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Isolation and Characterization of Serum Immunoglobulins from kalbasu (*Labeo calbasu*) (Hamilton, 1822)

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Abstract

The Ig (Ig) from the serum of kalbasu (*Labeo calbasu*, Cyprinidae) was isolated and characterized during the present investigation. Kalbasu of 500-800 g size were immunized with Bovine Berum Albumin (BSA) to prepare immune sera. The Ig was isolated from the immune sera by an immunoaffinity column of BSA-Sepharose 4B. The purity and homogeneity of the isolated sample were confirmed by observing a single band in native gradient polyacrylamide gel electrophoresis. This also indicated the presence of only single type of Ig in kalbasu. The molecular weight of native Ig molecule was determined from the gel to be ~857 kDa. In Sodium dodecyl sulphate polyacrylamide gel electrophoresis (E), the constituent heavy (H) and light (L) polypeptide chains of the Ig molecules were identified. There were only one type of H chain with a molecular weight of ~78 kDa and two types of L chain with molecular weights of ~27 kDa and ~26 kDa. The antiserum against the kalbasu Ig was raised in a rabbit and adsorbed with 10% kalbasu liver tissue homogenate in order to enhance its specificity. By an indirect ELISA standardized using this adsorbed rabbit antiserum, the normal serum Ig concentration in kalbasu was estimated to be ~2.82 $mg.ml^{-1}$ (n=22), which is around 8% of the total serum proteins.

Introduction

Fish like higher vertebrates synthesize immunoglobulins (Ig) upon antigenic stimulation, which is secreted into blood circulation and also present in the bile and the skin mucus. The Ig have been isolated and specific structural characteristics investigated in a number of fish species (Pilstrom and Bengten 1996). In general, there is only one class of immunoglobulin in fish, which is considered analogous to mammalian IgM. Cartilagenous fishes (elasmobranchs) have been shown to possess a pentameric as well as a monomeric form of IgM; whereas, in bony fishes (teleosts) the molecule is tetrameric in nature, although low molecular weight IgM has also been reported in some species (Clem and McLean 1975). Presence of another class of immunoglobulin, homologue to mammalian IgD has also been reported from gene sequence studies in few teleostean

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species (Wilson et al. 1997; Hordvik et al. 1999; Stenvik and Jorgensen 2000). The tetrameric Ig in teleosts like mammalian IgM consist of heavy (H) and light (L) polypeptide chains. However, variations in both H and L chains as isotypes (Lobb et al. 1984; Lobb and Olson 1988; Sanchez and Dominguez 1991) and molecular weight variants (Watts et al. 2001; Swain et al. 2004; Grove et al. 2006) have been reported.

India is endowed with a wide variety of fish species. Carps constitute the bulk of the inland fish resources of India and kalbasu (*Labeo calbasu*) forms one important component in carp polyculture system. However, there are only limited studies on the immunoglobulin molecules of few indigenous species. Considering the importance of the species and the lack of information on its immune response, the present investigation was undertaken to isolate the serum Ig of kalbasu to characterize the molecules biochemically and produce rabbit antisera against these molecules for further application in immunological investigations.

Materials and methods

Production of immune serum in kalbasu

Experimental fish and their maintenance

Twenty-five numbers of adult kalbasu (*Labeo calbasu*) weighing 500-800 g were collected from the Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar, India fish farm and maintained in tanks for immunizations. The animals were acclimatized for seven days prior to immunization.

Immunization of fish

Fourty mg of bovine serum albumin (BSA) was dissolved in 5 ml of tris buffered saline (TBS, 0.02 M Tris HCl with 0.15 M NaCl, pH 7.4) and emulsified with equal volume of Freund's complete adjuvent. Fishes were injected intraperitoneally with 800 µg BSA in 0.5 ml emulsion. Booster doses of BSA with Freund's incomplete adjuvant were administered on 14th and 28th days following the primary immunization. The fishes were bled through caudal vein on 42nd day of first injection in order to collect serum. The serum samples collected were pooled, aliquoted to 2 ml and preserved at -20 °C for further experiments.

Isolation of kalbasu serum Ig by affinity chromatography

Kalbasu Ig was isolated by affinity chromatography on an immuno affinity column of BSA-sepharose 4B (Genei, India), according to the procedure of Swain et al. (2004). In brief, 4 mL of column matrix was packed and washed thoroughly with equilibration buffer (TBS). Three ml of of pooled kalbasu serum was filtered through a 0.45 µm filter, diluted 1:4 with TBS and slowly loaded into the column. The loaded serum sample

was then allowed to pass through the column slowly at a flow rate of 10 mL.h^{-1} and the column was washed with TBS till the absorbance of the flow at 280 nm dropped to baseline. The bound Ig in the column was then eluted with 10 ml of 0.1 M Glycine NaOH, pH 11.0 and was collected as a single fraction. The pH of the collected sample was immediately neutralized with 2 M Tris HCl, pH 2.5 and was dialysed against TBS over night at 4°C temperature. After dialysis the sample was concentrated by a centrifuge filter, vectaspin 3 (cut off 30 kDa) (Whatman, U.K) at 4000 x g at 4 °C. The isolated sample was aliquoted and preserved at -20 °C after determining the protein concentration.

Protein estimation

The total protein concentration of isolated immunoglobulin samples and serum samples were estimated by the dye binding method of Bradford (1976), using BSA as the standard.

Native gradient polyacrylamide gel electrophoresis (Native PAGE)

A native polyacrylamide gel electrophoresis on 2.8 to 22.5% acrylamide gradient was run to check the purity of the isolated Ig. The gels were analyzed with GS-800 densitometer and quantity one software (Bio-Rad, USA) and the molecular weight of the native kalbasu Ig was determined from six samples by comparing it with known molecular weight markers in the gel. Different molecular weight markers used were bovine IgM (950 kDa), thyroglobulin (660 kDa), apoferritin (480 kDa), catalase (250 kDa) and lactate dehydrogenase (146 kDa).

Sodium dodecyl sulphate polyacrylamide gel electrophoesis (SDS-PAGE)

SDS-PAGE was carried out according to the method of Laemmli (1970), in order to determine the types of heavy (H) and light (L) polypeptide chains present in kalbasu Ig molecules. The electrophoresis was run on a separating gel of 12% and a stacking gel of 5% acrylamide concentration. The molecular weight analysis was similarly carried out as for native gradient PAGE.

Production of anti-kalbasu Ig serum in rabbit

One healthy male rabbit procured from a local farm weighing around 1 kg was immunized with three injections of kalbasu Ig (300 µg·injection^{-1}) to raise anti-kalbasu Ig serum. The immunization schedule followed was similar to that mentioned earlier for immunization of fish. The rabbit was bled through the ear vein and the separated serum stored at -20°C.

Western blotting

The specificity of rabbit anti-kalbasu Ig serum was checked in western blotting.

Kalbasu serum and the purified Ig run in SDS–PAGE were electroblotted to a nitrocellulose paper (NCP) (Bio Rad, USA) at 100 V for 1 h. Half of the nitrocellulose paper was stained for 2 min in 0.1% amido black protein staining solution containing 25% isopropanol and 10% acetic acid, and destained in a solution of 25% isopropanol and 10% acetic acid, to assess the transfer success. The other half of NCP to be processed for immunostaining with rabbit anti-kalbasu Ig serum diluted to 1:2000 in TBST and goat anti-rabbit IgG alkaline phosphatase conjugate (Genei, India) at a dilution of 1:1000 in TBST. Since the rabbit antiserum showed cross-reactions with some unrelated serum proteins, the antiserum was adsorbed overnight with kalbasu liver tissue homogenate (10%) at 1:1 ratio. The serum was centrifuged to remove the precipitate. The specificity of this adsorbed antiserum was checked again in western blot and used in subsequent ELISA experiment.

Enzyme-linked immunosorbent assay (ELISA)

An indirect ELISA was performed to determine the normal immunoglobulin concentrations in kalbasu serum samples. ELISA plates (Tarsons, India) were coated with 50 µl·well^{-1} of individual kalbasu serum at 1:64000 dilution (determined earlier by using one serum sample in dilutions of $2x10^3$ to $512x10^3$) in TBS. One dilution series of purified kalbasu Ig (1000 ng·ml^{-1} to 4 ng·ml^{-1}) was put in each plate as a reference standard. Each sample and standard was used in triplicate wells. After 3 h of incubation at 30°C, the plates were washed thrice with TBST (TBS with 0.05% Tween 20) at 5 min interval. Blocking agent (5% skim milk powder in TBS) was added at 100 ml per well and incubated at 4°C over night. The plates were washed again and anti-kalbasu Ig rabbit serum was added at 1:10000 dilution (determined earlier by checker board titration) in 50 µl volume and incubated for 2 h at 30°C. The plates were washed again with TBST as mentioned before. Subsequently, goat anti rabbit IgG HRPO (horseradish peroxidase) conjugate (Genei, India) was added at 1:5000 dilution. After 2 h of incubation at 30°C and washing with TBST, 100 µl of substrate, trimethyl benzidine/hydrogen peroxide (TMB/ H_2O_2) (Genei, India) was added to each well. After 10 min of incubation the colour reaction was stopped by adding 1 N H_2SO_4 at 50 µl per well. The absorbance was read at 450 nm in an automated ELISA reader (Model Sunrise, Tecan, Austria) against substrate control (only TMB/H_2O_2 substrate solution) and the results were analyzed by Magellan software. Absorbances obtained with standard Ig dilutions were plotted against the log concentration of Ig to get a standard curve. The Ig concentrations of individual serum samples were determined from the standard curve. A total number of 22 serum

samples from kalbasu (100-300 g size) were analyzed for Ig concentration. The total protein concentration of these serum samples were also estimated by the Bradford procedure mentioned earlier.

Results

Isolation of kalbasu Ig

The kalbasu Ig was eluted from BSA affinity column as a single continuous peak. The eluted fraction contained a protein concentration of 600-800 mg recovered from 3 ml of kalbasu immune sera.

Analysis of kalbasu Ig in native gradient PAGE

The purified kalbasu Ig sample showed a single band in native gradient PAGE analysis. The molecular weight of the kalbasu Ig was determined to be 857.09 ± 12.18 (SE) kDa (Fig.1).

Analysis of the polypeptide chains of kalbasu Ig in SDS-PAGE

In SDS-PAGE four bands were observed (Fig.2).

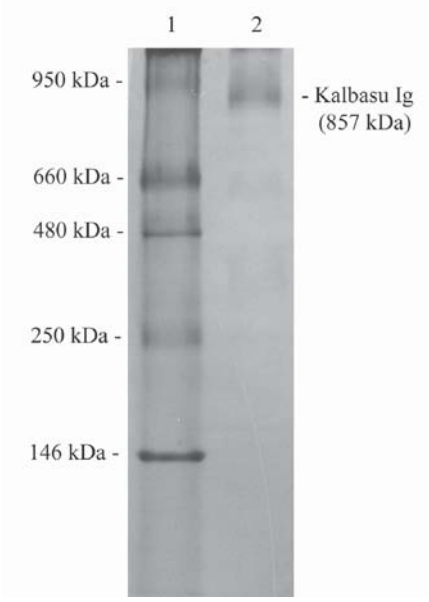

Figure 1. Analysis of purified kalbasu Ig in native gradient page: Lane 1. Molecular weight markers, Lane 2. kalbasu Ig.

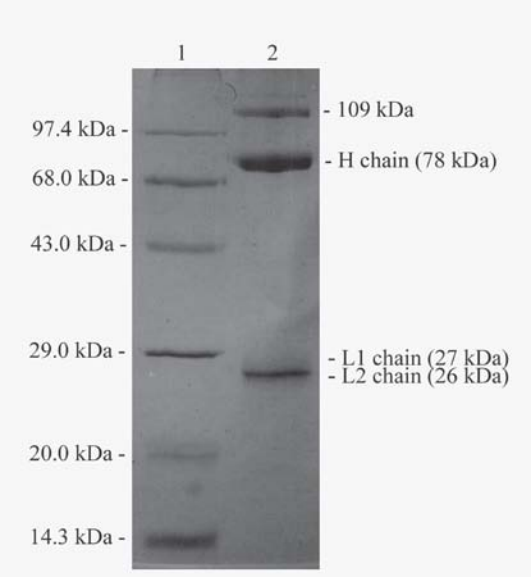

Figure 2. Analysis of kalbasu Ig in SDS-PAGE: Lane 1. Molecular weight markers, Lane 2. kalbasu Ig.

Based on the distance of migration one of these bands was identified as heavy (H) chain and the two other as light (L) chains. The molecular weights of these bands were calculated from the gel and found to be 78.31 ± 0.58 (SE) kDa for the heavy (H) chain and 27.39 ± 0.08 (SE) kDa and 25.94 ± 0.079 (SE) kDa for the two light chains. There was also a faint band observed above the heavy chain, whose molecular weight was determined to be 109.20 ± 0.81 (SE) kDa.

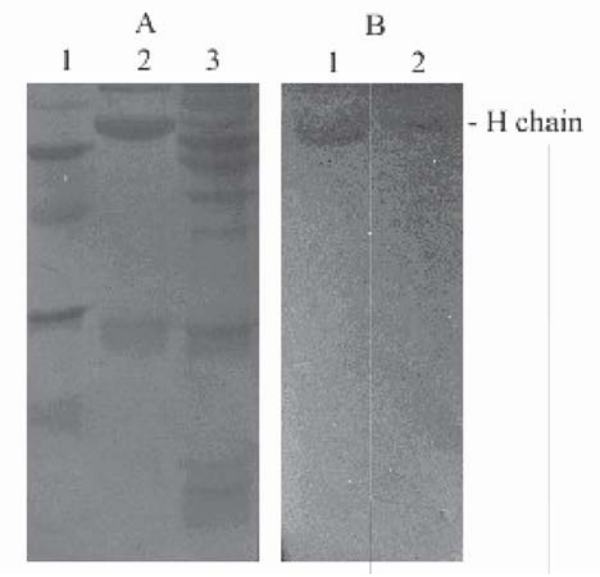

Figure 3. Specificity testing of adsorbed rabbit anti kalbasu Ig serum in western blotting: A. Amido black staining of the blot. Lane 1. Molecular weight marker, Lane 2. Purified kalbasu Ig, Lane 3. Kalbasu serum, B. Immunostaining of the blot. Lane 1. Purified kalbasu Ig, Lane 2. Kalbasu serum.

Specificity of rabbit antiKalbasu Ig serum in western blot

In western blot, the successful transfer of protein bands from SDS-PAGE gel could be assessed by staining the nitrocellulose paper with amido black (Fig. 3A). Immunoblotting with adsorbed rabbit anti-kalbasu Ig serum showed specific reaction with H chain of kalbasu Ig (Fig. 3B).

Quantitation of immunoglobulin concentrations in normal kalbasu serum

An indirect ELISA was standardized to determine the Ig concentration in kalbasu serum samples. Graph plotted using absorbance of serial two-fold dilution of normal kalbasu serum and purified kalbasu Ig showed parallelism (Fig. 4), which indicates the suitability of the system for determination of normal Ig concentration. The dilution of kalbasu serum samples was selected at 1:64000, so that the absorbance values fall within the linear part of the standard curve. The mean Ig concentration in 22 serum samples was quantitated to be 2.82 ± 0.22 (SE) $mg.mL^{-1}$. The average total protein concentration of these serum samples was estimated to be 36.22 ± 1.03 (SE) $mg.mL^{-1}$ and so, the normal Ig level constitutes approximately 8% of the total protein concentration.

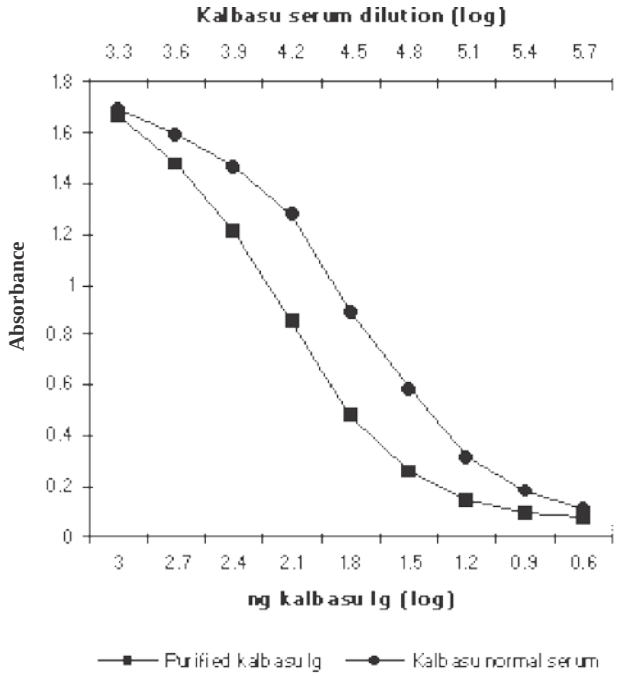

Figure 4. Standard curve used for quantitation of immunoglobulin in kalbasu serum by indirect ELISA.

Discussion

In the present study BSA affinity chromatography was used to isolate Ig from BSA immunized sera from kalbasu. BSA-immunoaffinity chromatography has been used successfully by several workers to purify Ig from other fish species (Phillips and Ourth 1986; Baldwin et al.1997; Swain et al. 2004). For isolation, the bound Ig from the column was finally eluted with a high pH buffer of 0.1 M glycine NaOH, pH 11.0 (Lim 1987), which gave a single continuous peak. The failure in initial attempt using low pH buffer prompted us using this high alkaline buffer, enabling elution of sufficient quantities (600-800 µg) of kalbasu Ig from the BSA affinity column.

In native gradient PAGE, the isolated Ig sample showed only one band that indicated the purity of the sample. The presence of the single band was also indicative of the existence of only single type of antibody molecule in kalbasu. In majority of the teleostean species, a single tetrameric serum Ig with a native molecular mass between 700 and 1000 kDa has been reported (Dacanay et al. 2006). In the present investigation also the kalbasu Ig was found to possess a molecular weight of ~857 kDa. Similar sized Ig have been reported in seabass *Dicentarchus labrax* (855 kDa) (Palenzuela et al. 1996). Based on the high molecular weight, the kalbasu Ig could be considered as IgM type, like other teleostean Ig.

In SDS-PAGE, the kalbasu Ig was reduced to produce one H chain type of ~78 kDa and two L chain types of ~27 kDa and ~26 kDa. Palenzuela et al. (1996) reported similar findings of a single H chain of ~78 kDa and two L chains of ~28.5 kDa and ~27.5 kDa for seabass (*D. labrax*). The molecular weight variants for L chain as found in our study, has also been reported for both H and L chains of several teleosts (Watts et al. 2001; Swain et al. 2004; Grove et al. 2006). Taking into account the molecular weights of H and L chains in the present study, the native kalbasu Ig of ~857 kDa possibly generates four monomeric immunoglobulin molecules of H_2L_2 configuration. Hence, the kalbasu Ig was confirmed to be tetrameric in nature as has been reported for other teleostean species (Ellis 2001). A faint band of ~109 kDa was also found in SDS-PAGE, which is possibly the unreduced H-L monomer, as could be calculated from the molecular weight of H and L chains.

In western blotting, the transfer efficiency could be demonstrated by amido black staining of the blot. In immunostaining of the membrane, the rabbit anti-kalbasu Ig serum reacted with the H chain as well as some non-specific serum proteins (not shown). After adsorption of rabbit serum to kalbasu liver cell homogenate, the specificity was found to be increased as it reacted only to the H chain of kalbasu Ig. Subsequently, this adsorbed antiserum was used for ELISA test.

In the present investigation, an indirect ELISA test was standardized with the rabbit anti kalbasu Ig serum to quantify the normal serum Ig concentration in kalbasu. The parallel sigmoid curves obtained with both purified Kalbasu Ig and normal kalbasu serum indicated that the test could be used to quantify Ig level in normal Kalbasu serum. ELISA has been used by others to measure the serum Ig concentrations in fish and its superiority over the traditional single radial immunodiffusion test has been implicated (Pomport-Castillon et al. 1997). An estimate of the normal serum Ig concentration in kalbasu weighing 100-300 g size was 2.82 $mg.mL^{-1}$, which was about 8% of the total serum protein concentration. In teleosts, the serum Ig concentration has been shown to vary between 2-7 $mg.mL^{-1}$ and the Ig concentration as percentage of total serum protein varies between 6-15 (Ellis 2001).

Conclusion

Kalbasu, *Labeo calbasu* Ig could be isolated to homogeneity by affinity chromatography and it could be proved that the kalbasu possesses only one type of high molecular weight tetrameric Ig similar to Ig of other teleosts. Polyclonal antisera raised against kalbasu Ig was found suitable for use in western blotting and ELISA and hence, may find applications in studying the immune response in kalbasu.

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References

Baldwin, T.J., L.A. Collins and J.C. Newton. 1997. Antigens of *Edwardsiella ictaluri* recognised by serum antibodies from naturally infected channel catfish. Fish and Shellfish Immunology 7**:** 261-271.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

Clem, L.W. and W.E. McLean. 1975. Phylogeny of immunoglobulin structure and function. VII. Monomeric and tetrameric Ig of the margate, a marine teleost fish. Immunology 29: 791-799.

Dacanay, A., B.E. Bentley, L.L. Brown, A.J. Roberts and S.C. Johnson. 2006. Unique multimeric immunoglobulin crosslinking in four species from the family Gadidiae. Fish and Shellfish Immunology 21: 215-219.

Ellis, A.E. 2001. The immunology of teleosts. In: Fish Pathology (3rd Edition) (ed. R.J. Roberts) pp. 133-150. W. B. Saunders, UK.

Grove, S., M. Tryland, C.M. Press and L.J. Reitan. 2006. Serum immunoglobulin M in Atlantic halibut (*Hippoglossus hippoglossus*): Characterization of the molecule and its immunoreactivity. Fish and Shellfish Immunology 20: 97-112.

Hordvik, I., J. Thevarajan, I. Samdal, N. Bastani and B. Krossoy. 1999. Molecular cloning and phylogenetic analysis of the Atlantic salmon immunoglobulin D gene. Scandinavian Journal of Immunology 50: 202-210.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lim, P.L. 1987. Isolation of specific IgM monoclonal antibodies by affinity chromatography using alkaline buffers. Molecular Immunology 24: 11-15.

Lobb, C.J. and M.O.J. Olson. 1988. Immunoglobulin heavy H chain isotypes in a teleost fish. Journal of Immunology 141: 1236-1245.

Lobb, C.J., M.O.J. Olson and L.W. Clem. 1984. Immunoglobulin light chain classes in a teleost fish. Journal of Immunology 132: 1917-1923.

Palenzuela, O., A. Sitja-Bobadilla and P. Alvarez-Pellitero. 1996. Isolation and partial characterization of serum Ig from sea bass (*Dicentrarchus labrax* L.) and gilthead sea bream (*Sparus aurata* L.). Fish and Shellfish Immunology 6: 81-94.

Phillips, J.O. and D.D. Ourth. 1986. Isolation and molecular weight determination of two immunoglobulin heavy chains in the channel catfish, *Ictalurus punctatus.* Comparative Biochemistry and Physiology 85B**:** 49-54.

Pilstrom, L. and E. Bengten. 1996. Immunoglobulin in fish - genes, expression and structure. Fish and Shellfish Immunology 6: 243-262.

Pomport-Castillon, C., C. Gasc, P. Rigobert and B. Romestand. 1997. Compared accuracy of two enzyme linked immunosorbent assay (ELISA) methods performed with polyclonal antibodies to measure immunoglobulin levels in *Platichthys flesus* L. 1758 (Flounder). Fish and Shellfish Immunology 6: 305-315.

Sanchez, C. and J. Dominguez. 1991. Trout immunoglobulin populations differing in light chains revealed by monoclonal antibodies. Molecular Immunology 28: 1271-1277.

Stenvik J. and T.Q. Jorgensen. 2000. Immunoglobulin D (IgD) of Atlantic cod has a unique structure. Immunogenetics 51: 452-461.

Swain, T., J. Mohanty and A.K. Sahu. 2004. One step purification and partial characteisation of serum immunoglobulin from Asiatic catfish (*Clarias batrachus* L.). Fish and Shellfish Immunology 17: 397-401.

Watts, M., B.L. Munday and C.M. Burke. 2001. Isolation and partial characterisation of immunoglobulin from southern bluefin tuna *Thunnus maccoyii* Castelnau. Fish and Shellfish Immunology 11: 491-503.

Wilson, M., E. Bengten, N.W. Miller, L.W. Clem, P.L. Du and G.W. Warr. 1997. A novel chimeric Ig heavy chain from a teleost fish shares similarities to IgD. Proceedings of National Academy of Sciences 94: 4593-4597.

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