	0.5	**0.8**	**1.0**	0.0	0.0
Halibut	0.5	**0.6**	**0.7**	**1.0**	**0.7**
Haddock	**1.0**	**0.9**	**1.0**	0.3	**0.6**
Cod	**0.6**	**0.9**	**1.0**	0.2	**0.6**

a) Results from scanning of ECL developed blots, maximum for each species being 1.0 (>60% of the maximum value is in bold type)—*Niðurstöður skönnunar á ónæmisþrykki, hámark hverrar tegundar var ákvarðað 1,0 (>60% af hámarki er feitletrað).*

Lectin analysis. Salmon IgM heavy chain reacted with three of the five lectins, showing a more limited reactivity than the other three species (Table 4). It reacted primarily with SNA and RCA indicating a relatively strong presence of terminal galactose and/or sialic acid. The other three IgM species reacted with all the five lectins. Halibut IgM heavy chain reacted primarily with DSA indicating a strong presence of GlcNAc β1–4 GlcNAc oligomeres. Haddock IgM heavy chain reacted relatively strongly with ConA, SNA and RCA which suggests the presence of oligomannose as well as complex types of N-linked glycans with terminal galactose and/or sialic acid. The reactivity of cod IgM heavy chain was similar to that of haddock IgM except the presence of oligomannose was relatively less marked (ConA).

DISCUSSION

The concentration of IgM in serum from several fish species has been surveyed (Israelsson *et al.*, 1991) and the results obtained in the present study fall within the range reported (0.7–17 mg ml^{-1}). Several studies have also demonstrated considerable individual variations in serum IgM levels amongst fish. This may be related to size/age (Matsubara *et al.*, 1985; Klesius, 1990; Magnadóttir *et al.*, 1999b), the environmental conditions (Olesen and Vestergard Jørgensen, 1986; Klesius, 1990; Magnadóttir *et al.*, 1999a) or the disease status (Magnadóttir *et al.*, 1995). It must be stressed therefore, that the IgM concentration levels of each species obtained in the present study only represent the mean of 2–3 healthy individuals of similar size and kept under similar environmental conditions. Compared to the other three species the IgM concentration in salmon serum was relatively low (<1 mg ml^{-1}). This was in agreement with previous analysis of healthy salmon (Håvarstein *et al.*, 1988; Magnadóttir and Guðmundsdóttir, 1992). The IgM concentration in cod serum, on the other hand, was comparatively high and about twice the amount reported by Israelsson *et al.* (1991). A recent study has shown that the serum IgM level of cod increases with increasing size (Magnadóttir *et al.*, 1999b), 8–10 kg cod having about 12–16 mg IgM ml^{-1} serum which is comparable to the values obtained in the present study. The cod examined by Israelsson *et al.* (1991) weighed just under 1 kg which probably explains the discrepancy between the two findings.

The difference between the IgM concentration in salmon serum compared to the IgM serum level in cod is of interest in that these species differ significantly in their immune response. Salmon is able to produce a strong antibody response of high specificity although of limited diversity (Håvarstein *et al.*, 1990; Magnadóttir *et al.*, 1995), whereas cod is known for its high level of natural antibodies but limited ability to produce specific antibody response (Pilström and Petersson, 1991; Bjørgan-Schrøder *et al.*, 1992).

With the exception of halibut IgM, analysis by gel filtration indicated tetrameric structure (832–870 kDa). EM examination also revealed tetrameric halibut IgM, in spite of the unusually large size suggested by the gel filtration analysis (933 kDa).

Analysis of unreduced IgM by SDS-PAGE in a thin gel gave lower values than gel filtration analysis especially in the case of halibut,

haddock and cod IgM. Several factors may contribute to this discrepancy. The most important is probably the amino acid composition resulting in different hydrophobicity and the degree and nature of glycosylation (See and Jackowski, 1989). The fact that the heavy and light chains of these four IgM molecules were similar in size indicated that the overall physical nature and biochemical composition of IgM contributed more to the variation in the overall molecular size than did the subunit sizes.

The gel filtration analysis showed that in aqueous solution, and hence in serum, only the tetrameric form of IgM was present. The presence of dimeric and monomeric forms demonstrated by SDS-PAGE in thin gel showed, however, that at least in case of the salmon, halibut and cod IgM, a proportion of the IgM molecule lacked the disulphide bridges and non-covalent forces maintained tetrameric configuration. A similar phenomena has been described in IgM of diverse species (Mikoryak and Steiner, 1984; Partula and Charlemagne, 1993; Whittington, 1993). The reason for the banded appearance of unreduced salmon IgM in thin gel is not clear but suggests heterogeneous molecular weight not seen in the other three species.

Isotypic salmon IgM, with respect to the overall charge observed in the present study has been described before by Håvarstein *et al.* (1988). Hordvik *et al.* (1992) have since demonstrated that the isotypes have genetic origin and represent two different forms of salmon heavy chain. Cod IgM was homogeneous in this respect and its genetic arrangement indicates a single heavy chain form (Bengtén *et al.*, 1991). The present results suggest that haddock IgM, like salmon IgM, may have two forms of heavy chains whereas halibut IgM has a single heavy chain like cod IgM.

The four IgM species studied showed different sensitivity to trypsin digestion, salmon IgM being most resistant, haddock and cod IgM being most susceptible.

A species variation has been observed in the fragmentation of IgM and different optimum temperature for trypsin digestion has been reported by several authors (Beal and van Dort, 1982; Klapper *et al.*, 1971; van Ginkel *et al.*, 1991; Glynn and Pulsford, 1993). This reflects important differences in the IgM structure of different species including the four species studied here, probably mainly due to conformational changes in the second constant domain of the heavy chain (CH2). In this context it may be pointed out that the CH2 domain (as well as the CH3 domain) shows more limited amino acid sequence homology from species to species than the other CH domains (Bengtén *et al.*, 1991; Lee *et al.*, 1993; Anderson and Matsunaga, 1993).

Comparison of the amino acid sequence of salmon IgM and cod IgM heavy chains (Hordvik *et al.*, 1992; Bengtén *et al.*, 1991) shows that the number and position of the trypsin sensitive amino acids, arginine (R) and lysine (K), are different. This could account for the different trypsin sensitivity of salmon and cod IgM. The amino acid sequence of halibut and haddock IgM heavy chain has not been described.

Another study has shown that the carbohydrate moiety of salmon IgM protects the immunoglobulin to some extent against trypsin digestion (Magnadóttir *et al.*, 1997). Whether this is the case of cod IgM is not yet certain, but the lectin analysis showed that the nature of the carbohydrate moiety of cod and salmon IgM was different.

Examination of the amino acid sequence also shows that the five likely sites of glycosylation (the sequence asparagine - x - threonine/serine, where x can be any amino acid except proline) were differently distributed on the heavy chain of salmon and cod (Hordvik *et al.*, 1992; Bengtén *et al.*, 1991).

The carbohydrate moiety of IgM from all four species was N-linked and associated with the heavy chain. It constituted from about 8–12% of the molecular weight of tetrameric IgM. The results of the lectin analysis showed that the nature of the carbohydrate moiety varied

between the four species. Interpretation of the results was, however, hampered by the relatively broad specificity of the lectins used. Complex type of N-linked oligosaccharides seemed more in evidence. Only haddock IgM showed a maximum reaction with the oligomannose sensitive ConA lectin. In general these results were consistent with the available data.

All immunoglobulin classes are glycosylated but the structure and the functional role played by these glycans is both complex and varied (Varki, 1993). IgM is characterised by relatively high carbohydrate content (≥10%) compared to, for example, IgG (1–2%). In the case of mammalian IgM there are commonly five glycosylation sites which are restricted to the constant region of the heavy chain. This carbohydrate moiety is of N-linked nature and both oligomannose and complex types have been identified (Anderson *et al.*, 1985).

In conclusion, the results indicated several common characteristics of fish IgM, i.e. the tetrameric form in serum, the molecular weight of the heavy and light chains and the N-linked nature and restriction to the heavy chain of the carbohydrate moiety. Characteristics that varied between these four species were: (1) the IgM concentration in serum, especially between salmon and the other three species, (2) the number and proportion of non-covalently bonded subunits, (3) the overall charge which was heterogeneous in salmon and haddock IgM but not in halibut and cod IgM, (4) the sensitivity to trypsin, and to some extent PNGase, digestion, and (5) the amount and composition of the carbohydrate moiety.

It would be of interest to examine whether these variable factors affect the functional role of IgM and also, bearing in mind the importance of proteases and glycosidases in the pathology of certain bacteria, to examine whether the variable enzyme sensitivity of the four IgM species correlates with difference in disease susceptibility.

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