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Anti-immunoglobulin antisera used in an ELISA to detect antibodies in barramundi *Lates calcarifer* to *Cryptocaryon irritans*

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ABSTRACT: Immunoglobulins (Ig) in serum from barramundi vaccinated with bovine serum albumin (BSA) were purified by ammonium sulphate precipitation and affinity chromatography using BSA as the ligand. The BSA-binding activity of eluted putative Ig fractions was assessed by enzyme-linked immunosorbent assay (ELISA) before being pooled and characterised by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Double affinity purification did not improve the purity of the Ig preparation compared to single affinity purification. Barramundi Ig were injected into sheep to produce anti-Ig antisera which were assessed in an indirect ELISA as the secondary antibody to detect serum Ig in barramundi vaccinated with *Cryptocaryon irritans* theronts. Affinity-purified Ig induced a more specific reagent for use as secondary antibody in ELISA than did normal whole-barramundi sera. The heavy (H) chain of barramundi Ig had an apparent molecular weight of 70 kDa while that of the light (L) chain was 27 kDa in SDS-PAGE studies. Under non-reducing conditions 2 putative populations of Ig were identified, at 768 and 210 kDa. The N-terminal sequence of the barramundi Ig H chain showed 78% homology with channel catfish *Ictalurus punctatus* Ig H chain sequence.

KEY WORDS: *Cryptocaryon irritans* · Antibodies · ELISA · Serology · Immunoglobulin · Barramundi · *Lates calcarifer* · Disease · Teleost

INTRODUCTION

Cryptocaryon irritans (Brown, 1951) is a holotrichous ciliate parasite and is the marine equivalent of *Ichthyophthirius multifiliis*, which causes white spot disease. The infective stage of the parasite (theront) penetrates into the epithelium of the skin and gills of marine fishes where it feeds and increases in size over several days, forming visible white spots on the host epithelium (trophont stage). The mature trophonts leave the host, sink to the substrate and encyst to form tomonts. After several divisions the cyst ruptures to release several hundred actively swimming theronts which must find a fish to continue the life cycle. White spot disease kills fish in marine aquaria throughout the world (Nigrelli & Ruggieri 1966, Andrews et al. 1988, Sindermann 1990) and is also of concern in commercial mariculture, affecting a range of cultured species, including brown-spotted grouper *Epinephelus tauvina* (Rasheed 1989); gilt-head sea bream *Sparus aurata* and European sea bass *Dicentrarchus labrax* (Diamant et al. 1991); grouper *Epinephelus malabaricus* and seabass *Lates calcarifer* (Leong & Wong 1990); and snapper *Lutjanus johni* (Leong 1992).

Fish can acquire resistance to *Ichthyophthirius multifiliis*, the cause of white spot disease in freshwater fishes. Hines & Spira (1974) found that mirror carp *Cyprinus carpio* were refractory to re-infection following exposure to sublethal doses of *I. multifiliis*, while Clark et al. (1987) demonstrated similar responses from channel catfish *Ictalurus punctatus*. Colorni (1987) noted that fish that had survived several infections of *Cryptocaryon irritans* acquired a certain degree of immunity, indicating it may be possible to protect fish by vaccination.

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The present investigation was part of a larger study conducted to investigate the humoral immune responses of barramundi *Lates calcarifer* following exposure to *Cryptocaryon irritans* antigens. This research necessitated the production of anti-barramundi antiserum for use in enzyme-linked immunosorbent assay (ELISA), as this reagent was not available commercially. Barramundi were chosen as an experimental host because this species is important in commercial aquaculture in Australia, is affected by white spot disease in Southeast Asian aquaculture (Seng 1987, Leong & Wong 1990), and is euryhaline and because hatchery raised fish are readily available for experimental purposes.

MATERIALS AND METHODS

***Cryptocaryon irritans* propagation and collection of theronts.** An isolate of *C. irritans* was obtained from infected bream *Acanthopagrus australis* caught in the Brisbane River, Queensland, Australia, then propagated by serial passage using naive barramundi. Tomonts were collected from tanks of infected fish, cleaned with several washes of filtered seawater (FSW) and incubated at 28 to 30°C in FSW at 30‰ salinity. Theronts to be used as antigen were collected after excystment from the tomonts, cleaned with FSW, sonicated and assayed for protein concentration by a modified Lowry assay (Markwell et al. 1978).

Immunisation of barramundi. Barramundi (30 to 35 cm total length), kept at 27 to 29°C, were immunised by intraperitoneal (IP) injection of 0.1 ml doses of vaccine given at least 3 wk apart. Four fish were injected with a total of 1.25 mg of bovine serum albumin (BSA) from 4 injections and 3 fish were injected with about 735 µg of sonicated *Cryptocaryon irritans* theronts from 3 injections. The primary vaccines were emulsified 1:1 with Freund's complete adjuvant (FCA) and the 2 booster injections were prepared by emulsifying antigens 1:1 with Freund's incomplete adjuvant (FIA). Fish were bled periodically from the caudal vein, blood was allowed to clot overnight at 4°C, then centrifuged at $1000 \times g$ for 10 min and serum was stored at -20°C.

Immunisation of sheep. Merino-cross sheep less than 1 yr old were given 3 intramuscular (IM) injections over 6 wk of 2 ml doses of vaccine containing either normal whole-barramundi serum or purified barramundi immunoglobulins (Ig) mixed with Quil A at 1 mg ml^{-1} in phosphate buffered saline (PBS), pH 7.2. Two sheep were each injected with a total of 650 µg of normal barramundi serum and 1 sheep was injected with a total of 400 µg of purified barramundi Ig. Sheep were bled from the jugular vein; blood was allowed to clot as before, then was centrifuged as before and serum was stored at -20°C.

Purification of barramundi Ig. Ig was precipitated at room temperature (RT) from 2 ml of both normal barramundi serum and serum from barramundi injected with BSA by slow dropwise addition of saturated ammonium sulphate, while constantly stirred, to a final concentration of 50% v/v. The precipitate was separated from the suspension by centrifugation at $10\,000 \times g$ for 10 min, then the pellet was resuspended in 2 ml 0.1 M phosphate buffer. The precipitation procedure was repeated twice, after which the final resuspended precipitate was dialysed at 4°C against 0.1 M phosphate buffer then stored at -20°C.

Affinity chromatography columns were prepared by coupling BSA (10 mg ml^{-1} of gel) to 10 ml of CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions and equilibrated with 0.1 M phosphate, 0.15 M NaCl buffer, pH 7.4.

Ammonium sulphate purified Ig from 2.4 ml total volume of either normal barramundi serum, used as a control or barramundi anti-BSA serum were incubated on the column for 1 h at RT. Bound material was eluted with 0.1 M glycine buffer, pH 2.8 and fractions were neutralised with 2.75 M Tris. The protein concentration was determined by absorbance at 280 nm and then fractions were buffer exchanged to 0.15 M PBS using Microcon microconcentrators (Amicon) and stored at -20°C.

Affinity-purified Ig from barramundi anti-BSA serum were subjected to a second affinity purification procedure using the method described above and final concentrations of protein in all purified samples were estimated using the modified Lowry assay mentioned previously.

ELISA. A standard protocol for ELISA was followed after optimisation of the concentrations of antigen, test sera, secondary antibody and conjugate. Typically 1 or 2 of the reagents were titrated on the ELISA plates using doubling dilutions while the other reagents were kept at a selected concentration. From plots of optical density (OD) versus dilution of reagents, optimal concentrations were selected so that antigen, anti-barramundi antisera and conjugate were not limiting and there was at least a 3-fold difference in OD between immunised and non-immunised fish sera.

BSA and sonicated *Cryptocaryon irritans* theront antigens were diluted in 60 mM carbonate buffer, pH 9.6, and coated on to polystyrene 96-well plates (Disposable Products Cat. no. 23148) overnight at 4°C, with 100 µl volumes of diluted antigen added to each well. Plates were blocked with 1% w/v gelatin in carbonate buffer, 150 µl $well^{-1}$, and incubated at 37°C for 1 h. After washing 3 times with 0.15 M PBS with 0.05% v/v Tween 20 (PBS-T), test samples of fish sera

diluted in PBS-T were added, 100 µl well^{-1}, to the plates and incubated as before. Plates were washed and fish antibodies were detected with either the sheep anti-barramundi serum or the sheep anti-barramundi Ig serum, diluted in PBS-T, and then incubated as before. Plates were washed and rabbit anti-sheep IgG conjugated to horseradish peroxidase (Cappel), diluted in PBS-T, was added and incubated as before. Plates were washed and 2,2'azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) chromogen in 50 mM citrate buffer, pH 4.0, 0.1 mM hydrogen peroxide was added. Plates were read at 405 nm, when OD values for positive control wells reached 0.6, on either a Titretek Multiscan (Flow Laboratories) or a Biorad Microplate reader (Model 3550) linked to Microplate Manager III (Biorad).

SDS-PAGE. Proteins in normal barramundi serum and single and double affinity-purified barramundi Ig preparations were separated, according to Laemmli (1970), in a 10% reducing sodium dodecyl sulphate polyacrylamide gel, containing 37.5:1 acrylamide:bis-acrylamide. After electrophoresis, the gel was stained with Coomassie brilliant blue (CBB). The apparent molecular weights (MW) were estimated from MW standards (Low Range Biorad) and purified Ig from mullet *Chelon labrosus* (gift from Peter Burgess) which were run concurrently.

Affinity-purified barramundi Ig were analysed under non-reducing conditions in a 3% gel, with samples diluted in buffer that did not contain β-mercaptoethanol. After electrophoresis, the gel was stained with B/T Blu (BT Scientific Technologies, San Diego). The apparent MW of unreduced Ig and subunits were estimated from the following standards run concurrently: human IgM (Sigma), bovine thyroglobulin (Sigma) and bovine IgG (Sigma).

N-terminal sequencing. Proteins in single affinity-purified barramundi Ig were separated on a 10% reducing gel as described above except that the running buffer also contained 0.1 mM thioglycolate. Separated proteins were electrophoretically transferred to 0.2 µm PVDF membrane (Biorad), which was then stained with CBB. Stained bands were cut from the membrane, sequenced using an ABI 473A pulsed liquid protein sequenator and then compared to sequence data from GenBank and SWISS-PROT databases using the Australian National Genomic Information Service (ANGIS) FastA program.

RESULTS

Calibration of ELISA and response of fish to immunisation

Optimisation of ELISA by titration of reagents led to the selection of the following concentrations: BSA antigen coated to plates at 5 µg ml^{-1}; barramundi anti-BSA sera diluted 1:200; sonicated *Cryptocaryon irritans* theront antigen coated to plates at 15 µg ml^{-1}; barramundi anti-*C. irritans* theront sera diluted 1:400; sheep antiserum against normal barramundi serum and sheep antiserum against purified barramundi Ig diluted 1:3200; and HRP-conjugated rabbit anti-sheep serum diluted 1:1600.

The 4 barramundi injected with BSA had variable responses to immunisation, with 1 fish showing a minimal response (Fig. 1). Peak responses were at Week 8, 2 wk after the third injection. Serum samples from the most responsive fish, L6, which had OD levels twice that of the second most responsive fish, were pooled for purification of barramundi Ig.

Affinity chromatography of precipitated barramundi Ig

Ammonium sulphate precipitation reduced the total serum protein from normal and immune barramundi serum by 42% and 47–57% respectively.

Ig precipitated from normal barramundi serum did not bind to the BSA column, while Ig from vaccinated fish purified either once or twice by affinity chromatography were eluted from the column after application of glycine buffer. Anti-BSA Ig activity was

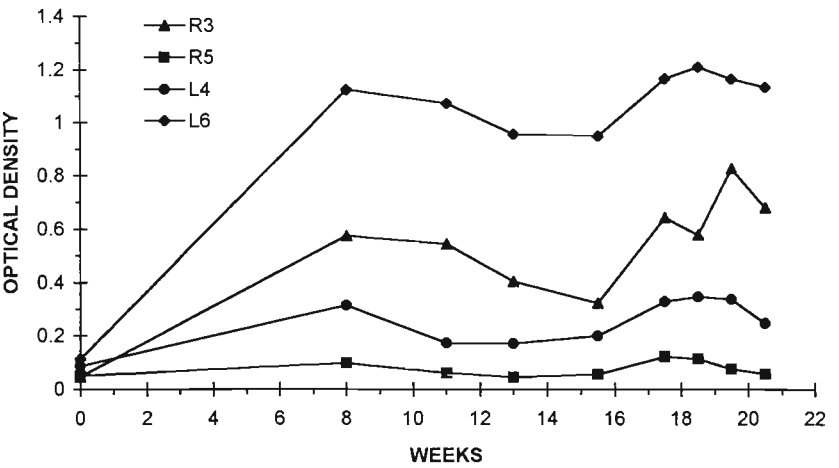

Fig. 1. *Lates calcarifer*. Humoral antibody responses, measured by ELISA against bovine serum albumin (BSA), from 4 barramundi injected with BSA. Fish were injected at Weeks 0, 3, 6 and 15.5 so that each fish received a total of 1.25 mg of BSA. Pre-bleed (PB) sera samples were taken before vaccination and immune-bleed (IB) sera samples were taken at intervals after the third injection

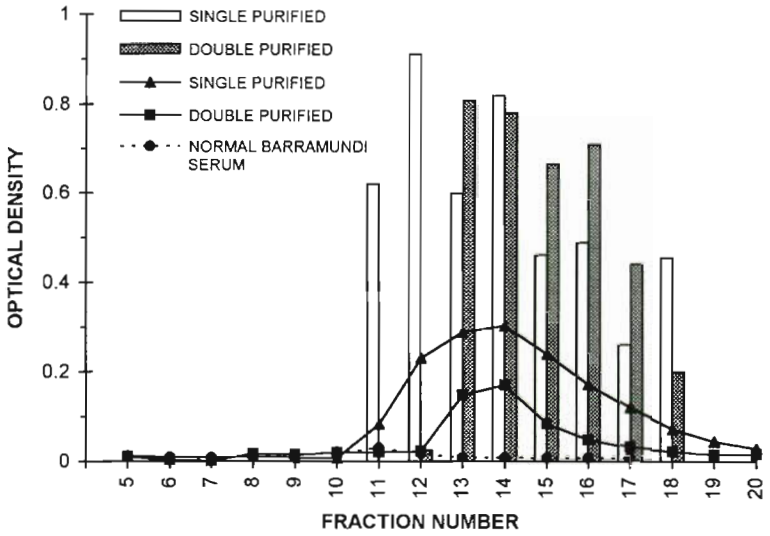

Fig. 2. Protein levels (optical density [OD] at 280 nm) in fractions eluted from an affinity chromatography column, with BSA as the ligand, following single affinity purification of normal barramundi serum, single affinity purification of barramundi anti-BSA serum and double affinity purification of barramundi anti-BSA serum. Columns represent anti-BSA immunoglobulin levels, measured by ELISA (OD at 405 nm), in eluted fractions of single affinity-purified barramundi anti-BSA serum and double affinity-purified barramundi anti-BSA serum corresponding to the protein profiles related by ELISA to these protein profiles (Fig. 2). The highest yield of purified barramundi Ig was obtained from eluted fractions of single affinity-purified antibodies which when pooled had a protein concentration of 140 µg ml^{-1} for a total volume of 15.6 ml. The yield of Ig was 2184 µg from the 2 ml sample of barramundi anti-BSA sera.

SDS-PAGE

Normal barramundi serum had >10 protein bands between 14.4 and 97.4 kDa and 4 bands >97.4 kDa (Fig. 3). In contrast, affinity-purified (both single and double) barramundi Ig loaded in high concentration had only 2 major bands at approximately 70 and 27 kDa. Resolution of the 70 kDa band indicated the possibility of several components. There was little difference in apparent purity between single and double affinity-purified samples. The purified mullet Ig, run concurrently, had 2 major bands at approximately the same MW as the purified barramundi immunoglobulin, but was of lower concentration.

Under non-reducing conditions a sample of affinity-purified barramundi Ig had 2 major bands (Fig. 4). From the calibration curve prepared from the relative mobilities of the controls that were run concurrently, the MW of these bands were estimated to be 768 and 210 kDa.

N-terminal sequencing

The putative barramundi Ig heavy (H) chain, corresponding to the major band of approximately 70 kDa on reducing SDS-PAGE, had a sequence of 'EQLTPASVTVQPG'. When compared with other reported protein sequences, it was found to have 78.6% identity in a 14 amino acid overlap with channel catfish *Ictalurus punctatus* Ig H chain V region, 78.6% identity in a 14 amino acid overlap with *I. punctatus* Ig H chain mRNA V region and 76.9% identity in a 13 amino acid overlap with human Ig rearranged light (L)-chain mRNA V region (Table 1).

Assessment of two sheep antisera as marker antibodies in ELISA

Sheep anti-barramundi Ig serum had greater specificity than sheep anti-barramundi whole serum. OD levels for pre-immunisation bleed (PB) sera from 3 barramundi injected with *Cryptocaryon irritans* theronts were 0.243, 0.22 and 0.182 while those for post-immunisation bleed (IB) sera were 0.54, 0.42 and 0.82 when sheep anti-barramundi

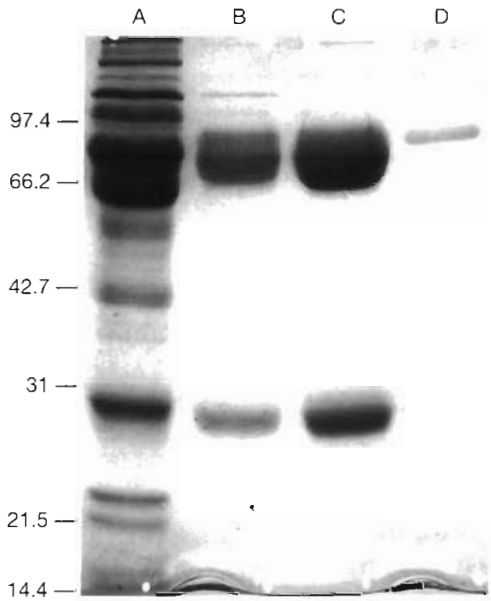

Fig. 3. SDS-PAGE analysis in a 10% gel under reducing conditions comparing normal barramundi serum (lane A), single affinity-purified barramundi immunoglobulin (lane B), double affinity-purified barramundi immunoglobulin (lane C) and purified mullet immunoglobulin (lane D). The gel was stained with Coomassie brilliant blue (CBB)

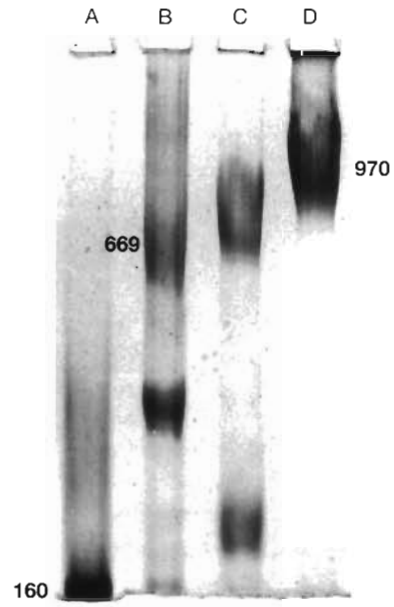

Fig. 4. SDS-PAGE analysis in a 3% gel under non-reducing conditions comparing bovine IgG, approx. molecular weight 160 kDa (lane A); bovine thyroglobulin, approx. molecular weight 669 kDa (lane B); affinity-purified barramundi immunoglobulin (lane C); and human IgM, approx. molecular weight 970 kDa (lane D). The gel was stained with B/T Blu

Ig serum was used as the secondary antibody. The corresponding OD levels for the same fish sera when sheep anti-barramundi whole serum was used were 0.432, 0.447 and 0.36 for PB sera and 0.557, 0.49 and 0.763 for IB sera. The mean difference in OD between pre- and post-immunisation sera was increased 2-fold for the 3 fish injected with *C. irritans* theronts. Similarly, for the most responsive fish injected with BSA the OD level for IB serum was increased by approximately 0.36 when the sheep anti-barramundi Ig antisera was used (Fig. 5).

Table 1. Comparison of N-terminal amino acid sequence of affinity-purified barramundi Ig heavy (H) chain with protein sequences sourced through the Australian National Genomic Information Service (ANGIS). B28835: channel catfish *Ictalurus punctatus* Ig H chain V region (3D11) (21 aa); ICTIGHVJ_1: *I. punctatus* Ig H chain mRNA V region, cl (132 aa); HUMIG1L_1: *Homo sapiens* Ig rearranged light (L) chain mRNA V region (106 aa).

		10
Barra Ig	EQLT-PASVTVQPG	
B28835	EELTQPASMTVQPGQSTNDST	
	10	20
		10
Barra Ig	EQLT-PASVTVQPG	
ICTIGHVJ_1	LAAASYVHGEELTQPASMTVQPGQSLS	
10	20	30
		10
Barra Ig	EQLT-PASVTVQPG	
HUMIG1L_1	QSQLTQPASVSVSPGQTASITCSGD	
	10	20

Comparison of humoral responses of fish injected with BSA and with *Cryptocaryon irritans* theronts

The humoral Ig reponses from both groups of vaccinated fish were monitored over 15.5 wk for 4 fish injected with BSA and 17 wk for 3 fish injected with *Cryptocaryon irritans* theronts. All sera were diluted 1:400 and analysed on the same ELISA with the same positive and negative controls. The responses from both groups of injected fish showed peaks at 8 wk (2 wk after the third injections) with gradual declines

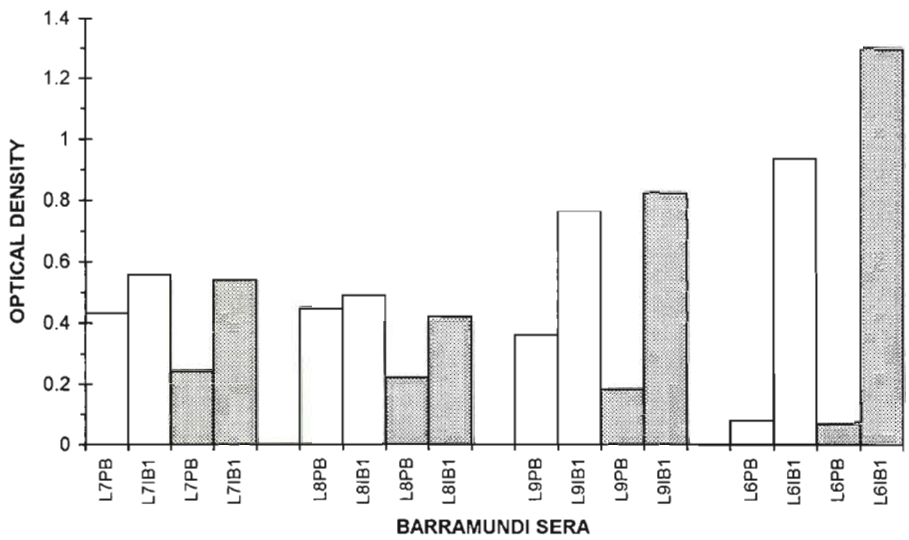

Fig. 5. *Lates calcarifer.* Humoral antibody levels, measured by ELISA against sonicated *Cryptocaryon irritans* theront antigen and BSA respectively, in the sera of each of 3 barramundi (L7, L8 and L9) injected with a total of 735 µg of sonicated *C. irritans* theront protein and of 1 barramundi (L6) injected with a total of 1 mg of BSA at 3 vaccinations 3 wk apart. Pre-bleed (PB) sera samples were taken before vaccination and immune-bleed (IB1) sera samples were taken 2 wk after the third injection. Fish antibodies were detected with antisera from sheep injected with normal barramundi serum (open bars) and sheep injected with affinity-purified barramundi immunoglobulins (shaded bars)

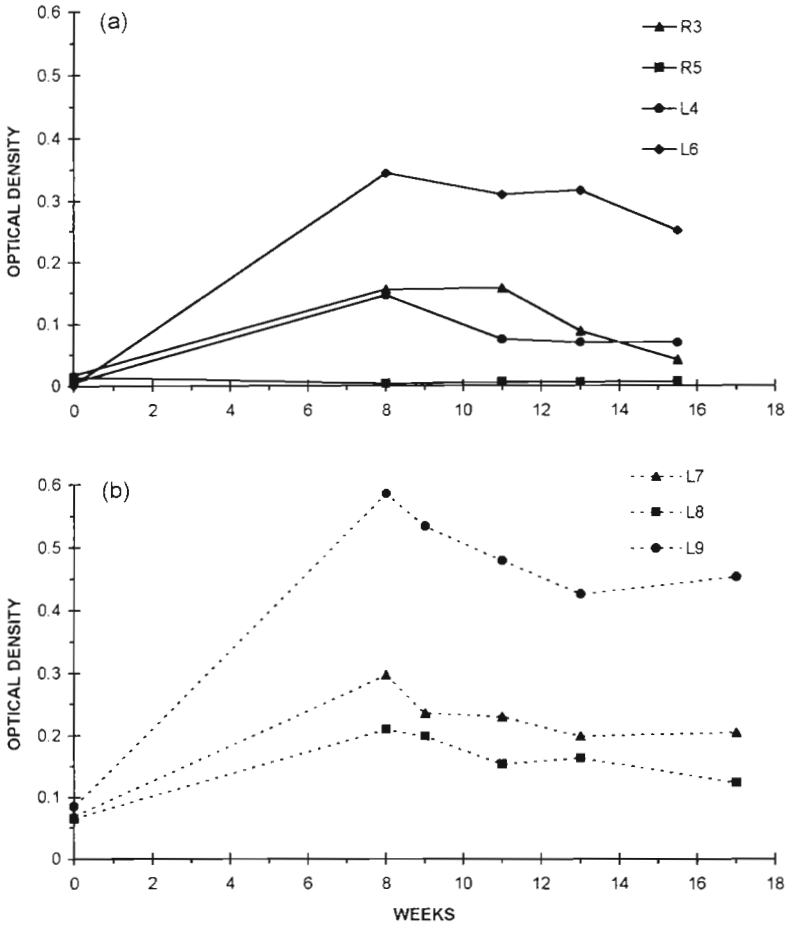

Fig. 6. Humoral antibody levels, measured by ELISA against (a) BSA and (b) sonicated *Cryptocaryon irritans* theront antigen, from the sera of 4 barramundi injected with a total of 1 mg of BSA and 3 barramundi injected with a total of 735 µg of sonicated *C. irritans* theront protein at 3 vaccinations 3 wk apart (Weeks 0, 3 and 6). Pre-bleed (PB) sera samples were taken before vaccination and immune-bleed (IB) sera samples were taken at intervals from 2 wk after the third injection

thereafter (Fig. 6). Both groups showed variations in response, with 1 fish injected with BSA being a non-responder

DISCUSSION

Almost half of the irrelevant serum proteins in immune barramundi sera were removed by ammonium sulphate precipitation before the anti-BSA Ig were purified by affinity chromatography. The yield of approximately 2 mg of purified Ig from just 2 ml of immune barramundi sera demonstrates the utility of this protocol. Ellis (1989) reported the concentration of Ig in teleost serum to generally be 2–7 mg ml^{-1} (6–15% of total serum protein); this would mean the affinity purification recovered 16–55% of the Ig.

The reduction in background levels on ELISA when using antisera from sheep injected with purified barramundi Ig compared to sheep injected with normal barramundi serum highlights the need to purify immunoglobulins in order to produce specific antisera for use as markers in ELISA. Similar results were reported by Whittington & Speare (1996) for antisera produced by rabbits to cane toad serum and purified cane toad immunoglobulin. From work to evaluate non-specific reactivity in the ELISA (authors' unpubl. data) it was concluded that barramundi serum components, including non-immunoglobulins, were binding non-specifically to *Cryptocaryon irritans* antigen and were thus being detected by the sheep anti-barramundi whole serum.

SDS-PAGE analysis of normal barramundi serum and of affinity-purified barramundi anti-BSA Ig showed that the protocol followed was very effective in purifying barramundi Ig. Double affinity chromatography did not appreciably improve the purity of the sample. The MW of the 2 principal bands present in purified barramundi Ig were indicative of putative H and L chains of IgM, which is the predominant immunoglobulin in the serum of teleost fish. Apart from MW, the intensity of staining was consistent with a characteristic of Ig in that the H chains, being bigger than L chains, take up more stain. Lobb & Clem (1983) characterised channel catfish *Ictalurus punctatus* Ig with H and L chains of 70 and 23 kDa, respectively. Havarstein et al. (1988) reported H and L chains of 72 and 27 kDa for Atlantic salmon *Salmo salar*, and Pilstrom & Petersson (1991) reported H and L chains of 81 and 27.5 kDa for Atlantic cod *Gadus morhua*. Similarly, Navarro et al. (1993) reported H and L chains of 70 and 25 kDa, respectively, for gilthead sea bream *Sparus aurata*; Whittington (1993) reported H and L chains of 72 and 28 kDa, respectively, for European perch *Perca fluviatilis*, and Estevez et al. (1994) reported H and L chains of 78 and 27 kDa for turbot *Scophthalmus maximus*.

Although the high concentration of protein may have contributed to broad diffuse banding on SDS-PAGE, there was a suggestion that the H and L chain bands were not homogenous populations. The broad bands in the H and L regions (Fig. 3) may indicate variations in migration of possible subclasses of antibodies. Isotypes of H chains have been reported in serum from sheeps-

head *Archosargus probatocephalus* (Lobb & Clem 1981); channel catfish (Lobb & Olson 1988); rainbow trout *Salmo gairdneri* (Sanchez et al. 1989); and European perch (Whittington 1993) Similarly, isotypes of L chains have been reported in channel catfish (Lobb et al. 1984) and rainbow trout (Sanchez et al. 1989, Sanchez & Dominguez 1991), and Whittington (1993) suggested this may also be the case for European perch.

Under non-reducing conditions the affinity-purified barramundi Ig were shown to consist of 2 distinct populations. The high MW (HMW) band, being of slightly lower MW than the pentameric human IgM, suggests a tetrameric structure which is characteristic of teleost IgM. The low MW (LMW) band suggests a monomeric structure slightly larger than bovine IgG, and as the MW is greater than a quarter of the HMW molecule, it may represent a unique Ig population and not just a dissociated portion of the tetramer. Something that must be borne in mind, however, is the difficulty of accurate MW estimation. Warr (1982) reported anomalies in the migration of standards of MW less than 100 kDa in non-reducing SDS-PAGE, and Butler (1983) also reported anomalous migration of Ig subunits in gels. Whittington (1993) observed that a proportion of HMW Ig from European perch may consist of tetramers formed by 4 non-covalently linked subunits which dissociate under conditions that would not cause dissociation of mammalian IgM. To test that this was also the case for barramundi Ig the LMW band would need to be analysed under reducing conditions looking for differences in MW of H chains compared to the HMW Ig H chains, which would indicate an Ig population unique from the HMW Ig.

The N-terminal sequencing results for the H chain band, to our knowledge the first reported sequence for barramundi immunoglobulin, reinforce the data from SDS-PAGE and confirm that the purified product is indeed immunoglobulin.

We found a wide range of responses among 4 fish immunised with BSA, with 1 fish showing a minimal response, while the most responsive fish had detectable antibody levels twice that of the second most responsive fish. Previously, Hodgins et al. (1967) and Avtalion et al. (1980) had concluded that BSA was a poor immunogen in rainbow trout and carp. Also, Whittington et al. (1994) found that a similar soluble antigen, ovalbumin, when emulsified with FCA and injected IP into rainbow trout appeared to be an unreliable immunogen. They reported inconsistent antibody responses to ovalbumin, with a longer induction phase compared with the response of fish injected with *Vibrio anguillarum*.

When sera from both vaccinated groups of barramundi were analysed on the same ELISA, we found that the 3 fish immunised with *Cryptocaryon irritans* theronts had responses similar to those from the 4 BSA-injected fish, with similar kinetics for the 2 antigens. The responses to both antigens had peaked by 2 wk after the third injections, after which responses from both groups decreased gradually, consistent with the results of Ellis (1989), who noted that the antibody titres in fish, compared to mammals, reach a plateau 20 to 30 d after antigen stimulation and are maintained for longer peroids. As the *C. irritans* antigen would consist of a mixture of lipids, carbohydrates and proteins in particulate and soluble forms, the amount of any 1 antigen in the mix would be far less than the amount of BSA given to the other group of fish, so it could be argued that the *C. irritans* antigen was the better of the two. Indeed, the BSA may be poorly immunogenic in barramundi, as ovalbumin was found to be in other fish, with the response augmented by an adjuvant effect. Further work is necessary to understand how fish metabolise and respond immunologically to foreign soluble proteins injected with adjuvants in order to establish effective vaccination programs.

The polyclonal anti-barramundi Ig antisera raised in sheep will enable further study of the humoral immune responses in barramundi to *Cryptocaryon irritans* antigens following successive exposure to the parasites and comparison of responses with those of vaccinated fish. If protection against the parasite can be provided by vaccination, commercial applications developed from this study could prevent debilitating outbreaks of cryptocaryoniasis in aquaculture facilities.

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