Bull. Eur. Ass. Fish Pathol., 20(5) 2000, 174

Elution of snapper, *Pagrus auratus* (Bloch and Schneider) Ig from a protein A affinity chromatography column yields contamination from the binding ligand

Richard N. Morrison and Barbara F. Nowak

School of Aquaculture and CRC for Aquaculture, University of Tasmania, Tasmanian Aquaculture and Fisheries Institute, Locked Bag 1-370, Launceston, Tasmania, Australia, 7250.

Abstract

Staphylococcal protein A (SpA) affinity chromatography was used to single step purify immunoglobulins (Ig) from snapper (*Pagrus auratus*) serum. Elution of Ig was successful, with analysis of the eluent using SDS-PAGE under fully reducing conditions and PAGE under native conditions revealing that the product was of high purity. Polyclonal antisera to SpA purified snapper Ig was produced in rabbits. Probing of reduced purified Ig with the rabbit-anti-snapper Ig antisera in a Western blot demonstrated that the antisera were most reactive with the heavy (H) chains but also with an unknown protein of approximately 65 kDa in molecular weight. Non-immune rabbit serum (pre-bleed) was not reactive with the H chains of the reduced Ig however, reaction with the unknown protein (65 kDa) occurred. Further, probing of purified Ig in a Western blot with a suite of control sera and antisera demonstrated that the unknown protein was reactive with all mammalian sera and antisera tested. SpA affinity chromatography without prior application of snapper serum was performed. Eluent was probed in a Western blot by rabbit-anti-snapper Ig antisera and non-immune rabbit sera (pre-bleed) once more and were also reactive. In addition, elution buffer pH (3-5) had no effect on the elution of the unknown protein. In contrast, only H and light (L) chains of reduced snapper serum were positive when probed with the antisera. It was concluded that due to the non-specific binding in the Western blot the unknown protein was SpA contamination.

Introduction

Purification of teleost systemic Ig has been performed using a number of methods including ion exchange chromatography (Lobb and Clem, 1981; Sanchez *et al*., 1989), gel filtration (Glynn and Pulsford, 1990; Israelsson *et al*., 1991; Bourmaud *et al*., 1995), ammonium sulphate precipitation (Pilström and Peterson, 1991), immuno-affinity chromatography (Bryant *et al*., 1999; Palenzuela *et al*., 1996) and SpA affinity chromatography (Suzuki *et al*., 1990; Estévez *et al.* 1993; Estévez *et al.* 1994; Scapigliati *et al*., 1996). SpA purification of teleost Ig was first described by Zikan *et al*., (1980) and is a simple one step purification method. SpA has been shown to possess two binding receptors and up to four binding sites, to which mammalian IgG binds via the C_H2-C_H3 domains of the Fc region and IgM via the Fab region respectively (Inganäs, 1981; Ljungberg *et al*., 1993). However, it remains unknown as to how the teleost Ig binds to SpA. Here, we aimed to purify snapper (*P. auratus*) Ig using SpA affinity chromatogra-

phy in order to inoculate rabbits and mice for polyclonal and monoclonal antibody production respectively.

Materials and Methods

Purification of Ig

Ig was single step purified using an SpA affinity chromatography kit (Bio-Rad; 732-2020) (Suzuki *et al.*, 1990). The column was prepacked with 2 ml of affi-gel® SpA coupled to agarose beads. Column operation was as specified by the manufacturer with modifications. Briefly, 3 ml of serum was prepared using a desalting column previously equilibrated with 20 ml of binding buffer (pH 9.0). An SpA column was equilibrated with 10 ml of binding buffer and the prepared sample applied to the column 5 times to maximise yield of Ig. The column was washed with 20 ml of binding buffer with the effluent absorbance (A_{280}) monitored to prevent contamination. Protein was eluted with 10 ml of elution buffer (pH 3.0) with absorbance (A_{280}) of 1 ml fractions analysed. Fractions with highest absorbance were pooled and desalted using a desalting column equilibrated with sterile 0.1 M phosphate buffered saline (pH 7.4). Control eluent was collected using the technique described without the addition of snapper serum to the protein A column. In this case elution buffer pH was 3.0, 3.5, 4.0, 4.5 or 5.0.

Native PAGE and Reducing SDS-PAGE

Snapper Ig purity was determined by polyacrylamide gel electrophoresis under both reducing (SDS-PAGE) conditions and native (PAGE) conditions (Laemmli, 1970). Samples were applied to the wells and electrophoresed using an SE 250 Mighty Small II vertical electrophoresis unit. Gels were then silver stained (Harlow and Lane, 1988). Low molecular weight standards (Novex and Bio-Rad) and high molecular weight standards (Pharmacia) including human IgM (Sigma) were included on gels under reducing and native conditions respectively. Molecular weights were estimated as described by Bollag *et al.*, (1996).

Western Blotting

SpA purified Ig, snapper serum or control eluent were electrophoresed under reducing conditions as described and electrotransferred (SemiPhor TE70; Hoefer) to 0.45 mm nitrocellulose membrane (Pharmacia Biotech) under semi-dry conditions (Bjerrum and Schafer-Nielsen, 1986). Membranes were washed with tris buffered saline (TBS; pH 7.5) and blocked with 1% non-fat skimmed milk ($w.v^{-1}$) in TBS (SM-TBS) for 1 hour at room temperature (RT). Membranes were then washed with TBS, tween-TBS (0.05%, $v.v^{-1}$) and TBS and probed with rabbit anti-snapper Ig serum diluted in SM-TBS (1:500) for 1.5 hours at RT. Again, membranes were washed as described and incubated in conjugated goat anti-rabbit HRP (Sigma) diluted 1:1000 in SM-TBS for 1.5 hours at RT. After another washing cycle, positive bands were visualised using diaminobenzidine and urea-peroxide (Sigma) dissolved in sterile distilled water. Control probing of transferred reduced Ig included;

(1) Omitting the primary antiserum,
(2) Omitting the secondary antiserum,
(3) Omitting both primary and secondary antisera,
(4) Substituting the primary antiserum with control rabbit serum (pre-bleed) of rabbits immunised with snapper Ig,
(5) Substituting the primary antiserum with

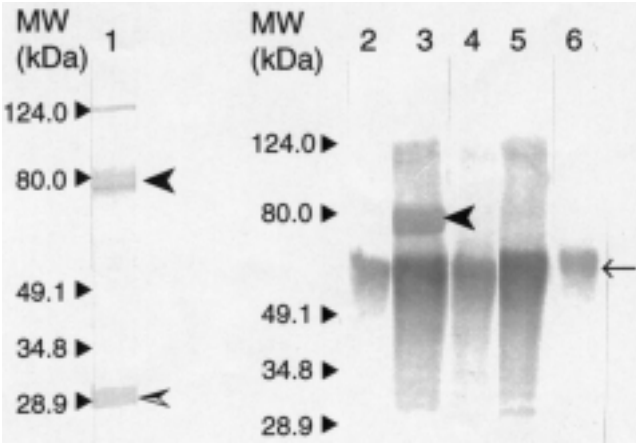

Figure 1. Western blots of snapper serum and SpA purified snapper Ig showing heavy chains (solid arrowhead) and light chains (open arrowhead) and putative SpA contamination (arrow). Lanes:(1) Snapper serum probed with polyclonal rabbit anti snapper Ig(1:500). (2) SpA purified snapper Ig probed with polyclonal rabbit anti-snapper Ig (1:500). (3) SpA purified snapper Ig probed with pre-bleed rabbit serum (anti-barramundi Ig, 1:500). (4) SpA purified snapper Ig probed with pre-bleed rabbit serum (anti-barramundi Ig, 1:500). (5) SpA purified snapper Ig probed with polyclonal rabbit anti barramundi Ig (1:500). (6) SpA purified snapper Ig probed with no primary antisera.

control rabbit serum (pre-bleed) of rabbits immunised with barramundi (*Lates calcarifer*) Ig, and

(6) Substituting the primary antiserum with rabbit anti-barramundi (*L. calcarifer*) Ig antiserum.

Results

Snapper Ig were successfully purified from serum using an SpA affinity chromatography column. Analysis of the purified Ig by SDS-PAGE under reducing conditions showed that two H and two L chain variants were present with minimal contamination from serum proteins. Probing of reduced Ig in Western blot using rabbit anti-snapper Ig polyclonal antisera elicited a positive reaction with the H chains as well as an unknown protein of approximately 65 kDa (Figure 1). The pre-bleed rabbit serum did not react with the H chain but, was reactive with a similar unknown protein to that described. A battery of control sera and antisera including polyclonal antisera from an independent project were used to probe the purified Ig with the unknown protein positive on all occasions when mammalian sera or antisera were added (Figure 1). Further, a controlled elution of the protein A column (without prior application of snapper serum) was performed (pH 3-5), with the unknown protein again present in Western blots when both pre-bleed and immunised rabbit sera were used as probes (Figure 2).

Discussion

Successful elution of snapper Ig from an SpA affinity chromatography column was demonstrated by SDS-PAGE analysis of the eluted product. However, in subsequent Western blot analyses, a contaminant that appeared to be SpA from the agarose-SpA affinity chromatography column was identified. Since the contaminant was used as part of an inoculating emulsion for production of polyclonal

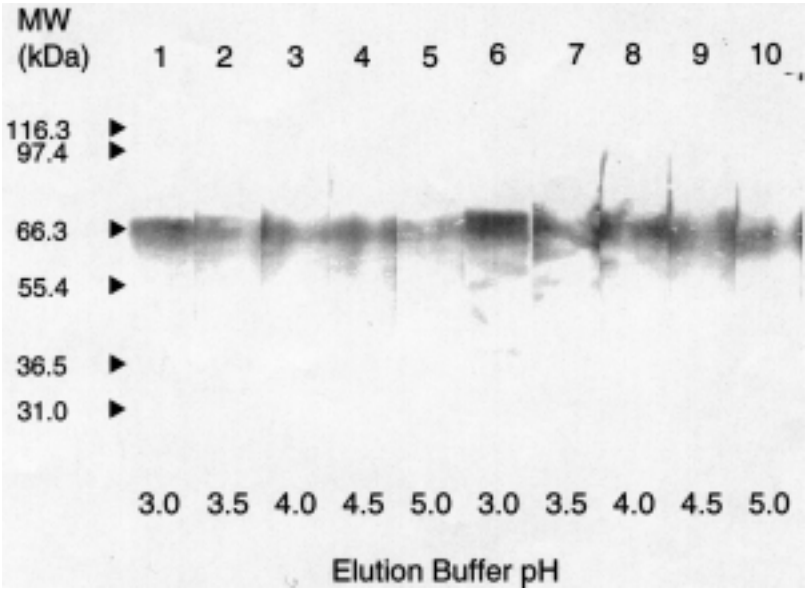

Figure 2. Western blot analysis of control eluent (without prior application of snapper serum to column), using non-immune rabbit serum (Lanes 1-5)(anti-snapper Ig, 1:500) and polyclonal rabbit anti-snapper Ig(lanes 6-10)(1:500) as the primary antiserum. The contaminating protein reacted with both immune and non-immune sera at every elution buffer pH analysed.

antisera, reactivity with the contaminant would be expected (if immunogenic). However, in control Western blot assays, it was demonstrated that not only the polyclonal antisera reacted with the contaminant, but also a number of other control sera. Further, elution of the affinity chromatography column without prior application of snapper serum, was performed. Probing of the control eluent in a Western blot demonstrated that the contaminant was column borne and also reactive with non-immune rabbit serum. Although definitive confirmation of SpA contamination using specific antisera (Knicker and Profy, 1991; Godfrey *et al*., 1992; Steindl *et al*., 2000) was not performed, results demonstrated here strongly suggest that the contaminating protein was SpA.

SpA is a 42 kDa protein, however the contaminating protein identified in this trial was approximately 65 kDa, suggesting that the ligand itself did not lose affinity to the support, but was cleaved toward the agarose beads. Although the binding arm may not have accounted for the difference in the expected molecular weight of SpA (42 kDa cf 65 kDa seen here), it may have affected the electrophoretic mobility, thus producing the observed anomalous result. Alternatively, contaminating SpA may have bound with constituent domains of the snapper Ig, thus affecting the electrophoretic mobility. SpA in the affinity chromatography column used here was coupled with N-hydroxysuccinimide esters of a derivatised cross-linked agarose support. According to the manufacturer, the product is resistant to heat, solvents and pH extremes (pH 2-11). In an independent control experiment, elution was performed at vary-

ing pH (3-5), which did nothing to inhibit ligand leakage (Figure 2). This suggests that the leakage observed is inherent.

Contamination from SpA has been reported previously, however quantification has been difficult due to the problems associated with competitive binding of the detecting antibodies and eluted IgG with the contaminating SpA. SpA purified monoclonal antibodies are commonly administered to human patients and since SpA has been shown to be potentially toxic (Bensinger *et al*., 1984), requisite quantification of contaminating SpA must be performed. Capture ELISA assays to detect SpA in eluent have been developed, using specific antisera to SpA in a capture ELISA (Knicker and Profy, 1991; Godfrey *et al*., 1992; Steindl *et al*., 2000). These techniques maybe useful to assess the quantity of SpA contamination (if required).

SpA affinity chromatography is widely used to purify teleost Ig (Suzuki *et al.*, 1990; Estévez *et al.* 1993; Estévez *et al.* 1994; Scapigliati *et al.*, 1996), however to the best of our knowledge, contamination of eluent with SpA has not been reported previously. This suggests that immuno-assays such as ELISA utilising SpA purified Ig (teleost or mammalian) require visual validation by immunoblotting to prevent SpA as a confounding factor.

Acknowledgments

The authors wish to thank S. Fielder and D. Barker at NSW Fisheries for providing serum. To P. Crosbie for technical assistance and polyclonal antisera and M. Watts who donated MW markers. Dr B. Munday provided valuable discussion on the possible origin of the anomalous band in the Western blot. This project was funded by the CRC for Aquaculture and R.N.M. was supported by an Australian Postgraduate Award.

References

Bensinger, W.I., Buckner, C.D., Clift, R.A., Thomas, E.D., (1984). Clinical trials with staphylococcal protein A. *J. Biol. Response Modif.* 3, 347.

Bjerrum, O.J. and Schafer-Nielsen, C., (1986). Buffer systems and transfer parameters for semidry electroblotting with a horizontal apparatus. In: *Electrophoresis*, Ed. M.J., Dunn, VCH, Weinheim, Germany, p315-327.

Bollag, D.M., Rozycki, M.D. and Edelstein, S.J., 1996. *Protein methods*. Wiley-Liss, New York.

Bourmaud, C.A-F., Romestand, B. and Bouix, G., (1995). Isolation and partial characterization of IgM-like seabass (*Dicentrarchus labrax* L. 1758) immunoglobulins. *Aquaculture*, 132, 53-58.

Bryant, M.S., Lee, R.P., Lester, R.J.G. and Whittington, R.J., (1999). Anti-immunoglobulin antisera used in an ELISA to detect antibodies in barramundi *Lates calcarifer* to *Cryptocaryon irritans*. *Dis. Aquat. Org*., 36, 21-28.

Estévez, J., Sánchez, C., Domínguez, J., Leiro, J., Sanmartín, M.L. and Ubeira, F.M., (1993). Protein-A binding characteristics of rainbow trout (*Oncorhynchus mykiss*) immunoglobulins. *Comp. Biochem. Physiol.* 106B(1), 173-180.

Estévez, J., Leiro, J., Santamarina, M.T., Domínguez, J. and Ubeira, F.M., (1994). Monoclonal antibodies to turbot (*Scopthalmus maximus*) immunoglobulins: characterization and applicability in immunoassays. *Vet. Immunol. Immunopath.*, 41, 353-366.

Glynn, P.J. and Pulsford, A.L., (1990). Isolation and partial characterisation of the serum immunoglobulin of the flounder, *Platichthys flesus*. *J. Mar. Biol. Ass. U.K.*, 70, 429-440.

Godfrey, M.A.J., Kwasowski, P., Clift, R., Marksa, V., (1992). A sensitive enzyme-linked immunosorbent assay (ELISA) for the detection of staphylococcal protein A (SpA) present as a trace contaminant of murine immunoglobulins purified on immobilized protein A. *J. Immunol. Methods*, 149, 21-27.

Harlow, E. and Lane, D., (1988). *Antibodies. A laboratory manual.* Cold Spring Harbor Laboratory, U.S.A.

Inganäs, M., (1981). Comparison of mechanisms of the interaction between protein A from *Staphylococcus aureus* and human monoclonal IgG, IgA and IgM in relation to the classic Fcg and the alternative $F(ab')_2$Œ protein A interactions. *Scand. J. Immunol.*, 13, 343-352.

Israelsson, O., Petersson, A., Bengtén, E., Wiersma, E.J., Andersson, J., Gezelius, G. and Pilström, L., (1991). Immunoglobulin concentration in Atlantic cod, *Gadus morhua* L. serum and cross-reactivity between anti-cod-antibodies and immunoglobulins from other species. *J. Fish Biol.*, 39, 265-278.

Knicker, S.M., Profy, A.T., (1991). Immunoassay to measure staphylococcal protein A in the presence of murine immunoglobulins. *J. Immunol. Methods*, 142, 53-59.

Laemmli, U.K., (1970). Cleavage of structural proteins during the assemblage of the head of bacteriophage T4. *Nature*, 227, 680-685.

Ljungberg, U.K., Jansson, B., Niss, U., Nilsson, R., Sanberg, B.E.B. and Nilsson, B., (1993). The interaction between different domains of Staphylococcal protein A and human polyclonal IgG, IgA, IgM and $F(ab')_2$: separation of affinity from specificity. *Mol. Immunol.*, 30(14), 1279-1285.

Lobb C.J. and Clem, L.W., (1981). Phylogeny of immunoglobulin structure and function-X. Humoral immunoglobulins of the sheepshead, *Archosargus probatocephalus*. *Dev. Comp. Immunol.*, 5, 271-282.

Palenzuela, O., Sitjà-Bobadilla, A. and Álvarez-Pelliteo P., (1996). Isolation and partial characterization of serum immunoglobulins from sea bass (*Dicentrarchus labrax* L.) and gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol.*, 6, 81-94.

Pilström, L. and Peterson, A., (1991). Isolation and partial characterization of immunoglobulin from cod (*Gadus morhua* L.). *Dev. Comp. Immunol.*, 15, 143-152.

Sanchez, C., Dominguez, J. and Coll, J., (1989). Immunoglobulin heterogeneity in the rainbow trout, *Salmo gairdneri* Richardson, *J. Fish Dis.*, 12, 459-465.

Scapigliati, G., Romano, N., Picchietti, S., Mazzini, M., Mastrola, L., Scalia, D. and Abelli, L., (1996). Monoclonal antibodies against sea bass *Dicentrarchus labrax* (L.) immunoglobulins: immunolocalisation of immunoglobulin-bearing cells and applicability in immunoassays. *Fish Shellfish Immunol.*, 6, 383-401.

Steindl, F., Armbuster, C., Hahn, R., Armbuster, C., Katinger, H.W.D., (2000). A simple method to quantify staphylococcal protein A in the presence of human or animal IgG in various samples. *J. Immunol. Methods*, 235, 61-69.

Suzuki, Y., Tanaka, M., Aida, K. and Hanyu, I., (1990). Affinity of fish immunoglobulin to protein A. *Nippon Suisan Gakkaishi*, 56(5), 831.

Zikán, J., Síma, P., Prokesová, L. and Hädge, D., (1980). Binding of non-mammalian immunoglobulins to Staphylococcal protein A. *Fol. Biol.* 26, 261-266.