Development of Protocol for Production of Primary Antibody against Ovalbumin Protein in Chicken for Detection of the Protein through Western Blotting

K.K. Kanaka¹, R.N. Chatterjee¹, Renu Shukla¹, Pushpendra Kumar², Bharat Bhushan² and T.K. Bhattacharya¹*

¹ICAR-Directorate of Poultry Research, Rajendranagar, Hyderabad, INDIA
²Animal Genetics Division, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, INDIA

*Correspondence author: TK Bhattacharya; E-mail: bhattacharyatk@gmail.com

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ABSTRACT

Ovalbumin, a major protein of egg white plays many roles including providing nutrition to the developing embryo, acting as coagulating agent, folliculogenesis and angiogenesis in chicken and other animals. This protein is expressed mainly in magnum and then deposited over the yolk of the oocyte/zygote. Hence, it is important in formation of egg and is an essential target to measure. We cloned chicken ovalbumin CDS in pAcGFP-C1 vector and has been initially expressed in chicken primary magnum cell culture. The ovalbumin protein tagged with 6x Histidine was purified from cell culture and used for production of primary antibody in rat. The ovalbumin protein along with freund’s adjuvant was injected to the rat, booster was given, and finally, hyper-immune sera was collected from rat. The antisera was purified for isolation of IgG. The IgG was used as primary antibody for Western blotting. Through Western blotting, ovalbumin protein isolated from chicken magnum was detected and the protocol was established to detect chicken ovalbumin protein.

Keywords: Ovalbumin, chicken, IgG, primary antibody, protocol

Ovalbumin is a member of SERPIN family and has been the major protein of the egg white in chicken. This protein is expressed mainly in magnum and has a molecular weight of 44.5 kDa comprising of 385 amino acid residues. It is a storage protein and major source of amino acids for the developing embryo (Mine and Shahidi, 2006). Other functions of ovalbumin are: acting as factors for coagulation/ fibrinolysis cascades (D’Alessandro et al., 2010), role in folliculogenesis and angiogenesis (Mann, 2008), serving as a source of amino acid for embryo and function on developing tissues (Sugimoto et al., 1999) and playing crucial role in calcium carbonate formation and amorphous calcium carbonate stabilization i.e. biomineralization (Schwahn et al., 2008). The N-terminus of ovalbumin is acetylated and contains four sulphydryl groups and one disulfide bridge (Cys74-Cys121), which are inaccessible in the native state (Iametti et al., 1998). Ovalbumin secondary structure has various motifs including α-helix (41%), β-sheet (34%), β-turns (12%), and random coils (13%) (Ngarize et al., 2004). The conserved reaction centre is located at Ala358- Ser359 (Stein et al., 1990) while the heterogeneous carbohydrate peptide chains contain a common core of mannose β (1-4) glcNAc β (1-4) glcNAc (Huntington and Stein, 2001; Guérin Dubiard et al., 2006). Types of ovalbumin protein are differentiated by the degree of phosphorylation with two, one and zero phosphorylated sites, respectively, which are located at serine residues 69 and 345 (Mine and Shahidi, 2006). Thus, activity of this protein is based on its phosphorylation sites in chicken and other animals. Detection of protein is normally accomplished by various techniques such as Western blotting or immuno-blotting and Enzyme linked immune-sorbent assay (ELISA). For detection of protein in these techniques, it is essential to have primary antibody which is detected by secondary antibody.
antibody conjugated with HRP conjugate and detection dye. In this study, we have developed protocol for production of primary antibody against chicken ovalbumin protein.

MATERIALS AND METHODS

Cloning of ovalbumin cDNA

An adult bird of IWK layer chicken line maintained at ICAR-Directorate of poultry Research, Hyderabad was sacrificed following the guidelines of Institute Animal Ethics Committee (IAEC) of the Institute. The adult birds are maintained by providing ad lib feeding and watering following standard management protocol (Chatterjee et al., 2008). The infundibulum tissue was collected and kept in 0.1% DEPC treated sterile polypropylene tube.

The total RNA was isolated from magnum tissues using Trizol (Amresco), according to the manufacturer’s instruction. The magnum tissues were homogenized with 1 mL Trizol/50 mg of tissue and total RNA was isolated following standard protocol (Bhattacharya et al., 2011b). The concentration and purity of the RNA was determined in Genova plus Nano Drop. The RNA sample showing the OD260:280 value in between 1.9 to 2.2 was considered as good quality and was used further. The RNA sample was treated with DNaseI (Fermentas) for removal of possible genomic DNA contamination. First strand cDNA was synthesized by using Revert Aid First Strand cDNA Synthesis Kit (Thermoscientific) (Bhattacharya et al., 2012).

For amplification of whole cDNA of ovalbumin (1161 bp), a pair of primers (F: GGAT CATG CATC ATCA CCAT CACC ACAT GGGC TCCA TCGG CGCA GCA and R: TTAA GGGG AAAC ACAT CTGC CAAA GAAG CTT) was designed by using DNASTAR software from the available ovalbumin gene sequence (NM_205152.2). The whole cDNA was amplified at 58°C annealing temperature and purified using gel purification kit (Qiagen).

The PCR amplified product was digested with BamHI and HindIII restriction enzymes and was cloned into an Expression vector, pAcGFP1-C1 (Clonetech) for expression of the gene following protocol of Bhattacharya et al., 2014.

SDS-PAGE

The 12% SDS-PAGE was prepared following standard protocol (Bhattacharya et al., 2011a) to analyse the purified protein. About 2µl protein was loaded in the gel and was run at 5V/cm of gel and the voltage was increased to 10V/cm. The gel was stained with Coomassie brilliant blue stain and visualized.

Preparation and immunization of antigen

The purified protein was mixed with complete Freund’s Adjuvant (CFA) and the mixture was injected subcutaneously on day1 at 4 sites of the rat. A total of 3 male Wistar rats (2 for target and 1 as control) of 3 months age were included in immunization schedule throughout period. The detailed Immunization protocol for rat polyclonal antibody production was as shown in Table 1. On 21st day, blood (having raised antibodies) was collected from retro-orbital sinus/plexus of rats and serum was collected. The IgG was purified from the hyper-immune sera with IgG purification kit (Protein A based, GeNei™). The purified IgG was used as a primary antibody for western blotting.

Western blotting

The protein isolated from magnum tissues was run on SDS-PAGE following standard method (Bhattacharya et al., 2019). The proteins separated in SDS-PAGE were
transferred into 0.45µm polyvinylidene fluoride (PVDF) membrane. The blotted PVDF was immersed in 3% BSA blocking buffer with primary antibody (1:1000 dilution in TBS Tween 20) and incubated at 4 °C for overnight. The membrane was washed with the TBS Tween 20 and incubated with anti-rat IgG HRP conjugate diluted to 1:1000 in TBS Tween 20 buffer for 1.5 hours with constant agitation. The PVDF membrane was incubated in DAB substrate solution for 5-30 minutes until the color development. Soon after the appearance of brown color, substrate solution was drained, and the reaction was stopped by adding distilled water.

**Table 1:** Immunization protocol for rat polyclonal antibody production. CFA= Complete Freund’s adjuvant; IFA= Incomplete Freund’s adjuvant

| Procedure          | Protocol day | Description                                                                 |
|--------------------|--------------|------------------------------------------------------------------------------|
| Control serum      | Day 0        | Pre-immune bleed (0.5ml per rat)                                             |
| Primary Injection  | Day 1        | Immunize with 0.1mg antigen with CFA, (sub-cutaneous injection at 4 sites)    |
| Booster Injection  | Day 14       | Boost with 50µg antigen with IFA, (sub-cutaneous injection at 4 sites)        |
| Blood collection   | Day 21       | Primary antibody production                                                  |

**RESULTS AND DISCUSSION**

The first strand cDNA were synthesized from total RNA obtained from magnum part of oviductal tissue of Iwk White Leghorn chicken. A 1161 bp coding sequence of ovalbumin gene was amplified using gene specific primers. Reaction conditions as well as cycle parameters of the PCR protocol was optimized to ensure the amplification of the specific fragment with good yield. The amplified PCR products were checked in 1% (W/V) agarose gel electrophoresis. A single band without any non-specific amplification was noticed at the expected position on the gel.

After successful amplification and gel elution, the amplified product was subjected to restriction digestion with BamHI and HindIII restriction enzymes. Consequently, the ready to use inserts were cloned to pAcGFP-C1 expression vector and transformed into DH5α *E.coli* competent cells. Then, we screened for positives colonies (Fig. 1) and positive clones were sub-cultured from which Plasmid DNA was isolated and further sequenced for confirmation of the insert.

The 1161 bp coding sequence of ovalbumin encodes 386 amino acid residues of ovalbumin protein, which was in agreement with the findings of Ishimaru, *et al.* (2018). Ovalbumin protein has a molecular weight of 44.5 kDa and is a monomeric phosphor glycoprotein with known complete amino acid sequence of 386 residues. Although it is a secretary protein, ovalbumin is lacking an N-terminal leader sequence. Transmembrane location is instead mediated by an internal sequence signal located within hydrophobic residues (Huntington and Stein, 2001).

The recombinant vector containing ovalbumin open reading frame was transfected into primary magnum cell culture by electroporation. The transfected cells were subcultured and harvested after 6 days. The crude protein was isolated from the cell lysate. The crude protein was subjected to column chromatography using His-Spin Protein Miniprep™ to purify Histidine tagged ovalbumin protein. The His-tagged protein was detected in the SDS-PAGE having single band.

The purified ovalbumin protein along with Freund’s adjuvant was injected into growing Wister rat on day one and booster was given on day 14 and the antibodies developed against the protein was isolated from the serum on day 21st. Blood samples (having raised antibodies) was
collected from retro-orbital sinus/plexus of rats and serum was collected. The IgG was purified from the hyper-immune sera with IgG purification kit. The purified IgG was used as a primary antibody for western blotting to detect ovalbumin protein.

The crude protein was collected from magnum tissues. The crude protein was analysed in SDS-PAGE and proteins were separated. Then, through Western blotting, the ovalbumin protein was detected in the magnum tissues indicating the efficiency of primary anti-body developed against ovalbumin protein in chicken (Fig. 2). This primary antibody may be used to develop Western blot assay as well as ELISA assay for detection of ovalbumin protein in the tissues.

**Fig. 2:** Western blot of ovalbumin protein expressed in magnum tissues of 2 birds and a control bird with muscle tissue. Magnum tissues of bird 1 and bird 2 showed presence of ovalbumin protein while muscle tissue of control bird did not show ovalbumin protein in muscle

**CONCLUSION**

The results revealed that the recombinant ovalbumin protein may be used to develop primary antibody against ovalbumin protein for further use to develop assay for detection of the chicken ovalbumin protein.

**CONFLICT OF INTEREST**

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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