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Production, characterization and application of monoclonal antibodies against chicken IgY

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ABSTRACT

Monoclonal antibodies (Mabs) were produced against chicken IgY purified from egg yolks. Purity of the IgY was established using immunoelectrophoresis and reducing and non-reducing polyacrylamide gel electrophoresis. Five stable hybrid clones were characterized and Mabs, all belonging to IgG_1 isotype, were found to bind to the IgY heavy chain in Western blots. These clones did not cross-react with IgM in enzyme-linked immunosorbent assay (ELISA). One of the Mabs was used as a capture antibody in ELISA for estimation of IgY purified from egg yolk by different methods. Caprylic acid precipitation method gave the highest recovery of IgY and with high purity.

Key words: chicken IgY, monoclonal antibodies, enzyme-linked immunosorbent assay, polyacrylamide gel electrophoresis, caprylic acid

Introduction

Chicken immunoglobulin (Ig) G, or as it is also known, IgY, is phylogenetically distinct from mammalian antibodies and has very different properties. It does not bind bacterial Fc receptors such as staphylococcal protein A or streptococci protein G or mammalian Fc receptors, as do most mammalian Igs (KRONVALL et al., 1974). Chicken IgY is the major serum

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antibody, however. The antibody is also transported to the egg similarly to placental transfer of IgG in mammals. The antibodies in the yolk protect the newly hatched chicken.

Chickens have become the preferred choice of animal for antibody production, since their antibody responses are better when mammalian antigens are used due to their phylogenetic distance (SHIMIZU et al., 1995; WOOLEY and LANDON, 1995). Chicken IgY purified from yolk avoids the invasive method of drawing blood from the animal. Due to present-day constraints regarding animal experimentation, chicken yolk IgY offers an easy and acceptable alternative for production of antiserum. Moreover, the active transport of IgY from serum to the egg occurs in a higher concentration than in serum. Thus, more antibodies can be produced per month than in rabbits (GOTTSTEIN and HEMMELER, 1985; HATTA et al., 1993). IgY is very stable under normal conditions. LARSSON et al. (1993) stored IgY preparations for 10 years at 4 ^{0}C, for 6 months at room temperature and for 1 month at 37 ^{0}C without any antibody loss.

Chicken IgY isolated from egg yolk has a molecular weight (180 KDa) and upon reduction dissociates into heavy chains (60 KDa) and light chains (25 KDa). Several methods for extraction of antibodies from egg yolk have been described (JENSENIUS et al., 1981; BADE and STEGEMANN, 1984; POLSON et al., 1985; HATTA et al., 1990).

The pioneering work of KÖHLER and MILSTEIN (1975) on *in vitro* synthesis of monoclonal antibodies (Mabs) by hybrid cell lines provided a method potentially superior to conventional techniques in respect of purity, specificity reproducibility and permanent availability of antibodies for use in any assay system.

The aim of the present study was to produce and characterize Mabs against chicken IgY. These Mabs were then applied in a capture enzyme-linked immunosorbent assay (ELISA) for estimation of IgY purified from egg yolk using different methods.

Materials and methods

Chicken IgY. Chicken IgY was purified from specific-pathogen-free (SPF) chicken eggs (Venkateshwara Hatcheries, Pune, India) using a

commercial kit (EGGstract purification system, Promega, U.S.A.) following manufacturer's procedures. The protein concentration of the purified IgY was determined by Lowry's method using a kit (Bangalore Genei, India).

Antisera to chicken serum. Polyclonal anti-sera were raised by immunizing rabbits with chicken serum, 4 times at 2-weekly intervals with Freund's adjuvant. This polyclonal serum was used for confirmation of the purity of chicken IgY in immunoelectrophoresis (IE) assays.

Immunoelectrophoresis. IE was performed in agarose gels using the purified IgY as antigen in the wells and rabbit anti-chicken serum in the troughs. The gels were run in barbital buffer (pH 8.2) for 4 hrs at 8 mA current according to the method HUDSON and HAY (1989).

Polyacrylamide gel electrophoresis (PAGE). The purified IgY samples were run on 10% sodium dodecyl sulphate (SDS)-PAGE as described by LAEMMLI (1970) under reducing or non-reducing conditions. The gels were stained with Coomassie blue and the molecular weights of the proteins resolved estimated in comparison to the molecular weight markers.

Production of monoclonal antibodies. The Mabs used in this study came from several fusions for which BALB/c mice were immunized at least five times at 15-day intervals with about 100 μg of IgY purified by the EGGstract purification system (Promega, U.S.A.) in phosphate buffered saline (PBS) or emulsified in Freund's complete (for the first priming) or incomplete (for subsequent priming) adjuvant. The mice were boosted intravenously with 50 μg of purified IgY (without adjuvant) 3 days prior to fusion. Spleen cells were fused with mouse myeloma cells, Sp2/0 following conventional procedures (GALFRE et al., 1977). Supernatants from wells containing hybrid cells were tested for IgY specific antibodies by ELISA. Positive clones were then sub-cloned twice and used for further characterization. Antibody isotypes were determined by ELISA using an isotyping kit (Sigma, U.S.A.)

ELISA. Plates were coated with IgY (10 μg/well) in carbonate-bicarbonate buffer (0.01 M, pH 9.6) overnight at 4 ^{0}C. The plates were washed with PBS containing 0.05% 20 (PBST) between each step. Throughout the test, reagents were added in 100 μl volumes. After blocking

the wells with PBS containing 3% bovine serum albumin (BSA), culture supernatants from hybridomas were incubated for 1 hr at 37 ^{0}C. The plates were then washed and goat anti-mouse IgG peroxidase conjugate (Sigma, U.S.A.) was added at optimum pre-determined dilution. After one hr incubation at 37 ^{0}C and washing, a substrate solution containing 0.022% of 2, 2' diazino bis (3 ethyl) benz-thiazoline-6 sulfonic acid (ABTS) in sodium citrate buffer, pH 4.2 containing 0.015% of H_2O_2 was added. The reaction was stopped with 5% SDS and optical density (OD) was read at 405 nm in an ELISA reader (Biotek, U.S.A.)

Western blots. The western blot analysis to determine the specificity of the Mabs produced was carried out according to the method described by ERHARD et al. (1992), with certain modifications. The purified IgY separated on SDS-PAGE under reducing conditions was transferred onto nitrocellulose membranes in an electroblot apparatus (Biorad, U.S.A.) for 1 hr at 350 mA. After blotting, the membranes were blocked with PBS and 3 % BSA for 1 hr at 37 ^{0}C. The membrane was then washed in PBST for 10 minutes in 3 changes, followed by the addition of tissue culture fluid from positive clones. Following overnight incubation at 4 ^{0}C the membrane was washed with PBST and anti-mouse biotin conjugate (Sigma, U.S.A.) was added at 1:10,000 dilution and the membranes incubated for 1 hr at 37 ^{0}C. The membranes were washed again and streptavidin-peroxidase conjugate (Sigma, U.S.A.) at 1:1000 dilution was added. After incubation and washing, the substrate solution containing diamino benzidine (DAB) was added onto the membrane for colour development, and then examined for presence of specific bands.

Cross-reactivity of IgY Mabs with chicken IgM. The specificity of IgY Mabs was determined by assessing cross-reactivity in ELISA using Sephadex-G 200 fractionated IgM and chicken IgY as coating antigen. All the anti-IgY positive tissue culture fluids were tested against IgM.

Purification of IgY from chicken egg yolk

i) Kit method. The yolk IgY was purified using a commercial kit (Promega, U.S.A.) following manufacturer's instructions.

ii) Polyethylene glycol (PEG) method. This method was similar to the kit method except that 4% PEG solution was used instead of precipitation

solution A (of the kit) and 46% solution of PEG in place of precipitation solution B.

iii) Organic solvent method. The procedure followed was similar to that described in BADE and STEGEMANN (1984). Briefly, Igs from yolks were precipitated with isopropanol and the lipid materials were removed with isopropanol and acetone. The precipitate was extracted with PBS and then used.

iv) Caprylic acid method. The procedure followed was similar to that used with chicken serum by BHANUSHALI et al. (1994). Briefly, 10 ml of egg yolk was diluted with 4x volume of acetate buffer (60 mM pH 4.0) and the pH was adjusted to 4.5 with 1N sodium hydroxide. Caprylic acid (Sigma, U.S.A.) was then slowly added with constant stirring to a total of 25 µl per ml of diluted yolk. The solution was stirred for an additional 30 min and the insoluble material removed by centrifugation at 10000 xg for 30 min at 4 ^{0}C. All steps prior to centrifugation were carried out at room temperature. Subsequent steps were carried out at 4 ^{0}C.

The supernatant was collected and filtered through a gauze cloth to remove any fine particles. The supernatant was then mixed with 1:10 its volume of 10× PBS with 20 ml of 100 mM EDTA per litre of 10× PBS, pH 7.4) and the pH was adjusted to pH 7.4 with 5N sodium hydroxide. The second precipitation was carried out by slow addition of saturated ammonium sulphate at 50% level. The mixture was stirred for 30 min and centrifuged at 10000×g for 30 minutes. The pellet was re-suspended in 1 ml of 1× PBS and dialyzed against PBS with at least 2 changes of buffer.

Development of sandwich ELISA for yolk IgY estimation. Mabs against chicken IgY were coated using carbonate-bicarbonate buffer onto ELISA plates and incubated overnight at 4 ^{0}C. Following washing and blocking, commercial IgY standards (Promega, U.S.A.) were added at different concentrations, together with IgY purified through different methods. After incubation for 1 hr at 37 ^{0}C and washing, anti-chicken IgY peroxidase conjugate (Bangalore Genei, India) was added at 1:2000 dilution. Colour was developed using ABTS substrate and OD values read. The ODs of the standard and their concentrations were used to develop a regression equation in MS Excel. Their IgY concentrations were estimated using this equation and the OD values of the samples.

The IgY purified using the 4 different methods was checked for their recovery in the capture ELISA and purity in SDS-PAGE.

Results

The IgY purified from yolk using the commercial kit was used as an immunogen for the production of Mabs against it. The recovery of IgY using this method was 89%. Purity was checked by IE and SDS-PAGE. On IE, the IgY gave a single precipitin line with rabbit anti-chicken serum and a single band of about 180 KDa under non-reducing conditions of SDS-PAGE. Under reducing conditions, 2 bands of 60 and 25 KDa were seen, with no other contaminating bands (Fig. 1.).

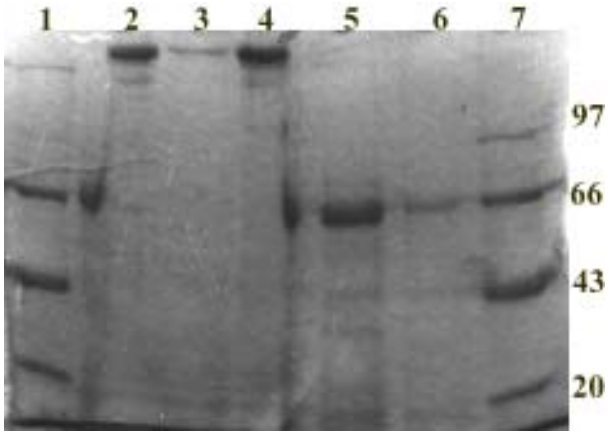

Fig. 1. PAGE profile of chicken IgY extracted from yolk - Reduced and Non-reduced. Lane 1: molecular weight markers; lane 2 and 5: non-reduced and reduced forms of IgY extracted using commercial kit (Promega, U.S.A.); lanes 3 and 6: non-reduced and reduced commercial standard IgY (Promega, U.S.A.); lane 4: non-reduced IgY extracted by the PEG method; lane 7: molecular weight markers. Note bands of about 180 KDa of non-reduced IgY and 6 and 25 KDa in reduced forms of IgY.

Nine clones positive for antibodies to yolk IgY were obtained after initial screening by ELISA. These clones had initial ODs in a range of 1.07 to 1.25. After sub-cloning twice, 5 clones were strongly positive, namely C11E4, C11E5, C11F2, C10B4 and C10B11, their OD values being 1.073, 1.192, 1.090, 0.622 and 0.621, respectively. All 5 clones were of IgG1 isotype.

Table 1. Cross-reactivity of IgY Mabs with IgM in ELISA

Clone	Mean (± SD) OD with IgY	Mean (± SD) OD with IgM
C11E4	1.073 ± 0.008	0.102 ± 0.006
C11E5	1.192 ± 0.103	0.081 ± 0.008
C11F2	1.090 ± 0.050	0.080 ± 0.019
C10B4	0.622 ± 0.004	0.076 ± 0.016
C10B11	0.621 ± 0.003	0.102 ± 0.015
Myeloma (SP2/0)	0.089 ± 0.006	0.089 ± 0.005

When these five Mabs were tested on reduced IgY on Western blots, they showed staining of the 60 KDa bands, which is probably the heavy chain. Light chain staining was not seen. No bands were seen with negative culture supernatants (data not shown). When IgM was used as the coating antigen in ELISA, these five culture supernatants showed no cross-reaction with it (Table 1).

Table 2. Estimation of IgY content in samples purified by different methods using Mabs against IgY as capture antibodies in ELISA

Technique	Total IgY	Purification (fold)	Recovery (%)
Commercial kit	160 mg/120 mg	5.0*/5.3	89**/76
PEG method	160 mg/120 mg	5.0/5.3	73/74
Caprylic acid method	160 mg/120 mg	4.0/4.3	97/90
Organic solvent method	160 mg/120 mg	1.0/1.1	69/58

* The final volume of sample divided by the initial volume of yolk used.
** The IgY content in the purified sample divided by the original IgY content in yolk (column 2).

The two figures indicate the results of two different trials.

Using one of these Mabs from a fast growing clone, C11F2, a capture ELISA was developed. The correlation coefficients of the IgY standards with their respective ODs were always higher than 0.93 using regression analyses. Using this capture ELISA, the recovery of yolk IgY by different methods were assessed and is shown in Table 2. The purity of the yolk IgY purified by different techniques was also checked on SDS-PAGE (Fig. 2). The caprylic acid method gave the highest recoveries of yolk IgY consistently, which was also the most pure.

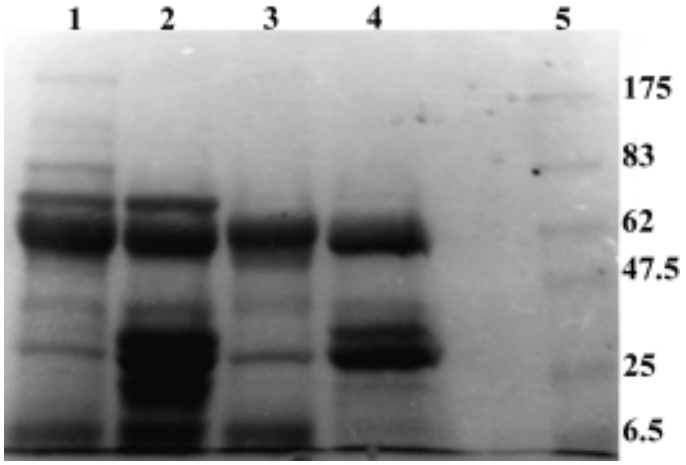

Fig. 2. PAGE profiles of chicken IgY extracted with different methods. Lane 1: reduced IgY extracted using the kit, lane 2: reduced IgY extracted by PEG, lane 3: reduced IgY extracted by caprylic acid precipitation, lane 4: reduced IgY extracted using organic solvent, lane 5: molecular weight markers.

Discussion

The aim of this work was to produce Mabs against chicken IgG and apply them to estimate the IgY content in yolk. Mabs were produced by conventional techniques using yolk IgY purified using a commercial kit as an immunogen. The purity of yolk IgY was established in IE and PAGE applying both reducing and non-reducing conditions. Several clones were obtained and stabilized clones were used for further characterization. The

constant region of the heavy chains determines the isotype of an Ig. Hence, it is known that Mabs specific to chicken IgY must be against an epitope on the heavy chain constant region. This was confirmed by Western blotting wherein the heavy chain of IgY was stained. In addition, lack of cross-reactivity to chicken IgM was confirmed in ELISA assays. Mabs produced against chicken IgM were used as controls (data not shown, manuscript in preparation).

These Mabs could be useful in various immunoassays, especially ELISA-based ones. It could be used in place of anti-chicken IgG whole molecule peroxidase conjugates raised in heterologous species and also for estimating total Ig content in serum of chickens. Such assays are used for measuring the immune status in different inbred lines of chickens. They can also be used for preparation of immunoaffinity columns for purification of chicken IgY, especially since IgY does not bind to protein A or protein G. It can also be used for measurement of antigen-specific IgG responses only. In this study we have used the Mabs for estimating IgY content in egg yolks purified by different techniques.

The first step in the isolation of yolk IgY from eggs yolks involves the extraction of soluble proteins from non-aqueous materials such as lipids and lipo proteins. Methods for this include the precipitation of the non-aqueous material by diluting the yolk in buffer or water and with the addition of a precipitant (JENSENIUS et al., 1981; SONG et al., 1985; AKITA and NAKAI, 1993). In addition, we have used the caprylic acid method of purification, which, to our knowledge has not previously been applied for the purification of yolk IgY. However, it has been shown to be a useful non-chromatographic method for purifying IgG from chicken serum (BHANUSHALI et al., 1994). Of the 4 methods compared, the caprylic acid method yielded the highest recovery and purity of the separated IgY, followed by the PEG method. A recovery rate of 85% of IgY from chicken eggs has been reported using T-gels (HENSEN et al., 1998).

The Mabs against IgY were used to estimate the total IgY content in the samples purified by different methods. It has been shown that IgY, especially the light chains, stain poorly with Coomassie blue dye (HENSEN et al., 1998). Thus, measurement of IgY concentrations by dye binding assays results in a substantial underestimation of IgY. In this context, the capture

ELISA developed using Mabs against IgY can be a useful alternative for estimating IgY content of samples with the specificity rendered by the Mabs, coupled with the ease of ELISA methodology.

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RAJ, G. D., B. LATHA, M. S. CHANDRASEKHAR, V. THIAGARAJAN: Proizvodnja, karakterizacija i primjena monoklonskih protutijela za pileći IgY. Vet. arhiv 74, 189-199, 2004.

SAŽETAK

Proizvedena su monoklonska protutijela za pročišćeni IgY iz žumanjka jajeta. Čistoća IgY dokazana je imunoelektroforezom te reducirajućom i nereducirajućom elektroforezom u poliakrilamid gelu. Obilježeno je pet stabilnih hibridnih klonova, a za monoklonska protutijela koja su pripadala izotipu IgG1 ustanovljeno je da su se vezala za teški lanac IgY u Western blotu. Ovi klonovi nisu unakrižno reagirali s IgM u imunoenzimnom testu (ELISA). Jedno od monoklonskih protutijela rabljeno je kao vezano protutijelo u imunoenzimnom testu za procjenjivanje pročišćenosti IgY različitim metodama. Metoda precipitacije s kaprilnom kiselinom bila je najosjetljivija u otkrivanju IgY visoke čistoće.

Ključne riječi: pileći IgY, monoklonska protutijela, imunoenzimni test, elelktroforeza u poliakrilamid gelu, kaprilna kiselina