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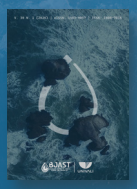

A NOVEL PURIFICATION PROCEDURE FOR *ACIPENSER BAERII BAERII* (BRANDT, 1869) SERUM IGM SUITABLE FOR RABBIT ANTISERA PRODUCTION TO BE USE AS SECONDARY ANTIBODY IN ELISA

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ABSTRACT

Acipenser baerii serum IgM was purified using a combination of precipitation and chromatography steps to produce anti-species antibodies. The purification procedure described herein is simple and easy to perform and the quality of the preparation obtained showed to be suitable enough to be used as an immunogen in rabbits. The anti-species antibodies produced were successfully employed in sandwich indirect ELISA to detect antibodies against Aeromonas *hydrophila* in serum samples from *A. baerii* and *A. gueldenstaedtii*, both cultured in Uruguay.

Keywords: *A. baerii*. Immunoglobulin. Purification.

1 Introduction

The economic importance of sturgeons lies in caviar and meat production. In Uruguay, two species were successfully introduced several years ago, and the country has become the main caviar producer in South America (Bronzi et al., 2019). Uruguayan caviar production is based on the culture of Siberian sturgeon (*Acipenser baerii*, Brandt, 1869) and Russian sturgeon (*Acipenser gueldenstaedtii*, Brandt & Ratzeburg, 1833). Despite the subtropical climate of Uruguay, these species have adapted well to aquaculture conditions; however, during summer, when water temperatures rise to around 30°C, fish mortality increases due to opportunistic pathogens (Castellano et al., 2020). In this context, the development of immunological tools to monitor fish health and immune responses is of increasing interest for sturgeon aquaculture.

Sturgeons are phylogenetically related to elasmobranchs and teleosts and possess an adaptive immune system. IgM is the main serum immunoglobulin produced by sturgeons, as no secreted form of IgD has been detected so far, although IgD gene sequences have been reported (Zhu et al., 2014; Zhu et al., 2016).

Serum IgM in sturgeons from the family *Acipenseridae* consists of polymeric immunoglobulins composed of heavy and light chains. Heavy chains have a molecular weight of approximately 70 kDa, while light chains range between 26 and 30 kDa (Partula & Charlemagne, 1993; Yektaseresht et al., 2018). Lundqvist et al. (1996), working on the light chain gene of *A. baerii*, concluded that sturgeons have a kappa-like organization of their IgL locus. This conclusion was based on the identification of a kappa recombination signal between the V and J segments in both teleosts and the sturgeon studied. The authors also suggested that the VL gene of both teleosts and the sturgeon studied is most similar to the kappa isotype, although they acknowledged the difficulties in classifying immunoglobulin light chains of non-mammalian vertebrates as either classical kappa or lambda isotypes.

In contrast, a comprehensive study of immune genes in *Acipenser sinensis* (Gray, 1835) by Zhu et al. (2016) described the immunoglobulin light chain gene as homologous to the lambda chain of cartilaginous fish. They did not identify any gene encoding a kappa light chain, to our knowledge.

Therefore, based on the available evidence, it is more likely that the light chains of sturgeon IgM, including those of *A. baerii*, predominantly belong to the lambda class. However, the presence of kappa chains cannot be completely excluded.

Albumin-like proteins, or albumins, have been identified in the blood of different fish lineages, although their concentrations vary widely among species. Sturgeons possess serum albumins that resemble those of higher vertebrates and consist of simple monomeric proteins with little variation in molecular mass depending on the species. Electrophoresis analyses under native conditions showed mobility corresponding to molecular masses ranging from 70 to 79.5 kDa, whereas under denaturing conditions all migrated similarly, corresponding to a molecular mass of approximately 67 kDa (Andreeva, 2010). Jafari et al. (2018) analyzed serum albumin concentrations in juvenile specimens subjected to different feeding strategies and reported variations ranging from 15 to 30 mg/mL. Total protein and globulin contents also varied among the feeding strategies studied. It is generally accepted that albumin concentration is affected by changes in salinity, temperature, muscle activity, and pre- and post-spawning periods, among other factors (Andreeva, 2010). Sturgeon albumins share some physicochemical properties with human serum albumin, particularly precipitation with ammonium sulfate and solubility in ethanol (Andreeva, 2010). Based on the information presented above, albumin may represent the main contaminant when purifying IgM from sturgeon serum.

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Several purification protocols for serum immunoglobulins from different fish species, including sturgeons, have been reported for a wide range of purposes, from basic research aimed at immunoglobulin characterization to applied studies requiring purified antibodies. These protocols involve methods ranging from protein precipitation under various conditions and/or using different precipitating agents to diverse chromatographic techniques, applied either individually or in combination. Examples include Partula & Charlemagne (1993), Smith et al. (1993), Kofod et al. (1994), Khoshbavar-Rostami et al. (2006), Rathore et al. (2006), Bag et al. (2009), Prokaeva & Novikov (2013), Yektaseresht et al. (2018), Mohanty et al. (2020) and Satkar et al. (2025), among others. Variations in preparation quality, yield, and processing time are strongly influenced by the method employed.

Obtaining the euglobulin fraction by direct dialysis against a low ionic strength buffer is a classical method broadly applied in immunology to separate IgM from other immunoglobulins across a wide range of species, from fish to mammals. In *A. baerii*, this method was applied by Partula & Charlemagne (1993). Although, the euglobulin fraction contains several serum proteins in addition to IgM, including proteins of the coagulation cascade, among others.

Other authors reported using a combination of ammonium sulfate precipitation or other precipitating agents together with size-exclusion chromatography, ion-exchange chromatography, or both (McCumber et al., 1976; Clerx et al., 1980; Adkison, Basurco & Hedrick, 1996; Prokaeva & Novikov, 2013).

Recently, a comparison between different chromatographic approaches for purifying fish immunoglobulins from *Clarias dussumieri* (Valenciennes, 1840) and *Etroplus suratensis* (Bloch, 1790) was reported by Satkar et al., 2025. They found that neither Protein A affinity chromatography nor Protein L affinity chromatography was appropriate. Both affinity matrices work well with mammalian immunoglobulins provided that: (a) the immunoglobulins are not polymeric—since Protein A binds IgM very weakly—and (b) the immunoglobulin light chains belong to the kappa variant, as Protein L binds this class of light chains. Protein A affinity chromatography was therefore unable to purify fish immunoglobulins, probably due to their polymeric nature, making this strategy unsuitable for sturgeon immunoglobulins. Although only a few studies have evaluated the ability of Protein A to bind fish immunoglobulins, Bromage et al. (2004), in a study of six teleost species, reported marked variability in binding. Specifically, they observed large differences in the proportion of Protein A–binding immunoglobulins among species, ranging from nearly complete binding in Mozambique tilapia to negligible binding in salmonids, which they attributed mainly to differences in immunoglobulin structure. Based on the knowledge about sturgeon light chains discussed above and on the findings of Satkar et al. (2025), Protein L affinity chromatography is unlikely to be effective for purifying IgM from *A. baerii*. These authors also tested ammonium sulfate precipitation followed by ion-exchange chromatography; however, the resulting immunoglobulin preparations still contained several contaminating proteins.

According to Satkar et al. (2025), antigen-affinity chromatography was the only method that yielded purified immunoglobulins suitable for downstream applications. Building on this strategy, antigen-affinity chromatography has been widely applied to purify fish immunoglobulins after antigen-specific immunization. Specific IgM fractions have been successfully obtained by several researchers from sturgeons (Khoshbavar-Rostami et al., 2006), tilapia (*Oreochromis aureus* (Steindachner, 1864)) (Smith et al., 1993), turbot (*Scophthalmus maximus* (Linnaeus, 1758)) (Kofod et al., 1994), African catfish (*Clarias gariepinus* (Burchell, 1822)) (Rathore et al., 2006), and Indian major carps such as mrigal (Cirrhinus mrigala (Hamilton, 1822)) (Bag et al., 2009; Mohanty et al., 2020). These examples illustrate the broad effectiveness of antigen-affinity approaches across diverse fish species for obtaining antigen-specific IgM and support the application of this strategy to sturgeons. Nevertheless, this strategy has at least two main disadvantages: (a) it requires prior immunization of the fish, and (b) only a small proportion of total immunoglobulin molecules can be purified, since the majority of serum immunoglobulins are not antigen-specific.

In this work, serum IgM from *A. baerii* was purified with the aim of producing a species-specific antibody for ELISA development. A purification procedure was developed to obtain an immunoglobccculin fraction suitable for immunization protocols. Polyclonal rabbit antibodies against purified IgM were then produced and applied in an ELISA to detect sturgeon antibodies against Aeromonas *hydrophila*. This approach provides a foundation for the development of immunological tools applicable to other sturgeon species and contributes to improved health monitoring in sturgeon aquaculture.

2 Materials and methods

2.1 Sequential fractionation of serum proteins by ammonium sulfate precipitation

A pooled serum sample from *A. baerii* specimens (5 mL), previously delipidated according to Sakagami & Zilversmit (1962), was used as starting material. Serum proteins were fractionated by sequential precipitation with increasing concentrations of ammonium sulfate.

First, a 100% saturated ammonium sulfate solution was added to the serum under stirring to reach 20% saturation. The mixture was stirred for approximately 10–15 min and then allowed to stand for 2h at 4°C to allow the precipitated protein aggregates to form. The precipitate and supernatant were separated by centrifugation at 10000×g for 15min at 4°C. The pellet was washed with a 20% saturated ammonium sulfate solution prepared in 1X phosphate-buffered saline, pH 7.2 (1X PBS) using a volume approximately equal to that of the supernatant. After washing, the precipitated material was resuspended in 1X PBS and dialyzed to remove residual ammonium sulfate.

The ammonium sulfate concentration of the supernatant was increased to 30% saturation under stirring, allowed to stand 2h at 4°C, and centrifuged under the same conditions. The pellet was washed as above, but using 30% saturated ammonium sulfate in 1X PBS, resuspended, and dialyzed.

Finally, ammonium sulfate was added to the supernatant to reach 50% saturation under stirring, allowed to stand 2h at 4°C, centrifuged, and the pellet was washed as above, but using 50% saturated ammonium sulfate in 1X PBS and resuspended. Residual ammonium sulfate was removed by dialysis against 1X PBS (20-fold excess) for 48h at 4°C, with one buffer change after 24h.

Protein composition of all fractions (20%, 20–30%, 30–50%, and 50% supernatant) was analyzed by SDS-PAGE using the Laemmli system (Laemmli, 1970) under reducing (12% resolving gels) and non-reducing conditions (7% resolving gels) with 4% stacking gels.

2.2 Purification of IgM from sturgeon serum

A pooled serum sample (16mL) from *A. baerii*, delipidated as above, was used. 100% saturated ammonium sulfate was added with continuous agitation to reach 33% saturation. The mixture was kept at 4°C for ≥2h without stirring.

The precipitate was removed by centrifugation at 10000×g for 20min at 4°C (Avanti J-30, Beckman, USA) and discarded. The supernatant was dialyzed against 5mM Tris, pH7.4, until sulfate ions were no longer detectable in the permeate (≥2 changes of 1L buffer). Absence of sulfate ions was verified by $BaCl_2$ precipitation after slight acidification.

After dialysis, the solution was clarified by centrifugation, and the resulting precipitate was resuspended in 1X PBS. Total protein concentration in fractions was determined using the bicinchoninic acid assay (BCA; Sigma-Aldrich, USA) with BSA as standard, following the manufacturer's instructions. Protein composition was analyzed by SDS-PAGE.

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2.3 Removal of fish serum albumin (FSA) by Blue-Sepharose affinity chromatography

FSA was removed using a 1mL HiTrap Blue HP column (Cytiva Life Sciences, USA) according to manufacturer instructions. The column was equilibrated with 10CV of 20mM sodium phosphate, pH 7.0.

A 12.5mL sample, diluted with an equal volume of binding buffer, was applied at 1mL min^{-1}, and the flow-through was collected. The column was washed with 10CV of binding buffer, and retained proteins were eluted with 2M NaCl in binding buffer. All fractions were analyzed by SDS-PAGE.

2.4 Analysis of protein fractions

Fractions from ammonium sulfate precipitation and Blue-Sepharose chromatography were analyzed by SDS-PAGE under reducing (with DTT) and non-reducing (without DTT) conditions. Non-reduced samples were run on 7 or 8% gels, reduced samples on 12% gels at 25mA at room temperature (RT) (Tall Mighty Small Vertical Slab Gel Unit, Hoefer, USA).

HMW and LMW SDS marker kits (Cytiva, USA) were included as appropriate. Protein bands were stained with Coomassie Brilliant Blue R-250. The band suspected to be the IgM heavy chain was excised and sent to Institut Pasteur–Montevideo (IP–Mon) for mass spectrometry analysis by MALDI-TOF/TOF mass spectrometry (ABI 4800, Applied Biosystems, USA).

2.5 Rabbit polyclonal antiserum against sturgeon IgM

One adult male New Zealand rabbit (~2.5kg) was immunized according to a protocol approved by the Animal Ethics Committee of the Facultad de Química, Universidad de la República (CEUA-FQ; protocol #101900-001846-19). For priming, 400µg purified *A. baerii* IgM in 1X PBS emulsified 1:1 (v/v) with Complete Freund's Adjuvant was administered intradermally at multiple sites on the back. Booster doses of 200µg IgM emulsified 1:1 with Incomplete Freund's Adjuvant were administered intramuscularly in the leg at 20–25 day intervals. Blood was collected 12 days after the third booster, and antibody production was assessed by Western blot.

2.6 Western blot reactivity control

A 12% SDS-PAGE under reducing conditions containing 5µg per well of *A. baerii* IgM fraction was transferred onto a Hybond nitrocellulose membrane (0.45µm pore size, Cytiva, US) using a semi-dry transfer unit (SemiPhor TE77, Hoefer, US) for 1h at 1.8mA cm^{-2} (Kyhse-Andersen, 1984). The membrane was blocked with an inert protein (1% (w/v) BSA in 1X PBS (PBS-BSA)) for 1 h at room temperature to prevent nonspecific antibody binding, and cut into 0.4mm-wide strips. After three thorough washes with 0.05% (v/v) Tween 20 in 1X PBS (PBS-T), using sufficient volume to completely cover the membrane strips (typically 5–10 mL per strip depending on strip size and container), one strip was incubated with rabbit hyperimmune antiserum against *A. baerii* IgM at 1:1000 dilution in 1X PBS, 0.05% (v/v) Tween 20, 0.1% (w/v) BSA (PBS-T-BSA) for 1 h at RT with gentle shaking. Two additional strips were incubated with either pre-immune rabbit serum or diluent only, following the same protocol. After three thorough washes with PBS-T (as above), the strips were incubated with anti-rabbit IgG (H+L) conjugated to alkaline phosphatase (Sigma, US), appropriately diluted in PBS-T-BSA, for 1h at RT with gentle shaking. Finally, a substrate solution of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (Harlow & Lane, 1984, pp. 471-510) was added after thorough washing as described above. Enzymatic reaction was stopped by rinsing with distilled water.

2.7 Purification of rabbit IgG specific for *A. baerii* IgM

Ten mg of purified *A. baerii* IgM was coupled to 0.3g CNBr-activated Sepharose (Sigma, USA) following the manufacturer's instructions and packed in a polypropylene cartridge (Pierce, USA). The column was equilibrated with 10CV of 0.1M glycine, 0.15M NaCl, pH 8.2. Rabbit hyperimmune IgG (37% ammonium sulfate precipitated fraction) conditioned in binding buffer was applied. Non-specific proteins were washed with 10CV of 0.1M glycine, 0.5M NaCl, pH 8.2. Specific IgG was eluted with 0.1M glycine, 0.5M NaCl, pH 2.5, and dialyzed against binding buffer. Concentration was measured at 280nm. Immunoreactivity was confirmed by Western blot using the protocol described previously with modifications as follows: *A. baerii* serum was separated by SDS-PAGE (5µg per well, 8% non-reduced gels) instead of *A. baerii* IgM fraction; affinity-purified rabbit IgG (1µg/mL in PBT-T-BSA) instead of hyperimmune rabbit serum. Controls to evaluate non-specific reaction –pre-immune rabbit antiserum and PBS-T-BSA– were included just as described previously.

2.8 ELISA using rabbit IgG specific for *A. baerii* IgM as a secondary antibody

Indirect ELISA (Voller et al., 1978) was performed to detect antibodies against *Aeromonas hydrophila*. Sturgeon serum samples from a previous vaccination trial, stored at −20°C, were kindly provided by Dr. A. Pereta (Instituto de Investigaciones Pesqueras, Facultad de Veterinaria, Universidad de la República). A cetyltrimethyl ammonium bromide (CTAB) extract of *A. hydrophila* (2.5µg/mL, 100µL per well) in 10mM glycine, 300mM NaCl, pH 9, was passively adsorbed overnight at 4°C. Wells were washed three times with 200µL of PBS-T per well to remove unbound antigen. Plates were blocked with PBS-BSA (200µL per well, 1 h, 37 °C) and washed as above. Serum samples from *A. baerii* or *A. gueldenstaedtii* (10 known positives, 10 known negatives) –without discriminating by species– were tested in duplicate at 1:500 dilution in PBS-T-BSA (100µL per well) for 1h at 37°C. After three washes with PBS-T to remove unbound antibodies (as above), affinity-purified anti-sturgeon IgM rabbit IgG in PBT-T-BSA (0.4µg/mL, 100µL per well) was added for 1h at 37°C. Anti-rabbit IgG(H+L)-peroxidase (Sigma, US) in PBS-T-BSA was added, incubated 30min at 37°C, washed as above, and substrate (H_2O_2 + o-phenylenediamine in 0.05M citrate-phosphate buffer) was added (Harlow & Lane, 1988; pp. 553-612). The reaction was stopped after 15min with 2N H_2SO_4 and absorbance read at 492nm.

2.9 Data presentation and analysis

ELISA absorbance values (normalized OD values) were summarized using box plots to illustrate their distribution within each sample group (positive and negative), using IBM SPSS Statistics (version 19) predictive analytics software. No formal statistical tests were performed, as the assay served as proof-of-concept for IgM detection. Values are presented as median ± interquartile range (IQR).

3 Results and Discussion

A preliminary exploration of serum protein separation by sequential ammonium sulfate precipitation was conducted. Four fractions were obtained: (i) proteins precipitated at 20% saturation, (ii) proteins precipitated between 20 and 30% saturation, (iii) proteins precipitated between 30 and 50% saturation, and (iv) proteins remaining soluble at 50% saturation. Electrophoretic analysis of these fractions under non-reducing and reducing conditions indicated that IgM was minimally precipitated at 30% saturation, remaining mainly in solution (Figure 1, panels A & B, lanes #2). Increasing ammonium sulfate to 50% saturation resulted in IgM precipitation. Thus, the fraction obtained between 30 and 50% saturation was enriched in IgM but also contained other serum proteins, particularly serum albumin (Figure 1, panels A & B, lanes #3). These behaviors of *A. baerii* serum proteins were similar to those observed in several mammalian species. In contrast, Yektaseresht et al. (2018) reported that sturgeon IgM precipitated directly from serum with 50% ammonium sulfate produced a preparation considered suitable for immunization. However, their characterization was limited to SDS-PAGE under reducing conditions and low protein concentration, as judged by the weak intensity of the heavy and light chain bands. The concentration of protein in the final preparation was notably low, making it difficult to evaluate contaminants, and no information was provided regarding the fraction that remained in solution.

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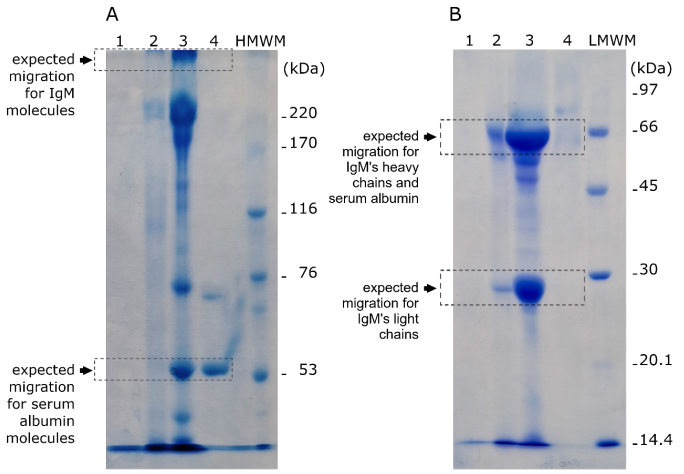

Figure 1. Sequential fractionation of *A. baerii* serum proteins using ammonium sulfate. (A) SDS–PAGE of non-reduced samples on a 7% acrylamide gel; (B) SDS–PAGE of reduced samples on a 12% acrylamide gel. (1) Precipitated fraction after addition of ammonium sulfate to 20% saturation; (2) precipitated fraction obtained between 20–30% saturation; (3) precipitated fraction obtained between 30–50% saturation; and (4) soluble fraction after the addition of ammonium sulfate to 50% saturation. HMWM: high molecular weight marker kit; LMWM: low molecular weight marker kit (Cytiva Life Sciences, USA). Gels were stained with Coomassie Brilliant Blue R-250.

Analysis of the material precipitated at 30% ammonium sulfate saturation showed that this value could be increased to promote the precipitation of contaminating proteins without precipitating most of the IgM. Accordingly, serum protein fractionation was performed at 33% ammonium sulfate saturation. As anticipated, several contaminating proteins precipitated, whereas IgM and albumin remained soluble (Figure 2, panels A & B, lanes 2 & 3). Subsequent IgM enrichment was achieved by dialyzing against low ionic strength Tris buffer (pH 7). The precipitation of IgM upon dialysis is consistent with previous studies on sturgeon immunoglobulins. Partula & Charlemagne (1993), working with *A. baerii*, obtained the euglobulin fraction directly by dialysis of serum against a low ionic strength Tris buffer. In contrast, Adkison, Basurco & Hedrick (1996), studying *Acipenser transmontanus* (Richardson, 1836), first precipitated serum proteins with 50% ammonium sulfate, then obtained the IgM-containing euglobulin fraction by dialysis, and finally applied DEAE ion-exchange chromatography to further purify IgM. In both studies, IgM was enriched while retaining the characteristic heavy and light chain composition. Our approach, with 33% ammonium sulfate precipitation followed by dialysis, is conceptually intermediate: it removes a portion of contaminating proteins while keeping IgM soluble until subsequent enrichment, thereby minimizing losses and preserving structural integrity.

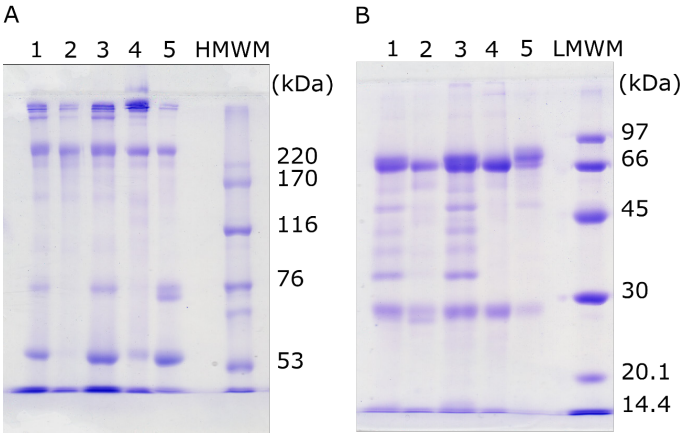

Figure 2. Presence of *A. baerii* IgM in the precipitated fraction under low ionic strength conditions. (A) SDS–PAGE of non-reduced samples on a 7% acrylamide gel; (B) SDS–PAGE of reduced samples on a 12% acrylamide gel. (1) *A. baerii* serum; (2) and (3) precipitated and soluble fractions, respectively, after addition of ammonium sulfate to 33% saturation; (4) and (5) precipitated and soluble fractions, respectively, after dialyzing the soluble fraction at 33% ammonium sulfate saturation against low ionic strength buffer. HMWM: high molecular weight marker kit; LMWM: low molecular weight marker kit (Cytiva Life Sciences, USA). Gels were stained with Coomassie Brilliant Blue R-250.

SDS-PAGE analysis of the fractions obtained at 33% ammonium sulfate saturation revealed complex patterns in the precipitated material under both reducing and non-reducing conditions. Regarding the soluble fraction, the two characteristic bands of immunoglobulin heavy and light chains (~70–75kDa and ~25–30kDa, respectively) were observed under reducing conditions (Figure 2, panel B, lane 2). Under non-reducing conditions, high-molecular-weight bands exceeding the 220kDa marker were visible (Figure 2, panel A, lane 2). Nonetheless, additional protein contaminants were still present in this soluble fraction. Dialysis of this fraction resulted in the precipitation of IgM, as shown by the expected band patterns under both reducing and non-reducing conditions (Figure 2, panels A & B, lanes # 4). A minor band migrating slightly above the 53kDa marker was observed under non-reducing conditions, consistent with serum albumin. To accurately distinguish IgM heavy chains (~70kDa) from albumin (~66kDa), analysis under both reducing and non-reducing conditions is necessary. In non-reducing gels, albumin migrates more compactly (~53kDa), whereas IgM remains as high-molecular-weight polymers that barely enter the gel, facilitating their differentiation. In reducing gels, the IgM heavy chain and albumin have similar molecular weights, making resolution more challenging. To remove residual albumin remaining after ammonium sulfate fractionation and dialysis, affinity chromatography using Blue Sepharose Hi-Trap was applied. This step effectively retained albumin (Figure 3, lane 2), while IgM was recovered in the flowthrough (Figure 3, lane 1), providing a simple and efficient final purification step for *A. baerii* IgM. Forty-five mg of IgM was obtained.

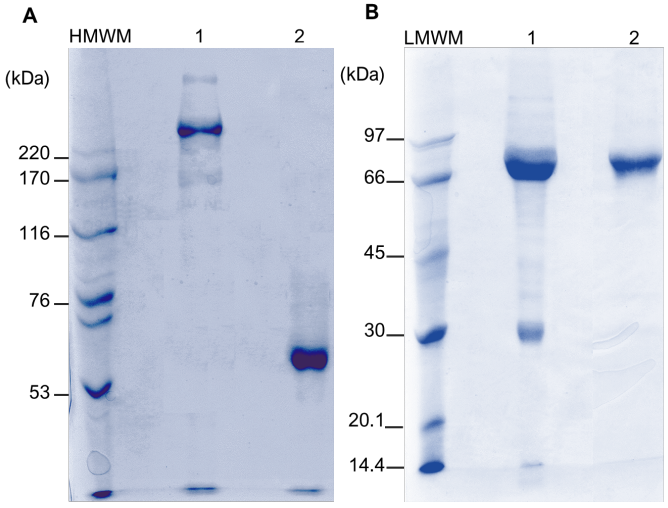

Figure 3. Removal of serum albumin from *A. baerii* IgM using Blue-Sepharose affinity chromatography. (A) SDS–PAGE of non-reduced samples on a 7% acrylamide gel; (B) SDS–PAGE of reduced samples on a 12% acrylamide gel. (1) Non-retained fraction (flow-through); (2) eluted fraction. HMWM: high molecular weight marker kit; LMWM: low molecular weight marker kit (Cytiva Life Sciences, USA). Gels were stained with Coomassie Brilliant Blue R-250.

Mass spectrometry analysis was performed on the band suspected to correspond to an immunoglobulin heavy chain (Blue-Sepharose flowthrough; SDS–PAGE under reducing conditions) at the Institut Pasteur–Montevideo (IP–Mon). Mascot analysis, conducted by IP–Mon staff, identified several peptides matching immunoglobulin heavy chains from different sturgeon species (see Table 1). Among these, four peptides were assigned to a complete *A. baerii* sequence (CAA73703.1) and were used to calculate the Mascot score (score: 70; expect: 1.1). Two additional peptides, initially assigned by Mascot to other sturgeon species, also matched to *A. baerii* sequences upon further analysis using *A. baerii* sequences from GenBank.

A search for *A. baerii* immunoglobulin heavy chain sequences in GenBank returned more than 40 entries with minor sequence variations; only two corresponded to complete sequences (CAA73703.1 and CAA73702.1), while the majority were partial sequences. In this second analysis, three of the four Mascot-assigned peptides also aligned with the second complete sequence (CAA73702.1). Moreover, this analysis showed that the two additional peptides, initially assigned by Mascot to other sturgeon species, matched a partial sequence (CAP71893.1), with one peptide completely overlapping the other.

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Table 1. MALDI-TOF/TOF mass spectrometry analysis. Peptide sequences matching *A. baerii* immunoglobulin heavy chain sequences are listed. Peptides matching immunoglobulin heavy chains from other sturgeon species are also included. All protein sequences used in the analysis were retrieved from GenBank.

MALDI-TOF/TOF–detected peptides matching sturgeon immunoglobulin heavy chain sequences (GenBank)	
A. baerii (Mascot score: 70, Expect 1.1)	Other sturgeons
KQTDSLPSPRV *	
KVTLEEWARG *	KEFETGTPEISNVNGYFTAVSKL [*Huso huso*]
KLKVTLEEWARG *	KWASETKEFETGTPEISNVNGYFTAVSKL [*H. huso*]
RTINSTSIIDCPGNVHGCISPPSVEEIFLRK *	KSTVPTAQWTDGGIYTCVVYHETIQPPTRM [*H. huso*]
RFINTAAVEDSDGHFSAYSLLTATKE	RFINTAAVEDSDKHFSAYSILTATEEEWLGSEIKC [A. gueldenstaedtii]
RFINTAAVEDSDGHFSAYSLLTATKEEWLESEIKC	
* peptides assigned by Mascot to the immunoglobulin heavy chain complete sequence CAA73703.1	

Thus, a total of six peptides were mapped to *A. baerii* immunoglobulin heavy chain sequences (see Figure 4): four peptides from one complete sequence (CAA73703.1), three of which also align with the second complete sequence (CAA73702.1), and two peptides corresponding to the partial sequence (CAP71893.1). Although the two peptides mapping to the partial sequence did not contribute to the Mascot score, they provided additional support for the identification of the band as *A. baerii* IgM heavy chain.

```
complete sequence   CAA73703.1  MGILTALCIIMTALSSVQSDVVLTESGTAVIKPGESHKLSCKASGFTFSSYWMGWVRQAP   60
complete sequence   CAA73702.1  MGILTALCIIMTALSSVRSDVVLTESGPAVIKPGESHKLSCKASGFTFSSYYMNWVRQVP   60
partial sequence    CAP71893.1  ------------------------------------------------------------

complete sequence   CAA73703.1  GKGLEWVSTINNGGSGTYYAPSVQGRFTISRDDSNSLLYLQMNSLKTEDTAVYYCTRNSG  120
complete sequence   CAA73702.1  GKGLEWVAEISNSGGSTFYAQSVQGRFTISRDDSNNMLYLQMNSLKTEDTAVYYCARWGG  120
partial sequence    CAP71893.1  ------------------------------------------------------------
                                                        ▼
complete sequence   CAA73703.1  SYSGFDYWGKGTMITVTSRTPSPPTVFPLMESCCLSDISGPVATGCLATGFLPTPATFSW  180
complete sequence   CAA73702.1  -YAYFDYWGKGTLVTVSSATPSPPTVFPLMQACCSVDVTGPSATGCLATEFLPTPATFSW  179
partial sequence    CAP71893.1  ------------------------------------------------------------

complete sequence   CAA73703.1  TDQSGKAFQTDKSLQYPPVQTEGTYTTSTGQLAISDAEWQKAEYFYCTVENASGKNKVKVA  240
complete sequence   CAA73702.1  TDQTGKAFQTDKSLQYPPVQSGGTYTTSTGQLAISDAEWQTAEYFYCTVENASGKNKVKVA  239
partial sequence    CAP71893.1  ------------------------------------DAEWQKAEYFYCTVENASGKNKVKVA   26
                                                                     *****.********************

complete sequence   CAA73703.1  KCEVEPTFHPHVFIIPRLSEEIRANKTATIVCVARGFSPKTWSFKWSKDATAFDAKRFIN  300
complete sequence   CAA73702.1  KCDPD-VTHPHVFIIPPSIEEIRANKTATIVCVARGFSPKTWSFKWSKDAVAFDAKRLLN  298
partial sequence    CAP71893.1  KCEVEPTFHPHVFIIPPSSEEIRANKTATIVCVARGFSPKTWSFKWSKDATAFDAKRFIN   86
                                **: : . ********  ****************************.******::*

complete sequence   CAA73703.1  TAAVEDSDGHFSAYSLLTATEEEWLESEIKCEVIHGRETVIRTINSTS-IIDCPGNVHGC  359
complete sequence   CAA73702.1  TAAVEDSDKHFSAYSILTATEEEWLGSEIKCEVTFGSAKVFRTINSTSEPGDCPANVHVG  358
partial sequence    CAP71893.1  TAAVEDSDGHFSAYSLLTATKEEWLESEIKCELINGRETVIRTINSTSIPGECSANVHVG  146
                                ******** ******:****:**** ******:  *  .*:*******   :*..***

complete sequence   CAA73703.1  ISPPSVEEIFLRKEATLTCKATGLVSKDHLEIKWASETKEFETGTPEISSADGYFTAVSK  419
complete sequence   CAA73702.1  ISPPSVEEIFLRKEATLTCKATGLVSEDHLEIKWASETKEFETGTPEIISVNGYFTAVSK  418
partial sequence    CAP71893.1  ISPPSVEEVFLLLISFFYSIGATFAKVK----------------------------------  174
                                ********:**  : : . :: :.. .

complete sequence   CAA73703.1  LKVTLEEWARGDKFFCSVKQTDSLPSPRVAAYHRELDGPSHRPAVFLLSPSPQESSNGNG  479
complete sequence   CAA73702.1  LKVTLEEWARGDKFFCTVKQTDSLPSPRVAAYHRELDGPSHRPAVFLLSPSPQESSNGNG  478
partial sequence    CAP71893.1  ------------------------------------------------------------

complete sequence   CAA73703.1  EVSLTCFVKDFYPEEVYITWLHSDNSVPQTFYTTHQFDSQEKRKPLFSVYSKLTVPTAEW  539
complete sequence   CAA73702.1  EVSLTCFVKDFYPEEVYITWLHSDNSVPQTFYTTHQFDSQEKRKPLFSVYSKLTVPTAEW  538
partial sequence    CAP71893.1  ------------------------------------------------------------

complete sequence   CAA73703.1  TDGGIYTCVVYHETIQPPTRMITRNADSTGKQTLVNVGLTLPDKANPCSN            589
complete sequence   CAA73702.1  TDGGIYTCVVYHETIQPPTRMITRNADSTGKQTLVNVGLTLPDKANPCSN            588
partial sequence    CAP71893.1  ------------------------------------------------------
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Figure 4. MALDI-TOF/TOF mass spectrometry analysis. Three immunoglobulin sequences of *A. baerii* retrieved from GenBank are aligned (two complete sequences and one partial). Six peptides are mapped onto these sequences. The Mascot score (70; Expect = 1.1) was calculated using the four peptides covering one complete sequence (CAA73703.1). Three of these peptides also map to a second complete sequence (CAA73702.1), while two peptides are found only in the partial sequence (CAP71893.1). Peptides are highlighted in red to illustrate overlap and coverage. Underlined sequences indicate shorter peptides contained within larger ones. This distribution provides strong evidence confirming the successful purification of *A. baerii* polyclonal immunoglobulins. Sequences shown in bold black indicate heavy chain constant regions. The ▼ symbol denotes the first amino acid of the CH1 domain.

Taken together, the combination of SDS-PAGE under non-reducing and reducing conditions and mass spectrometry mapping of six peptides from the IgM heavy chain provides robust evidence that polyclonal immunoglobulins from *A. baerii* were successfully purified.

Finally, affinity-purified rabbit antibodies were used in an ELISA to detect sturgeon immunoglobulins against an antigen extract of *A. hydrophila*. To minimize non-specific reactions against conserved bacterial antigens, rabbit antibodies were first purified by antigen affinity chromatography and their reactivity verified by Western blot. Membrane strips containing *A. baerii* whole serum, separated by SDS-PAGE under non-reducing conditions, were used for verification. As shown in Figure 5, IgM was specifically recognized, whereas no reaction was observed against serum albumin or other abundant serum proteins. Controls with pre-immune rabbit serum or secondary antibody labeled with alkaline phosphatase showed no non-specific binding. This analysis confirmed the specificity of the rabbit antisera against serum IgM. ELISA results obtained with 20 sturgeon sera from *A. baerii* and *A. gueldenstaedtii* showed that IgG-purified rabbit antibodies against *A. baerii* IgM successfully detected antibodies against *A. hydrophila*, discriminating between positive and negative samples from both species indistinctly (Figure 6).

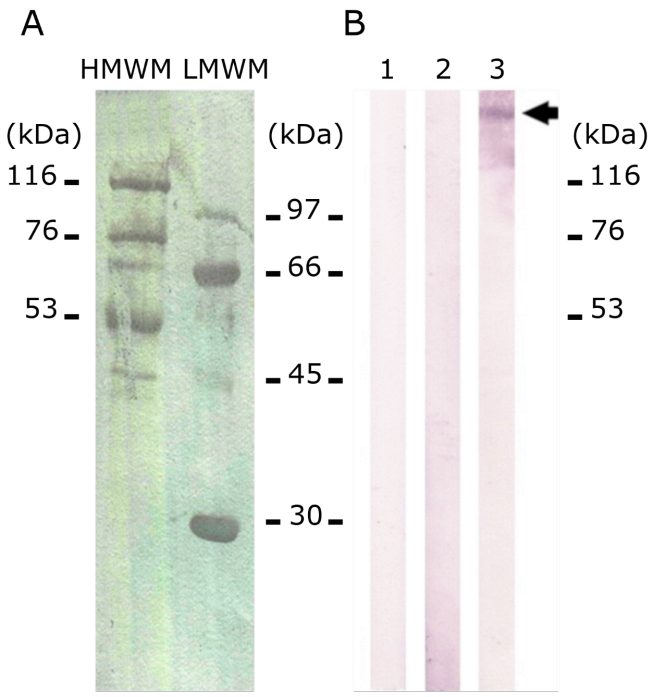

Figure 5. Western blot analysis of rabbit immunoglobulin-specific fractions against *A. baerii* IgM. (A) Blotted protein bands stained with India Ink (Harlow & Lane, 1988, pp. 471–510). (1) High molecular weight markers (116, 76, 53kDa); (2) low molecular weight markers (96, 67, 45, 30kDa). (B) Immunoblot with rabbit antiserum fractions. (1) Anti-rabbit IgG: Alkaline Phosphatase control; (2) rabbit pre-immune serum control (1:200 dilution); (3) affinity-purified fraction of rabbit antibodies specific for *A. baerii* IgM (1:1000 dilution). Substrate: NBT/BCIP. Arrows indicates electrophoretic migration of *A. baerii* IgM (A) and antibody recognition by the affinity-purified fraction (B). Note: Panels A and B correspond to the same blotted membrane. For India Ink staining, the membrane was cut to include lanes with HMWM and LMWM. For immunoblot staining, three additional strips (~0.4cm wide) were cut.

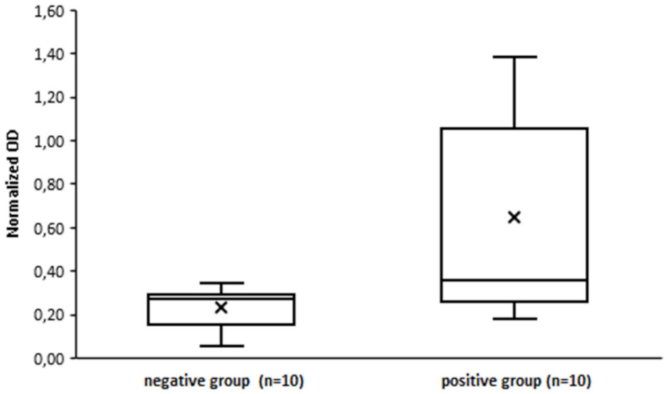

Figure 6. ELISA using rabbit immunoglobulins specific for *A. baerii* IgM as secondary antibodies. The graph shows ELISA results (normalized OD values) for sera from individuals with prior vaccination (positive group) and unvaccinated individuals (negative group) from a sturgeon farm in Uruguay, including *A. baerii* and *A. gueldenstaedtii*. Box-and-whisker plots illustrate a clear separation in signal between sera from vaccinated and unvaccinated animals.

4 Conclusion

A purification process combining precipitation and chromatography steps was developed, allowing the obtainment of a protein fraction highly enriched in serum IgM from *A. baerii* (Figure 7). The procedure is straightforward to perform, and the resulting material was suitable for immunization purposes. The elicited rabbit antibodies showed excellent performance for detecting antibodies in ELISA for *Acipenser* species.

10.14210/bjast.v30n1.21194

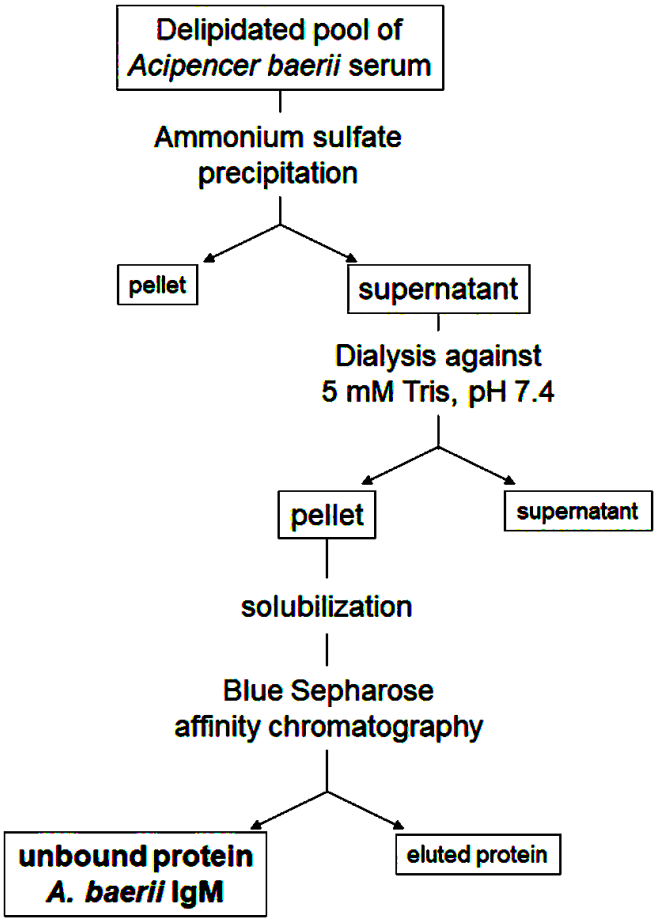

Figure 7. Schematic flowchart summarizing the proposed steps for *A. baerii* IgM purification.

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