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One-step purification of the major rainbow trout immunoglobulin

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ABSTRACT

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Salmonid immunoglobulin purification has been hampered by time-consuming, labour-intensive, conventional chromatographic procedures. In this report we describe the use of immunoaffinity chromatography for the purification of immunoglobulins from trout serum in a single step. An anti-heavy chain monoclonal antibody (1G7) which reacts with more than 85% of total trout immunoglobulins was used to purify the trout immunoglobulin. The purity achieved was higher than 95%, and the immunoglobulins recovered were fully immunogenic. This method served equally well in isolating immunoglobulins from the sera of other salmonids (coho salmon and chinook salmon). The procedure should be useful in the standardization of salmonid immunoglobulin reagents.

INTRODUCTION

The predominant serum immunoglobulin (Ig) in salmonids is a high-molecular weight, tetrameric IgM-like molecule (Voss et al., 1980). Other classes of Ig have not been reported in salmonids, although recent data (Sánchez et al., 1989) suggest the existence of antigenic heterogeneity in trout Ig, similar to the one described in catfish Ig (Lobb et al., 1984; Lobb and Olson, 1988). These studies, using monoclonal antibodies (mAb) to Ig from the channel catfish (*Ictalurus punctatus*), have shown the existence of two classes of light chains and at least four different heavy chains. The purification of trout Ig usually involves anion exchange chromatography on DEAE followed by gel filtration on Agarose 5M (Bio Rad, Richmond, U.S.A.) or Sepharose 4B (Pharmacia, Uppsala, Sweden). This approach is slow and labor-intensive, and results in only partial purification. Immunoaffinity chromatography is a powerful method of purification that involves specific interaction of antigen with an antibody molecule covalently attached to an insoluble support matrix

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(Cuatrecasas and Anfisen, 1971). The use of this method has increased rapidly since the advent of mAb, the use of which has solved problems of low binding capacity, harsh elution conditions and low specificity, frequently found when polyclonal antisera are used. In this report we describe the use of mAb 1G7 for the isolation of pure salmonid Ig by means of immunoaffinity chromatography.

MATERIALS AND METHODS

Production of monoclonal antibodies

Female BALB/c mice were injected intraperitoneally (i.p.) with 20 μg trout Ig emulsified in complete Freund's adjuvant. The Ig was purified by ion-exchange chromatography on Trisacryl M DEAE (LKB, Sweden) followed by gel filtration on Sephacryl 300 (Pharmacia, Sweden), as previously described (Sánchez et al., 1989). 25 days later, a second dose of 100 μg trout Ig, emulsified in incomplete Freund's adjuvant, was given i.p. Three days prior to fusion, on day 35, the mice received daily i.p. or intravenous injections of 60 μg trout Ig, according to the method of Stahli et al. (1983). The spleen cells were fused with the non-secreting X63Ag8.653 myeloma cell line, using the conventional method (Kohler and Milstein, 1975). Culture supernatants from wells with growing colonies were assayed by an indirect ELISA, as described below. Twenty two hybridomas were selected and cloned at least twice by limiting dilution. The class and subclass of the mAb were determined by the indirect ELISA technique, using rabbit antisera specific for mouse heavy and light chains and a peroxidase-conjugated goat anti-rabbit Ig (Bio-Rad). Ascites fluid was obtained as described previously (Coll, 1987).

Monoclonal antibody detection by enzyme immunoassay

M-129 B ELISA plates (Dynatech, Plochingen, F.R.G.) were coated with 2 μg per well of trout Ig, purified as described above, diluted in PBS and incubated overnight at 4°C. For blocking, the plates were incubated with 0.2 ml per well of 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), for 1 h at 37°C. Culture supernatants diluted in PBS containing 1% BSA, 0.2% Tween 20 and 0.01% merthiolate (dilution buffer) were added, and incubated for 1 h at room temperature. The plates were washed five times with tap water containing 0.1% Tween 20. Subsequently, 100 μl of rabbit anti-mouse Ig conjugated to peroxidase (Sigma), diluted 1/1000 in dilution buffer, was added and incubated for 1 h at room temperature. Finally, after five washes in tap water containing 0.1% Tween 20, 50 μl of the substrate buffer containing 1,2 o-phenylenediamine (2 mg/ml) in 0.1 *M* citrate buffer, pH 5.0, and 0.03% H_2O_2, were added. Colour development was stopped by adding 50 μl of 3 *N* H_2SO_4 after 15 min. The plates were read on a Titertek Multiskan (Flow, Irvine, Great Britain) at 492 nm.

Immunoprecipitation analysis

Purified trout Ig from a pool of 20 individual sera was radioiodinated by the modified chloramine T method (Johnstone and Thorpe, 1982). An aliquot of 10 μl trout Ig solution (1 mg/ml) was mixed with 10 μl of 0.25 *M* phosphate buffer, pH 7.5, and 200 μCi $Na^{125}I$. Ten μl of chloramine T (1 mg/ml) was added and the mixture was incubated at room temperature for 1 min. 500 μl of tyrosine in PBS (0.1 mg/ml) was added and gently mixed. Finally, 500 μl of a solution of 1 mg/ml potassium iodide in PBS containing 0.1% BSA was added, and the mixture was applied to a 30×0.5 cm column of Sephadex G-50 (Pharmacia), which had been pre-equilibrated with PBS–0.5% BSA.

The amount of trout Ig recognized by mAb 1G7 was determined by immunoprecipitation. A 100-μl aliquot containing 100 000 cpm of the radiolabelled trout Ig in PBS–0.01% BSA was incubated with 5 μl of a 1/20 dilution of 1G7 ascites fluid for 1 h at 4°C. Simultaneously, 100 μl of a 10% *w/v* suspension of *Staphylococcus aureus* (Calbiochem, CA) was incubated with rabbit antibodies specific for mouse Ig, (Serolab), diluted 1/10 in PBS–0.01% BSA, for 1 h at 4°C, with occasional mixing. After centrifuging the *S. aureus* cells in an Eppendorf centrifuge for 2 min, the supernatant was removed and the pellet resuspended in the solution containing the radiolabelled trout Ig and the mAb. They were incubated for 1 h at 4°C and, after three washes in PBS–0.5% Nonidet P-40, the total counts immunoprecipitated by the mAb were determined. The amount of trout Ig immunoprecipitated with sheep anti-trout Ig polyclonal antibodies (Seromed, Berlin, Germany) was considered as 100% antigenically active trout Ig. Non-specific precipitation, measured using an irrelevant mAb of the same isotype, was less than 3%.

Quantitation of trout Ig by enzyme immunoassay

M-129 B plates (Dynatech) were coated with 1 μg per well of sheep anti-trout Ig polyclonal antibodies (Seromed) or with 1 μg per well of mAb 1G7 purified by 40% saturated ammonium sulphate precipitation of ascites fluid. Blocking was done with PBS–1% BSA for 1 h at 37°C. 100 μl of the different fractions diluted 5-fold in dilution buffer was added to each well, and incubated for 1 h at room temperature. Unbound trout Ig was removed by five washes with tap water containing 0.1% Tween 20. The second antibody (polyclonal anti-trout Ig or 1G7), labelled with peroxidase according to the periodate method (Nakane, 1979), was added at 1/2000 and 1/100 dilutions in dilution buffer and incubated for 1 h at room temperature. The plates were washed five times in tap water containing 0.1% Tween 20, and the enzymatic reaction was developed as described above.

Immunoaffinity chromatography

The mAb was purified from ascites fluid by 40% saturated ammonium sulphate precipitation followed by two washes of the precipitate in 40% satu-

rated ammonium sulphate. The precipitate was dissolved in coupling buffer (0.1 *M* $NaHCO_3$, pH 8.3, containing 0.5 *M* NaCl) and dialysed against the same buffer. 16 mg of mAb in 5 ml of coupling buffer was mixed with 1 g of CNBr-activated Sepharose 4B (Pharmacia) and the mixture was rotated in an end-over-end apparatus for 3 h at room temperature. Excess ligand was washed away with PBS and the remaining active groups were blocked with 0.2 *M* glycine, pH 8, for 2 h. The gel was washed with PBS followed by 0.05 *M* diethylamine, pH 11, and again with PBS. It was then poured into a column (1.5×3 cm) and stored at 4°C. Trout serum (0.5 ml) was applied to the column, and the unbound fraction was washed away with PBS. Bound Ig was eluted with 0.05 *M* diethylamine, pH 11, and fractions of 1 ml were collected and immediately neutralized with 100 μl 2 *M* Tris–HCl adjusted with HCl to pH 3. The bound and unbound fractions were analysed by a sandwich ELISA for the presence and relative content of trout Ig. The percentage of Ig recovered in the bound fraction was calculated by the following formula: (Ig in the bound fraction)×100/(Ig in the bound fraction+Ig in the unbound fraction).

Purity of the isolated Ig was tested by 5–17% polyacrylamide gradient gel electrophoresis with sodium dodecyl sulphate (SDS–PAGE) and 2-mercaptoethanol (Maizel, 1969). SDS–PAGE high-molecular weight markers were purchased from BRL; they were myosin H-chain (200 000), phosphorylase b (97 400), bovine serum albumin (68 000), ovalbumin (43 000), α-chymotrypsinogen (25 700), β-lactoglobulin (18 400) and lysozyme (14 300).

Immunoblotting analysis

This was performed by the method of Towbin et al. (1979) with slight modifications. Trout serum proteins were resolved by a 5–17% polyacrylamide gradient SDS–PAGE in the presence of 2-mercaptoethanol. The gel was then equilibrated with 20% methanol in 25 m*M* Tris, 192 m*M* glycine (pH 8.3), and the proteins were electrophoretically transferred to a nitrocellulose membrane filter at a constant current of 400 mA for 3 h at 4°C. After transfer, the nitrocellulose membrane was washed with 0.5 *M* NaCl 20 m*M* Tris, pH 7.5 (TBS), dried, and cut into strips. The strips were incubated with TBS containing 2% non-fat dry milk (Molico, Nestlé) for 1 h at 37°C, then incubated in a 1/40 dilution of the ascites fluid in TBS–0.05% Tween 20 containing non-fat dried milk (TTBS), for 1 h at 37°C. After washing in TTBS, the strips were incubated with a 1/1500 dilution of an alkaline phosphatase-conjugated goat anti-mouse Ig (Bio-Rad) in TTBS for 1 h at 37°C. After washing with TTBS, the bands recognized by the mAb were developed by the BCIP technique (Ey and Ashman, 1986).

RESULTS

Of 22 mAb specific for trout Ig, mAb 1G7 was selected for use in affinity chromatography because it precipitated the highest percentage of radioiodinated trout Ig. The amount of Ig recognized by mAb 1G7 in a pool of 20 individual sera was 85% of the radioactive precipitated by polyclonal antisera against trout Ig. This mAb is of the IgG_1, k class, and reacts against the 70-kD heavy chains, as demonstrated in immunoblotting analysis of whole trout serum under reducing conditions (Fig. 1). Three bands at approximately 200 kD and a weak band at 45 kD were also revealed by mAb 1G7.

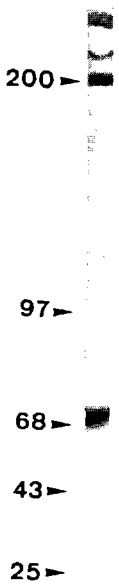

Fig. 1. Western immunoblot of the reaction of mAb 1G7 with trout serum proteins. Trout serum proteins were resolved under reducing conditions by a 5–17% polyacrylamide gradient SDS–PAGE and transferred to a nitrocellulose membrane. Numbers on the left are molecular weight markers in kD.

About 0.8 mg of trout Ig, representing 85% of the total Ig, as determined by ELISA with polyclonal antibodies, was recovered from 0.5 ml of trout serum using the immunoaffinity method (Fig. 2). Residual activity was detected in the unbound fraction with polyclonal antibodies but not with the mAb 1G7 by the ELISA assay. The purity of the isolated Ig was assessed by SDS–PAGE after treatment with 2-mercaptoethanol. Only bands corresponding to heavy and light chains were resolved in the gel when a sample of 50 μg was applied. As the limit of detection using Coomassie brilliant blue for a protein giving a sharp band is near 1 μg, this means than more of 95% of the protein was Ig.

Sera of chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*O. kisutch*), brown trout (*Salmo trutta*), goldfish (*Carassius auratus*), barb (*Barbus barbus*), black seabass (*Micropterus salmoides*) and gilthead bream

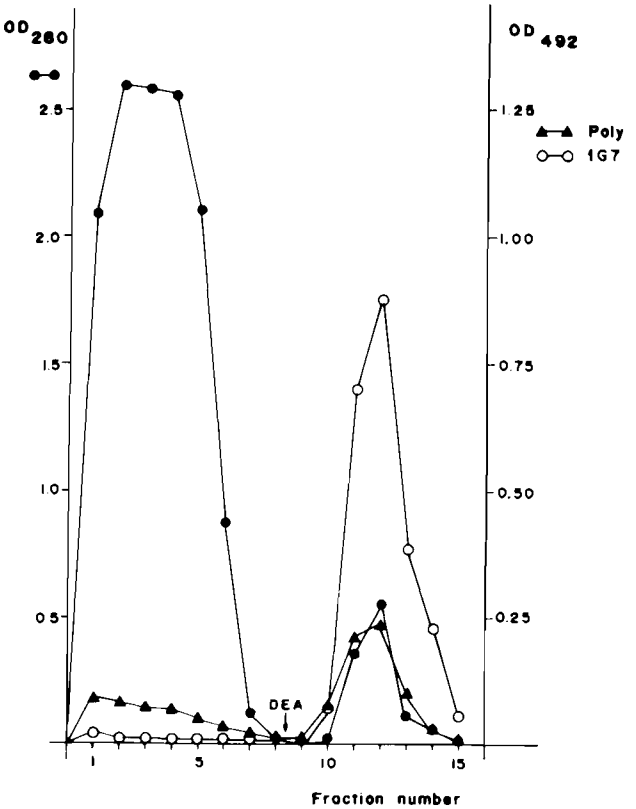

Fig. 2. Stepwise elution of Ig from total steelhead rainbow trout serum from a mAb 1G7-CNBr coupled to Sepharose 4B column. ●-●, OD_{280}; ▲-▲, sandwich ELISA absorbance values (OD_{492}), using the polyclonal anti-trout Ig as second antibody; ○-○, sandwich ELISA absorbance values (OD_{492}), using mAb 1G7 as second antibody. DEA, diethylamine. Fractions of 1 ml were collected.

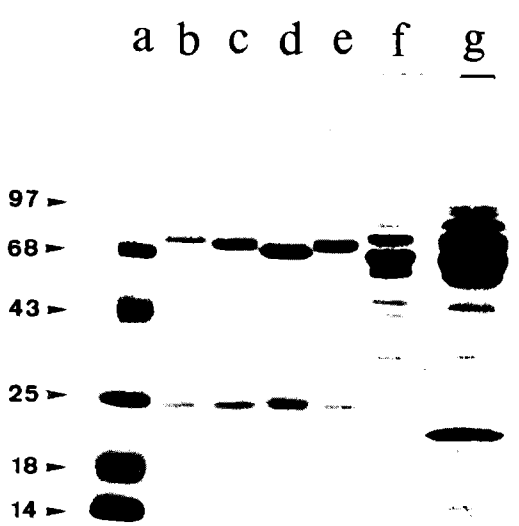

Fig. 3. 5–17% polyacrylamide gradient SDS–PAGE under reducing conditions of salmonid Ig purified by 1G7 affinity chromatography: a, molecular weight markers (kD); b, chinook salmon Ig: c, shasta rainbow trout Ig; d, coho salmon Ig; e, steelhead rainbow trout Ig; f, unbound fraction from steelhead rainbow trout serum; g, total steelhead rainbow trout serum.

(*Sparus aurata*) were screened by the sandwich ELISA technique, using mAb 1G7 both to coat the plates and to develop the ELISA. Only Ig of fishes of the family Salmonidae were recognized by this mAb in the ELISA assay. High-purity Ig from these salmonid species, showing 70-kD H chains and 25-kD L chains, were also easily isolated using the immunoaffinity column (Fig. 3). An additional band at 23 kD was also detected in the chinook salmon preparation.

DISCUSSION

Determination of total Ig levels can be used as a general measure of health in studies on environmental effects, or during diseases. Accurate quantitation requires the use of pure Ig standard preparations, as contaminating serum proteins could lead to erroneously high values. In this report, we describe the use of mAb 1G7 for the isolation of pure trout Ig by means of immunoaffinity chromatography. This method allows the purification of milligram amounts of Ig from small volumes of serum in a short period of time, and to 95% purity.

The mAb 1G7 recognizes an epitope on heavy chains of the trout Ig, as demonstrated by immunoblotting. The bands at approximately 200 kD observed in immunoblotting may be attributable to incompletely dissociated subunits of Ig that accumulate at the top of the gel; the band at 45 kD could

be a degradation product of the H chain, as it sometimes appears in purified Ig samples after long periods of storage at 4°C (data not shown).

In immunoprecipitation, mAb 1G7 binds 85% of total trout Ig, as defined with the polyclonal antisera. This result agrees with the recovery obtained by affinity chromatography. Since individually all the sera studied reacted with this mAb, the 15% of total Ig not recognized by it with respect to the polyclonal antiserum does not seem to be due to allotypic variation. This residual fraction may be explained by the existence of a minor subpopulation of Ig in trout, or alternatively by the presence of non-Ig serum components recognized by the polyclonal antiserum. The existence of different isotypes of Ig has been demonstrated in other teleost fishes (Lobb et al., 1984; Lobb and Olson, 1988). Furthermore, we have recently described another mAb against trout Ig, non-additive with mAb 1G7, which recognizes 15–30% of total Ig (Sánchez et al., 1989), supporting the existence of different Ig populations.

The reactivity of mAb 1G7 with Ig from other salmonids confirms the existence of common determinants among Ig molecules as previously demonstrated with polyclonal antisera (Kobayashi et al., 1982), and makes the mAb 1G7 immunoaffinity method suitable for isolation of these cross-reacting Ig species.

Since two L chains differing in molecular weight have been described in Atlantic salmon using silver-stained gradient gels (10–15%) (Havarstein et al., 1988) it was not surprising to find an additional minor band at 23 kD in the Ig isolated from chinook salmon. On the other hand, in other salmonids, as in rainbow trout, we recently demonstrated the existence of two L chains of 26 kD and 24 kD, that are independently recognized by two mAb, using radioiodinated Ig and 17–20% gradient gels (manuscript in preparation).

Preliminary analysis by ELISA indicates that mucosal Ig is also recognized by mAb 1G7. This method could therefore also be used to isolate Ig from these secretions, where, as they are minor components, it would also work as a concentration procedure.

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