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Production, characterisation and applicability of monoclonal antibodies to immunoglobulin of Japanese flounder (*Paralichthys olivaceus*)

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Abstract

Immunoglobulin (Ig) of Japanese flounder (*Paralichthys olivaceus*) was purified by a combination of salting-out and DEAE Sepharose Column chromatography. The purified immunoglobulin had an apparent molecular weight of 74 kDa (heavy chain) and 24 kDa (light chain) in SDS-PAGE. Eighteen hybridomas secreting monoclonal antibodies (MAbs) against Japanese flounder Ig were obtained by immunisation of Balb/C mice with purified Ig preparations, which were selected on the basis of the double indirect enzyme-linked immunosorbent assay (D-ELISA). Two of them designated as 2D8 and 2H1 were cloned by limiting dilution and characterised with western blotting, indirect immunofluorescence assay test (IIFAT) and fluorescence-activated cell sorter (FACS) analysis. Under reducing conditions in western blotting, both MAb 2D8 and MAb 2H1 were specific for the heavy chain of Japanese flounder Ig. MAb 2D8 was used to identify surface Ig-positive lymphocytes in the peripheral blood, spleen and pronephros of healthy Japanese flounder by flow cytometry. FACS analysis revealed that 40.48% of lymphocytes in the peripheral blood, 17.32% in the spleen and 9.67% in the pronephros were reactive to 2D8.

Keywords: Monoclonal antibodies; Immunoglobulin; Japanese flounder; *Paralichthys olivaceus*

1. Introduction

In the past 20 years, studies on the immune system of teleost fish have greatly benefited from the production of monoclonal antibodies (MAbs) to marine or freshwater fish immunoglobulin (Ig). MAb specifically reacting with Ig provides a powerful tool for determining the level of total and specific serum immunoglobulin and the number of B and plasma cells in different tissues in healthy, infected and vaccinated fish. As far as we know, MAbs have been produced to Ig of a number of teleost fish, including channel catfish (*Ictalurus punctatus*) [1,2], carp (*Cyprinus carpio*) [3], rainbow trout (*Oncorhynchus mykiss*) [4–6], sea bream (*Sparus aurata*)

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[7], gold fish (*Carassius auratus*) [8], red drum (*Sciaenops ocellatus*) [9], Atlantic salmon (*Salmo salar*) [10,11], turbot (*Scophthalmus maximus*) [12], European eel (*Anguilla anguilla*) [13], sea bass (*Dicentrachus labrax*) [14–16], tilapia (*Oreochromis niloticus*) [17], torafugu (*Takifugu rubripes*) [18]. Unfortunately, MAbs to Japanese flounder (*Paralichthys olivaceus*) Ig are not yet available, despite the fact that the fish is an economically important fish as a food and recently culturing has become popular in Asian countries such as Japan, Korea and China [19]. Furthermore, with the development of aquaculture for the fish, significant pathogens have emerged, especially lymphocystis disease virus (LCDV), which causes major losses in Japan and China [20,21].

In this study, to obtain MAbs to Japanese flounder Ig, the fish were immunized by inactivated purified lymphocystis disease virus (LCDV), purified Ig from the immune fish serum and with the Ig as antigen to immunize mice, and then produce hybridomas according to standard methods. The MAbs were characterised by double indirect enzyme-linked immunosorbent assay (D-ELISA), western blotting, indirect immunofluorescence assay test (IIFAT) and fluorescence-activated cell sorter (FACS).

2. Materials and methods

2.1. Fish immune sera preparation

Ten adult Japanese flounder (*Paralichthys olivaceus*), 450 ± 25 g in weight, were reared in a local fish farm (Huangdao, China). A total of 800 μg in 1 ml of sterile TNE buffer (50 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.4) of inactivated purified lymphocystis disease virus (LCDV), was given by intraperitoneal inoculation into each fish in four injections over a 4-week period. The fish were each inoculated with 200 μg of LCDV in 250 μl TNE buffer emulsified with an equivalent volume of Freund's complete adjuvant (FCA) on day 1. On day 15, the animals were boosted with the same dose of LCDV emulsified with Freund's incomplete adjuvant (FIA). On days 22 and 30, booster injections were given twice with 200 μg of LCDV. Five weeks after the first inoculation, blood was drawn from the caudal vein, and allowed to clot at room temperature for 1 h and stored overnight at 4 °C. Serum was separated the next day by centrifugation at 1000 × *g* for 20 min and stored at −20 °C until use.

2.2. Purification of immunoglobulin

Japanese flounder Ig was primarily purified from serum by precipitation with 50% saturated ammonium sulphate. The precipitate was pelleted by centrifugation at 14,000 × *g* for 30 min at 4 °C, resuspended in 0.02 M Tris–HCl buffer (pH 8.0) and dialyzed extensively against the same buffer. The dialyzed solution was clarified by centrifugation at 14,000 × *g* for 30 min at 4 °C.

The supernatant was fractionated by a DEAE Sepharose Column (Amersham) using protein purification system (AKTA prime, Amersham) according to the technique developed by Nishida et al. [22] with modifications. Briefly, the column was equilibrated with 30 ml of 0.02 M Tris–HCl buffer (pH 8.0) at a flow rate of 1 ml/min. After equilibrating the column, 10 ml of the above-mentioned dialyzed solution was applied to the column. The column was washed with equilibration buffer to eliminate the unbound proteins and bound proteins were then eluted using a stepwise elution with the same buffer containing NaCl at 0.05, 0.1, 0.15, 0.2 and 0.4 M. The absorbance of the effluent at 280 nm was continuously monitored and registered. The fraction eluted by NaCl at 0.1 M was concentrated by a freeze dryer (Heto-holten) and changed to 0.01 M phosphate buffered saline (PBS, 0.13 M NaCl, 2.7 mM KCL, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4).

The protein content was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [23], using a Mini-Protean unit (Bio-Rad). Samples were reduced and denatured by 5 min boiling in loading buffer (0.5 M Tris–HCl, pH 6.8, containing 5% SDS, 50% glycerol, 5% 2-mercaptethanol and 0.05% bromophenol blue). Samples were loaded onto wells of 4% stacking gels over 12% resolving gels, and electrophoresed for 1 h at 150 V in electrode buffer (25 mM Tris, 192 mM glycine and 0.1% SDS, pH 8.3). The gels were fixed and stained with Coomassie brilliant blue R-250, then destained with a solution of 7.0% (v/v) acetic acid and 5.0% (v/v) methanol in distilled water. The protein concentration of the sample was quantified by Coomassie brilliant blue dye-binding assay, in which bovine serum albumin (BSA) was used as the standard protein.