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Purification and Characterization of IgM-like Immunoglobulin from Turbot *(Scophthalmus maximus* L.)

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Kofod, H., K. Pedersen, J.L. Larsen and K. Buchmann: Purification and characterization of IgM-like immunoglobulin from turbot *(Scophthalmus maximus* L.). Acta vet. scand. 1994, 35, 1-10. – A total of 40 turbot *(Scophthalmus maximus)* were immunized 3 times during a 3 months period using DNP-HSA whereafter serum samples were collected and pooled. Specific immunoglobulins (Ig) were affinity purified on an agarose column with immobilized DNP-BSA and further purified by gel filtration whereafter monospecific rabbit anti Ig serum was generated. Size exclusion chromatography and non-reduced SDS-PAGE indicated a MW of 8-900 kDa of the dominant antigen binding proteins from turbot serum. Reduced SDS-PAGE showed this fraction to be composed of disulphide linked heavy and light chains with MWs of 79 and 27-29 kDa, respectively, indicating a tetrameric structure. Isoelectric focusing of the 800-900 kDa Ig showed several bands between pH 5.5 and pH 5.8. Mean Ig concentration in serum of 10 turbot was measured to 6.48 mg/ml (SD 5.4) using rocket immunoelectrophoresis. Low molecular weight antigen binding molecules were copurified with the dominating immunoglobulins with an estimated MW of 500 kDa. Reducing SDS-PAGE of this fraction revealed molecules with MWs of 97, 79, 57, 29,and 27 kDa.

affinity purification; gel filtration; fish; rabbit anti turbot Ig.

Introduction

Immunoglobulins of teleosts are generally described as IgM-like tetramers composed of heavy and light polypeptide chains *(Marchalonis* 1977). However, some variation in the structure and properties of these fish proteins has been demonstrated. Evidence of low molecular weight Ig forms, dimers and monomers, in this vertebrate group has been presented *(Clem & McLean* 1975, *Lobb & Clem* 1981, *Elcombe et al.* 1985, *Buchmann et al.* 1992). In addition the existence of isotypes of heavy chains *(Lobb & Olson* 1988) and light chains *(Lobb et al.* 1984) has been demonstrated. Of the numerous studies on humoral immune response in the teleost group only few and limited studies have been concerned with the immunoglobulins from flatfishes *(Pleuronectiformes) (Cottrell* 1977, *Secombes et al.* 1991, *Al-Harbi & Austin* 1993). Therefore the present paper describes the isolation of serum immunoglobulin from turbot *(Scophthalmus maximus)* by affinity purifica-

tion, its subsequent biochemical characterization and the generation of a monospecific rabbit anti turbot Ig serum.

Materials and methods

Experimental animals

Fourty turbots with body weights of 200 - 300 g were used in the experiments and kept under mariculture conditions in fiberglass ponds containing 8 m^3 oxygenated seawater. Salinity was approximately 25 ppt and water temperature increased from 10 to 18 °C during the immunization period. Before blood sampling the fish were anaestetized with benzocaine.

Rabbits for production of rabbit anti-turbot antisera were kept in 5000 cm^2 cages and fed a standard laboratory animal diet. Before blood sampling, animals were sedated with fluanisonum 10 mg/ml, fentanylum 0.2 mg/ml (Hypnorm, Janssen Pharmaceutica, Belgium).

Immunization and blood sampling

Specific antibodies to dinitrophenylated human serum albumin (DNP-HSA)(Sigma) were raised in turbots by intraperitoneal injection of 0.1 ml of an emulsion of DNP-HSA and Freund's complete adjuvant (Statens Seruminstitut, Copenhagen, Denmark) corresponding to 366 µg antigen/fish. Injections were repeated after 42 and 84 days using Freund's incomplete adjuvant (Sigma). Water temperatures were 10 °C, 16 °C, and 18 °C at first, second, and third immunization, respectively. Turbots were bled from the tail 33 days after the last immunization and the pooled blood samples were allowed to clot for 4 h at room temperature. After removal of the coagulum serum was collected by centrifugation at 3000 g for 20 min and stored at –20 °C.

Two rabbits were immunized 3 times during a 2 months period by subcutaneous injections of an emulsion of equal volumes of purified turbot immunoglobulin solution and Freund's complete adjuvant in first immunization and Freund's incomplete adjuvant in boosters, corresponding to 100 µg Ig per animal. Serum was prepared as described above.

Purification of immunoglobulin

Turbot serum was diluted 1:5 in phosphate buffered saline (PBS) pH 7.3, sterile filtered (Minisart, 0.2 µm pore size), and loaded onto a column containing agarose coupled with dinitrophenylated bovine serum albumin (DNP-BSA), (Mini-leak, medium activated, Kem-En-Tec, Denmark). After extensive washing with 0.5 M NaCl in PBS protein bound to the column matrix was eluted with 50 mM diethylamine (DEA) buffer, pH 11.6, in 0.15 M NaCl and further purified by gel filtration in a Sephacryl-300 Superfine (S-300) or a Sephacryl-400 High Resolution (S-400) (Pharmacia, Uppsala, Sweden) column eluted with PBS, pH 7.3, containing 0.001 % sodium azide. Concentrations of protein in collected fractions were determined by the bicinchoninic acid – copper(II) sulphate assay (BCA-1 kit, Sigma).

SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Mini Slab cell (Idea Scientific Company, Mn, USA) *(Matsudaira & Burgess* 1978). Electrophoresis under reducing conditions was carried out in 10% gels *(Laemmli* 1970) and under non-reducing conditions in 3.0 or 5.0% gels *(Weber & Osborn* 1969). After electrophoresis, gels were stained with Coomassie brilliant blue or subjected to immunoblotting (western blot).

Western blot

Proteins from turbot serum and gel filtered

fractions were separated by SDS-PAGE and blotted onto nitrocellulose membranes in a semi-dry blotter (Kem-En-Tec, Copenhagen, Denmark) *(Kyhse-Andersen* 1984). Blocking and subsequent incubations were carried out at room temperature in a blocking buffer consisting of PBS containing 1.0 % non-fat instant dry milk. Nitrocellulose filters were incubated with rabbit anti turbot antiserum diluted 1:200 - 1:51200, using a Convertible™ Filtration Manifold System (BRL Life Technologies, Md, USA), and thereafter peroxidase labeled swine anti-rabbit IgG (Dako, Glostrup, Denmark) diluted 1:2000. Between steps filters were washed 4 times in PBS containing 0.05 % Tween 80. Peroxidase activity was visualized with diaminobenzidine (DAB) tablets, 10 mg (Sigma), dissolved in 40 ml H_2O and added 20 µl 30 % H_2O_2.

Dot blot assay

Ten µl of DNP-HSA solution, 100 µg/ml in PBS, were added to nitrocellulose membranes using a Convertible™ Filtration Manifold System (BRL Life Technologies, Md, USA). Incubation with 2 fold dilution series of turbot immune and nonimmune sera, blocking and following steps were carried out as described above.

Isoelectric focusing

Isoelectric focusing was performed in agarose gels (IsoGel, FMC Bioproducts, Maine, USA) at 1500 V for 20 min according to the instructions of the manufacturer.

Immunoelectrophoresis

Crossed immunoelectrophoresis (CIE), Rocket immunoelectrophoresis (RIE) and fused rocket immunoelectrophoresis (FRIE) was carried out in 1 % agarose (Litex HSA, Denmark) in Tris-Barbital buffer, pH 8.6 *(Axelsen* 1983).

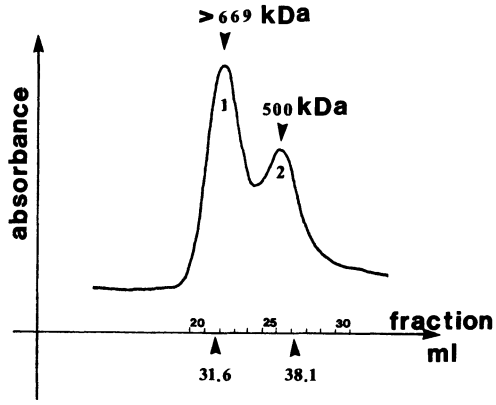

Figure 1. The UV-light absorbance profile of the S-300 filtration of the affinity purified turbot serum showing 2 peaks, corresponding to molecular weights of approximately > 669 and 500 kDa, respectively. Collected fraction numbers and ml eluate are indicated along the abscissa.

Double diffusion precipitation

Double diffusion precipitation test *(Ouchterlony* 1958) was carried out using 1% agarose (Litex HSA, Demnark) in Tris-Barbital buffer, pH 8.6.

Results

The response of the turbots to the hapten-carrier conjugate, DNP-HSA, was measured by both a double diffusion precipitation test and a dot-blot test. The double diffusion test showed a precipitation line with serum from the immunized fish and none with the serum from the controls.

The dot blot test revealed that pooled sera from non-immunized fish possessed a relatively high anti DNP-HSA titre, 1:128, but after 3 immunizations, the titre of pooled sera showed an increase to 1:512.

Antigen binding immunoglobulins were isolated by affinity chromatography on a DNP-

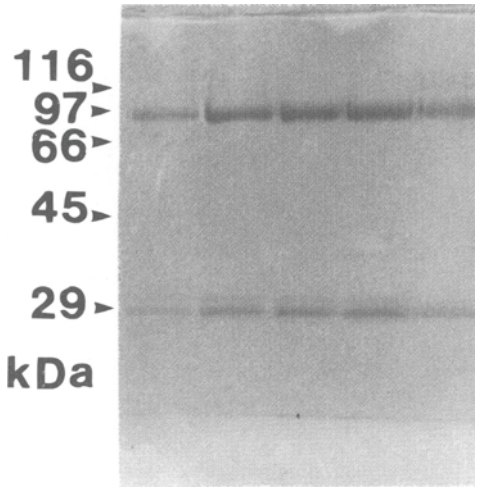

Figure 2A. SDS-PAGE of fractions from peak 1 run in a 10% polyacrylamide gel under reducing conditions showing 1 major band at 79 kDa and 2 minor bands at 29 kDa and 27 kDa. Lanes 1 - 5 correspond to fractions 20 - 24 from Fig. 1. Numbers to the left are molecular weight markers.

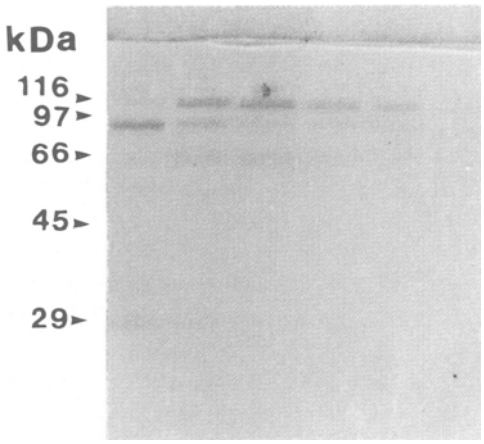

Figure 2B. SDS-PAGE of fractions from peak 2 run in a 10% polyacrylamide gel under reducing conditions containing an additional band at 97 kDa and smaller amounts of a protein of 57 kDa. Lanes 1 - 6 correspond to fractions 25 - 30 from Fig. 1.

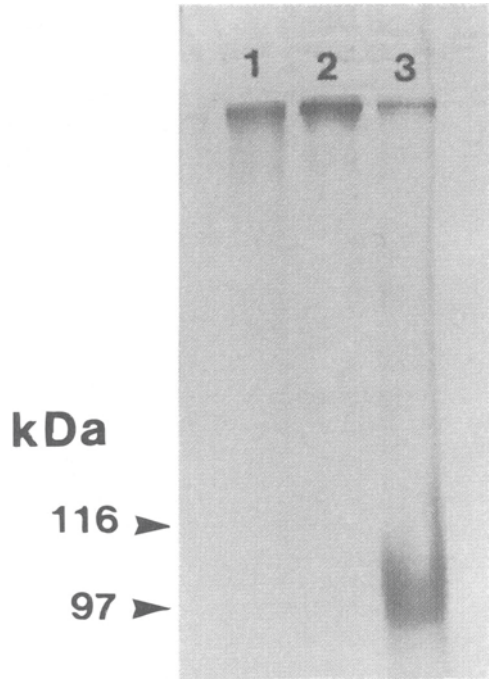

Figure 3A. SDS-PAGE run under non-reducing conditions with 3% stacking gel and 5% separation gel. Lane 1: purified salmon Ig, lane 2: fractions from peak 1, and lane 3: fractions from peak 2.
No major proteins were able to penetrate the gel matrix, except a protein in lane 3 of approximately 100 kDa.

BSA column. Proteins non-specifically adsorbed to the column were removed by thorough washing with 0.5 M NaCl in PBS before elution with DEA-buffer. The eluate was pooled and applied onto a S-300 column. The 280 nm absorbance profile of the gel filtrate revealed 2 closely positioned peaks corresponding to MW's of > 669 kDa (V_o) and approximately 500 kDa (Fig.1). When collected fractions were subjected to SDS-PAGE 10 % polyacrylamide under reducing conditions the first peak displayed 1 major band of 79 and 2 minor bands of 29 kDa and 27kDa. The 27 kDa protein was more dominant than that of

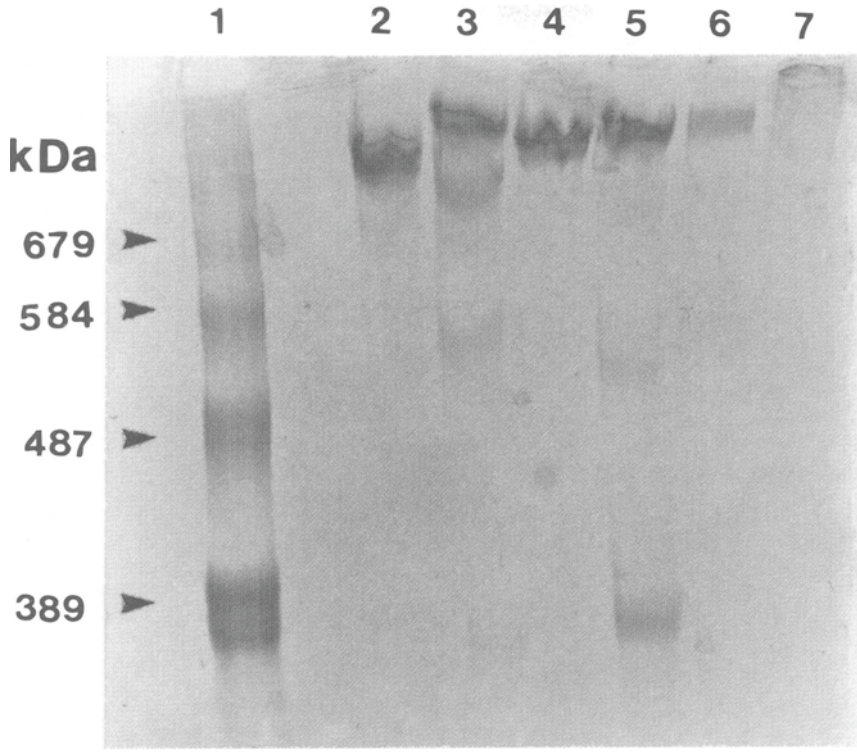

Figure 3B. SDS-PAGE run under non-reducing conditions in a 3% separation gel. Lane 1: molecular weight markers, lane 2: purified salmon Ig, lane 3: purified rainbow trout Ig, lane 4: fractions from peak 1, lane 5: fractions from peak 2, lane 6: a small fraction from peak 2, lane 7: purified IgM from mink (MW approximately 1000 kDa).

the 29 kDa protein (Fig.2A). The second peak contained the same structures but also an additional major band of 97 kDa and small amounts of protein of 57 kDa (Fig.2B).

No proteins of the first peak fraction were able to penetrate into a 5% SDS-PAGE gel run under non-reducing conditions, but the second peak was shown to contain a protein of 97 kDa and a non penetrating protein (Fig 3A). The proteins with the molecular weights of 79, 29 and 27 kDa were not demonstrated under these conditions.

SDS-PAGE in 3% gels run under non-reducing conditions, showed a protein from peak one of approximately 850 kDa, while the second peak contained proteins of 850 kDa and 400 kDa. In addition trace amount proteins of 500 kDa and an unseparated protein fraction with MW below 205 kDa, represented by the gel front, were found in peak two (Fig 3B).

Rabbits were immunized with protein from fractions containing only the high molecular weight component from the first peak.

The high molecular weight protein concentrated to 1700 µg/ml by ultrafiltration (Centriprep-30 Concentrators, Amicon Division, Beverly, MA, USA) was applied onto a Sephacryl S-400 column. The results indicated a

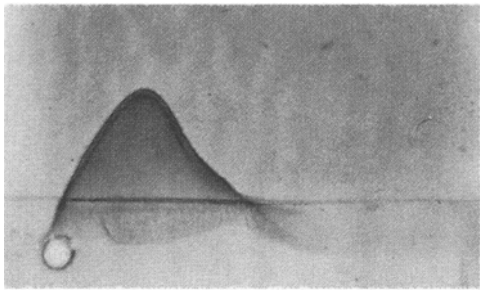

Figure 4. The reactivety and specificity of the rabbit anti turbot antiserum as judged by CIE. Total turbot serum was run in first dimension, and electrophoresed into the second dimension gel containing rabbit anti turbot antisera. The anode is to the right in the first dimension electrophoresis and at the top in the second dimension.

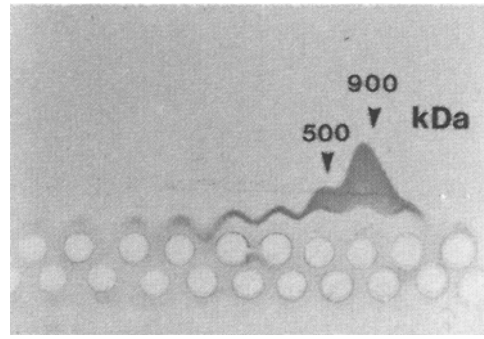

Figure 5. Fractions of turbot serum collected from the S-400 column subjected to FRIE demonstrated a monospecific rabbit anti turbot Ig antiserum with 2 peaks. The gel contained rabbit anti turbot Ig serum. Anode at the top.

molecular weight of approximately 850 kDa.
Isoelectric focusing of the high molecular weight proteins from peak one revealed several bands ranging between pH values of 5.5 and 5.8.
The reactivity and specificity of the rabbit anti turbot antisera was tested by CIE. A single precepitation arch with a double contour appeared (Fig. 4)
Turbot serum fractions collected from the S-400 gel filtration column were subjected to FRIE and RIE. A major precipitation arch and rocket occurred at an elution volume corresponding to MW of 850 - 900 kDa (Fig. 5).
In the western blot of total turbot serum, rabbit antisera in dilution below 1:1600 reacted to several bands, but 3 major bands were seen at 150, 79 and 57 kDa, and 2 distinct bands at 29 and 27 kDa. No reaction was noticed against the 97 kDa protein.
When rabbit antisera were diluted 1:3000 only 3 bands appeared on the blot, corresponding to 79 kDa and 27-29 kDa bands and in dilution 1:6400 only the 79 kDa band reacted (Fig 6A).
In western-blot made on a purified immunoglobulin fraction, specific reaction was recorded against 2 protein bands at 79 kDa and 27 kDa (Fig. 6B).
To analyse if the antiserum had affinity to the proteins from the S-300 fraction peak two, these proteins were investigated by a dot blot test. This revealed a positive reaction in dilutions up to 1:12800.
The Ig concentration in turbot serum was measured by RIE. Serum from 10 turbot contained a mean of 6.48 mg immunoglobulin per ml, standard deviation (s.d.) ± 5.40 mg/ml, while total serum protein contents measured by the BCA test gave a mean of 27.0 mg/ml, s.d. ± 9.2 mg/ml.

Discussion

As has been detected in other teleosts *(Marchalonis & Warr* 1978, *Vilain et al.* 1984, *Buchmann et al.* 1992) serum of non-immunized turbot contains natural antibodies to DNP, but anti-DNP-HSA titre in turbot serum rose after repeated immunization with DNP conjugated to HSA. The following affinity-purification of antigen-specific antibodies from immune sera on a DNP-agarose column

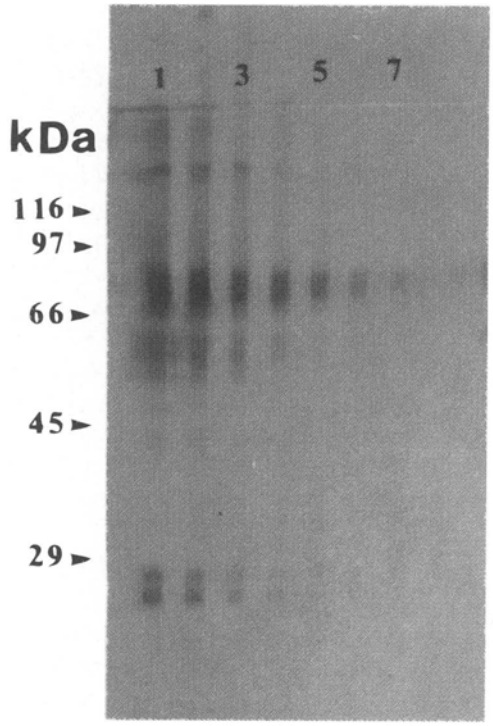

Figure 6A. Western blot of total turbot serum showed a reaction of the rabbit anti turbot antiserum to several bands depending on the dilution.
The dilution were twofold starting with 1:200 (lane 1) until 1:51200 (lane 9).

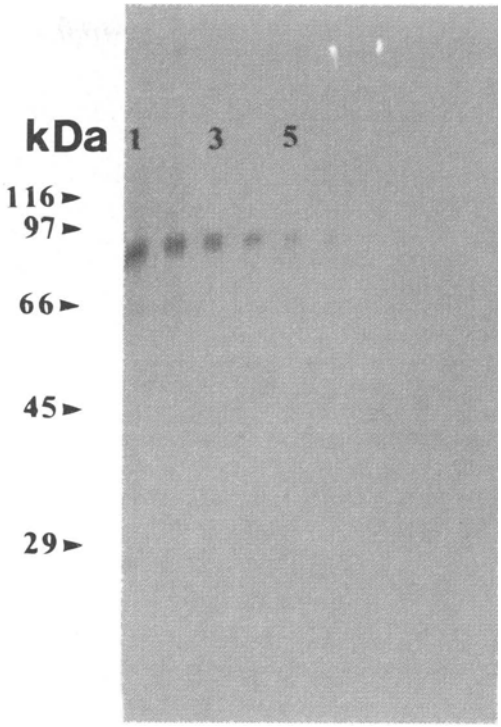

Figure 6B. Western blot of purified turbot Ig showed specific reaction of the rabbit anti turbot antiserum to the heavy chain in dilutions up to 1:3200 and to a light chain in dilution 1:200. Dilutions were twofold starting with 1:200 (lane 1).

recovered 2 groups of proteins with the dominating molecular weights of 800-900 kDa and 500 kDa respectively, as judged by S-400 and S-300 gelfiltration and non reduced SDS-PAGE. Reduced SDS-PAGE analysis of these fractions showed that the former and dominating fraction was composed of disulphide linked heavy (79 kDa) and light (27 and 29 kDa) chains, whereas the low molecular weight fraction also contained an additional protein band of 97 kDa and small amounts of protein of 57 kDa. As the high molecular weight protein fraction corresponded to the classical IgM-like immunoglobulin described from teleosts *(Shelton & Smith* 1970, *Pilström & Peterson* 1991) rabbits were immunized with this putative turbot immunoglobulin which resulted in the generation of a monospecific rabbit antiserum. Western blot analysis demonstrated this antiserum in a dilution of 1:6400 to react specifically with a 79 kDa turbot serum protein corresponding to the heavy chain of the immunoglobulin. The finding of 2 putative light chains of 27 and 29 kDa, respectively, suggest the presence of Ig light chain isotypes in turbot. The western blot also showed a reaction of the rabbit anti turbot serum to these 2 proteins in dilutions up to

1:3000. Isotypes of light chains have previously been reported in the channel catfish *(Ictalurus punctatus) (Lobb et al.* 1984). The isoelectric point of the high-molecular weight fraction was found in the range between pH 5.5 and 5.8 which is in accordance with values for Igs from salmon *(Håvarstein et al.* 1988) and cod *(Pilström & Peterson* 1991). Also the concentration of immunoglobulin in the serum of turbot (6.48 mg/ml) was of a magnitude normally detected in teleosts *(Israelson et al.* 1991). The recovered high molecular weight immunoglobulin is thus readily interpreted as a tetramer consisting of disulphide linked heavy and light chains but the nature of the low molecular weight fraction seems more complicated. The monospecific rabbit anti turbot serum also reacted with this fraction as shown from both dot blot analysis and FRIE and the presence of disulphide linked protein bands of 79 kDa and 27 kDa and 29 kDa in this fraction indicates the presence of immunoglobulin in this fraction. The amount of proteins of 800 kDa in the unreduced SDS-PAGE 3% polyacrylamide from peak 2 must be interpreted as residues of Igs from peak 1 due to tailing from the gel filtration column. Teleost immunoglobulins have not invariably been shown to consist solely of tetramers as also the presence of monomer *(Clem & McLean* 1975, *Elcombe et al.* 1985) and dimer *(Lobb & Clem* 1981, *Buchmann et al.* 1992) molecules have been suggested. Investigations of immunoglobulins from flatfishes have only suggested high molecular weight forms *(Cottrell* 1977, *Secombes et al.* 1992, *Al-Harbi & Austin* 1993) but the 400 kDa protein recovered from turbot serum in unreduced SDS-PAGE in 3% polyacrylamide gels in the present study could be interpreted as a dimer Ig molecule in accordance with the S-300 gel-filtration investigations. However, the presence of the additional 97 kDa and 57 kDa band in the low molecular weight fraction does not conform with a classical description of immunoglobulins. The function and properties as well as their possible association with immunoglobulins are at present unknown. Similar proteins copurified with putative low molecular weight antibodies were tentatively interpreted as a secretory component *(Lobb & Clem* 1981) or suspected to be a complement related factor *(Buchmann et al.* 1982). The molecular weights of the alpha and beta-chains of complement factor C3 in different vertebrates have been reported to range from 107-119 and 64-83 kDa respectively *(Alsenz et al.* 1992) thus the possible complement related nature of the copurified proteins from turbot should be further elucidated.

Acknowledgements

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Sammendrag

Oprensning og karakterisering af et IgM-lignende immunglobulin fra pighvar (Scophthalmus maximus *L.)*

I alt blev 40 pighvar immuniseret 3 gange i løbet af en 3 måneders periode med DNP-HSA, hvorefter sera blev opsamlet og poolet Specifikke immunglobuliner blev affinitets-oprenset på en agarose søjle med immobiliseret DNP-BSA og yderligere oprensning blev foretaget ved hjælp af gel filtrering. Størrelses chromatografi og ureduceret SDS-PAGE indicerede en MW på ca. 850 kDa af det dominerende antigenbindende protein fra pighvar sera. Reduceret SDS-PAGE viste, at denne fraktion bestod af en tung kæde med MW på 79 kDa samt to lette kæder på henholdsvis 27 og 29 kDa, bundet sammen med svovlbindinger. Det dominerende pighvar immunglobulin synes derfor at være en tetramer struktur. Isoelektrisk focusering af dette protein viste adskillige bånd med en pH værdi fra 5,5 til 5,8. Middelværdien af immunglobulin-koncentrationen i serum fra 10 pighvar blev målt til 6,48 mg/ml med en

standard afvigelse på ± 5,4 mg/ml ved hjælp af raket immunelektroforese.
En fraktion af antigenbindende molekyler med en lavere molekylvægt blev oprenset sammen med det dominerende immunglobulin. Reduceret SDS-PAGE af denne fraktion viste, at den indeholdt proteiner med molekylvægte på henholdsvis 97, 79, 57, 29 og 27 kDa.

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