Journal of Chromatography
Separation Techniques
ISSN: 2157-7064

Justiz Vaillant et al., J Chromat Separation Techniq 2012, 3:3
DOI: 10.4172/2157-7064.1000127

Research Article

Open Access

Purification of Immunoglobulin Y (IgY) from the Ostrich (*Struthio camelus*) by Staphylococcal Protein a (Spa) Affinity Chromatography

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Abstract

Immunoglobulin Y (IgY) is the major protein present in the avian egg yolk. This antibody fulfils important functions in the protection of Ostrich birds against infections. The aim of this study was to demonstrate the binding capacity of Staphylococcal proteins A (SpA) to Ostrich IgY and assess purification of the IgY by SpA affinity chromatography. Chloroform polyethylene glycol (Polson), affinity chromatography, Enzyme-Linked Immunosorbent Assay (ELISA) and Western blotting methods were used in the process. Results obtained revealed that Ostrich IgY has heavy chain of 70 kDa and light chain of 30 kDa confirming results by Western blot. In addition livetins (egg yolk proteins) were shown in the protein electrophoresis that preceded the Western blot. The binding capacity between SpA and Ostrich IgY is important because SpA can be used as a reagent in immunoassays for antibody detection against microbial agents that usually infect livestocks. This is the first time the use of SpA for purification of Ostrich IgY is being reported in literature.

Keywords: Protein A ; Protein LA ; Ostrich Immunoglobulin Y ; Affinity chromatography ; ELISA ; Trinidad & Tobago

Introduction

Immunoglobulin Y (IgY) is the major antibody produced by birds and offers many advantages over antibodies conventionally derived from other laboratory animals [1]. Egg yolk antibodies are important therapeutically [2,3] but™ also they have been used as immunological tool in immunodiagnostic assays, including radio-immunoassays for measuring anti-peptide antibodies [4] and enzyme-linked immunosorbent assays (ELISA) for quantitation of proteins [5,6]. The binding capacity between SpA and Ostrich (*Struthio camelus*) IgY is important because SpA can be used as a reagent in immunoassays for antibody detection against microbial agents. Among the microorganisms that can be diagnosed by the detection of specific ostrich IgY are *Campylobacter jejuni*, *Chlamidia psittaci*, Micoplasma sp, Megabacteria, and Newcastle disease virus [7]. The use of avian eggs in antibody production leads to less number of laboratory animals that are used for this purpose. In addition, immunized birds for example hens and ostriches produce larger quantities of antibodies than do rodents in the laboratory, they are considered to be farmyard animals and are therefore less expensive than laboratory animals such as rabbits [1]. Antibodies developed in birds recognize more epitopes on mammalian proteins. It is therefore more advantageous to use IgY in immunoassay, which detect mammalian proteins. This is especially true when the antigen is a highly conserved protein such as a hormone. This study reports on the purification of IgY from ostrich and its interaction with Staphylococcal Protein A (SpA) making this an important reagent that can be used in immunoassays for antibody detection against microbial agents usually infecting livestocks and humans. This is the first time the use of SpA for purification of Ostrich IgY is being reported in literature.

Materials and Methods

Materials

Peroxidase-labeled SpA conjugate, Peroxidase-labeled SpLA conjugate, SpA antibody purification kit, ELISA microtitre plates were obtained from Sigma-Aldrich Co, St. Louis Missouri, USA. All other chemicals were of reagent grade, obtained from the same company.

Methods

Immunoglobulin Y isolation: The IgY fraction was isolated from the egg yolks of the ostrich. The IgY fraction was isolated by the chloroform-Polyethylene Glycol (PEG) method and then further attempts of its purification was by using Protein-A affinity chromatography, where the ostrich IgY were purified from egg yolk [8]. Briefly, the eggs were washed with warm water and the egg yolk was separated from the egg white. The membrane was broken and the egg yolk collected and diluted 1:3 in Phosphate Buffered Saline (PBS), pH 7.4. To a third part (1/3) of the egg yolk mixture an equal volume of chloroform was added, the mixture was then shaken and centrifuged for 30 min (1000 x g, at room temperature). The supernatant was decanted and mixed with PEG 6000 (12%, w/v), stirred and incubated for 30 min at room temperature. The mixture was then centrifuged as previously described [8]. The precipitate containing IgY was dissolved in PBS (pH 7.4) at a volume equivalent to 1/6 of the original volume of the egg yolk and dialyzed against one litre (1L) of PBS (pH: 7.4 for 24 h at 4°C). The IgY was removed from the dialysis tubing and its concentration was determined by using the Bradford method [9]. IgY samples were stored at -20°C. Then using a commercially-available SpA antibody

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Received April 11, 2012; **Accepted** June 15, 2012; **Published** June 17, 2012

Citation: Justiz Vaillant AA, Akpaka PE, McFarlane-Anderson N, Smikle MP, Wisdom B (2012) Purification of Immunoglobulin Y (IgY) from the Ostrich (*Struthio camelus*) by Staphylococcal Protein a (Spa) Affinity Chromatography. J Chromat Separation Techniq 3:127. doi: 10.4172/2157-7064.1000127

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purification kit according to the manufacturer (Sigma – Aldrich Co, St. Louis Missouri) instructions, the ostrich IgY was further purified using a Protein-A affinity chromatography as previously reported [10].

Western blot analyses for characterization of ostrich IgY: The purity of purified antibodies was evaluated using Western blot analysis. Aliquots of 3-5 μg/μl of the sample were applied to the gel and run on a protein electrophoresis (SDS-PAGE) [11]. Gels were transferred to nitrocellulose membranes (Immobilon-Nc, pore size 0.45 μm, during 75 min at 40 mAmps using a semi-dry electroblotter, HEP-1 Model, Owl Scientific Inc). The running buffer contained 25mM Tris, 192mM glycine pH 8.3 and 20% methanol. The nitrocellulose membranes were blocked overnight in 10% non-fat skim milk in PBS with 0.05% Tween-20 pH 7.4 and then washed 4 times for 10 minutes with PBS-Tween 20. Peroxidase-labeled recombinant protein LA (SpLA) conjugate was diluted 1:5000. This conjugates contains the fusion of SpA and the protein L (SpL) [12], and this last one is a surface antigen from *Peptostreptococcus magnus*. The SpLA has a protein binding capacity to immunoglobulins higher than the SpA alone [13]. The peroxidase-labeled SpLA conjugate was added to membranes and incubated at 4°C overnight. Membranes were washed as above and then Tetra-Methyl-Benzidine (TMB) was added and the reaction was stopped with deionised water.

Direct ELISA: An ELISA was used to determine the presence of ostrich Immunoglobulins (IgY) with binding capacity to react to SpA as previously reported in literature [14]. 96 well microtitre plates were coated overnight at 4°C with 1 μg/ml in each well of ostrich IgY samples in carbonate-bicarbonate buffer pH 9.6. Plates were washed four times with 150 μl PBS-Teen 20 buffer. Then 50μl of a commercial Horseradish-Peroxidase Labeled Staphylococcal Protein A (HRP-SpA) conjugate diluted 1:5000 in PBS-non-fat milk was added to each well and incubated for 1h at Room Temperature (RT). The plates were washed four times with PBS-Tween. 50 μl of 4 mg/ml o-phenylenediamine solution was added and the plates were incubated 15 minutes at RT. The reaction was stopped with 25 μl of 3M sulphuric acid solution. The plates were read in a microplate reader at 492 nm using standardized procedures [14].

Results

The chloroform-PEG method was successful for isolation of the ostrich IgY. The affinity chromatography was able to further purify the ostrich IgY. The purification of ostrich IgY by SpA affinity chromatography was a confirmative test of the IgY binding capacity to SpA. It proved that SpA columns could be used when high level of ostrich IgY purity is required. The protein electrophoresis and the Western blot analysis revealed that the ostrich IgY is a protein with heavy chain of 70 KDa and light chain of 30 KDa as shown in Figure 1 below. The whole molecule of ostrich IgY has a molecular weight approximately of 200 KDa. In addition two livetins (egg yolk proteins) were shown in between the light and heavy chain of IgY, they were separated in the protein electrophoresis but did not interact with SpA. These two livetins were identified as a 54 kDa protein band which was alpha-livetin and a 40 kDa protein band which was a smaller subunit of the lipovitellin fraction, a phosphorus-containing nucleo albumin.

Western blot analysis using SpLA-HRP as a marker demonstrated that either SpA reacted with both Fc and Fab region of the IgY or SpA molecule reacted to the Fc region and the SpL portion interacted with the Fab region, which is a well-known binding capacity of both SpA [15] and SpL [11].

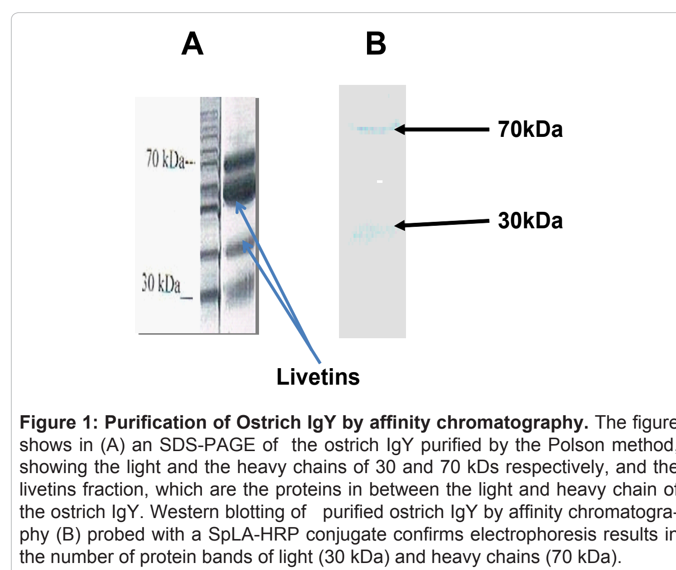

Figure 1: Purification of Ostrich IgY by affinity chromatography. The figure shows in (A) an SDS-PAGE of the ostrich IgY purified by the Polson method, showing the light and the heavy chains of 30 and 70 kDs respectively, and the livetins fraction, which are the proteins in between the light and heavy chain of the ostrich IgY. Western blotting of purified ostrich IgY by affinity chromatography (B) probed with a SpLA-HRP conjugate confirms electrophoresis results in the number of protein bands of light (30 kDa) and heavy chains (70 kDa).

The direct ELISA [14] demonstrated the interaction of SpA with the ostrich IgY. The positive control was human IgG and the negative control was chicken IgY which did not bind to SpA [15]. The cut-off point for the ELISA was 0.08; between 0.08 - 0.1 was considered as a weakly interaction, between 0.1 and 0.25 was considered as a moderate interaction and as a higher than 0.25 was considered a strong binding capacity. The test for SpA binding to ostrich IgY was 0.15 which had a moderate binding capacity.

Discussion

The purification of the ostrich immunoglobulins by SpA affinity chromatography has not previously been reported. The molecular weight of ostrich IgY was comparable to that previously reported [16]. The major problem in the isolation of egg yolk IgY is the removal of lipids, which are present in high concentrations. Separation of IgY requires extraction of the Water-Soluble Fraction (WSF) from egg yolk, followed by IgY purification from other proteins (livetins) present in the egg yolk [17]. To remove lipoproteins from the WSF, some methods have been reported including hydrophobic interaction chromatography [18]; water dilution method [19]; use of caprylic acid [20]; and filtration with hydrophobic membranes [21]. For the isolation of IgY from livetins (an egg yolk protein), several methods have been developed to improve the IgY quality and yield. These have included salt precipitation using ammonium sulphate or sodium sulphate followed by centrifugation [19,22]; alcohol precipitation [23, 24] and cation exchange chromatography [25, 26]. However, among the extensive variety of IgY isolation procedures, the use of chloroform combined with PEG-6000 is one of the most widely used methods (has a purity of up 85%). Its purity can be improved by the inclusion of an affinity chromatography step as we did in this study using SpA affinity chromatography, where the IgY purity is closed to 100%, as revealed by the western blotting.

SpA binds to the Fc fragment of IgG produced by several animal species including human, dog, rabbit, hamster, monkey [27], and birds such ostrich IgY as was proved in this study. The native SpA consists of five domains. Of these, four showed a high structural homology, contain approximately 58 aminoacids and have the capacity of binding to Fc regions of IgG [28]. In addition to the Fc gamma domains of IgG, SpA can interact with the Fab domains. It mediates conventional

antigen binding by Ig heavy-chains belonging to the VH3+ family [27], and SpA can bind to IgM of various species of animals.

In a previous study (unpublished results) we demonstrate the interaction between ostrich IgY and commercially-prepared peroxidase labeled anti-IgY conjugate, the one that reacted with a panel of 10 different birds suggesting that bird IgYs has probably a common ancestor. However, ostrich IgY failed to react with Protein G (SpG) from *Streptococci* and human Immunoglobulin G (IgG). We did not purify ostrich IgM from the egg white but we demonstrated a low to moderate binding affinity between ostrich egg white and SpA, which suggest that it is possible to purify ostrich IgM using SpA affinity chromatography. The use of specific enzyme-labeled ostrich IgY conjugates could be important for their use as markers in ELISAs for the detection of specific antibodies to Salmonella sp., *Escherichia coli* and Pseudomonas sp., which are microorganisms that infect ostriches instantly after hatching [7].

A major limitation to this study is the lack of data to adequately validate the information presented because of the novel nature of this work. This research will no doubt widely throw open doors for more future extensive experiments to determine SpA/IgY dissociation constant Kd, quantitative comparison of SpA/IgY verses anti-IgY/IgY interactions and so forth; which should further assist in delineating more linearity range and limit of detection, and binding capacity and efficacy of SpA for IgY isolation.

However, despite the above limitations, we still can conclude that the experiments described here suggest that SpA interaction with ostrich IgY could have importance in IgY protein purification. In addition, SpA conjugates could be used as a reagent in immunoassays for the diagnosis of infectious diseases in the ostrich, since SpA binds to ostrich IgY. Preparation of specific IgY to infectious gastrointestinal infectious agents could be developed to a large scale, because of the size of its egg aiding to substitute the use of antibiotics. The purification of the ostrich immunoglobulins and its interaction with SpA has biological value. It helped to understand structures and functions of these antibodies that play a major role in the mechanisms of defence of ostriches. And this work is the first time the use of SpA for the purification of ostrich IgY is report reported in literature.

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