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# Chicken Antibodies – Superior Alternative for Conventional Immunoglobulins

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The aim of this review is to deal with all the major aspects of chicken antibody (IgY) technology, report on the latest development on IgY purification procedures as well as ways of IgY application. From the point of view of the immunized animal welfare, the production of chicken antibody brings great benefit, since the antibody preparation is a non-invasive technique making use of collected eggs. Egg yolk is an abundant and every day source of IgY (about 100 mg IgY/yolk). In addition, the isolation of IgY from egg yolk is fast and simple. Chicken antibodies also offer a lot of advantages to the common mammalian antibodies when they are used e.g. for the detection of mammalian antigen. Due to the evolutionary distance chicken IgY will react with more epitopes on a mammalian antigen, which will give an amplification of the signal. Chicken antibodies can also be used to avoid interference in immunological assays caused by the mammalian complement system, rheumatoid factor or Fc-receptors. As the antibodies can be purified in large amount from egg yolks, these immunoglobulins are suitable for passive immunization against pathogenic microorganisms and toxins. Thus, IgY technology should be considered as a good alternative and/or superior substitute to conventional polyclonal antibody production in mammals.

**Key Words: Immunoglobulins, Chicken egg, Yolk antibody, Domain structure, IgY Application, Purification**

## Introduction

Antibodies are key proteins of organism's specific immune response. These immunoglobulins are widely used in clinical practice for determination of levels of either own antibodies (HIV test, IgE), proteins associated with various diseases (cancer markers) or low molecular weight compounds (progesterone). The great majority of clinical diagnostic tests have been converted to ELISA or strip tests for fast diagnostics. Besides the field of diagnostics, antibodies are applied as "antidote" in order to neutralize e.g. toxins (tetanus toxin, snake venom) or as a means of passive immunization against for example microbial or viral diarrhoea infection (*Escherichia coli*, rotavirus). Antibodies are also indispensable tool in protein science. They are frequently employed for detection and/or determination of various antigens (proteins) making use of techniques such as immunodiffusion, ELISA, and Western blotting. Inhibitory antibodies, blocking a ligand-receptor interaction or affecting enzyme activity, are exploited in biochemistry and enzymology. For example, for separation of a desired protein from a complex mixture (even when its concentration is extremely low), immobilized antibodies on a chromatography support are well suited for affinity purification.

Most frequently, the antibodies are obtained from blood of an experimental animal (e.g. mouse, rabbit, goat, pig, horse) collected either by repeated bleeding or heart puncture resulting in death of the animal. The other source of antibodies is mammalian colostrum. Mammalian antibodies, however, could be substituted with immunoglobulins obtained from avian eggs. Evidence is accumulating that egg antibodies possess properties comparable or in some regards even better than those of mammalian ones (Carlander et al.1999, Hodek 2000, Tini et al. 2002). One might expect that application of avian egg antibodies, namely those of chicken

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(*Gallus domesticus*), will gain wide acceptance as a appropriate substitute and /or superior alternative to mammalian ones. This review is devoted to publicize IgY technology and show its inherent advantages related to all crucial areas of polyclonal antibody application.

## Avian Immunoglobulins

Mammals protect their offsprings during the early postnatal period of life by passive immunization using colostrum which is rich in antibodies. These breast milk antibodies neutralize pathogens invading the gastrointestinal tract of the young. In contrast, birds make use of another protection strategy. During egg formation, blood immunoglobulins, corresponding to mammalian IgG, are concentrated in the yolk, while IgA and IgM plus other proteins are secreted into the egg white (Morrison et al. 2001). The concentration of IgG in egg yolk is 1.3-1.9 times higher than that in hen blood (Rose et al. 1974, Sunwoo et al. 1996). The newly-hatched chicken pulls the yolk sack into its body. Then the yolk sack serves as a nutrition source as well as a means of passive immunization of the young chick. While the yolk IgG is circulating in chicken blood, IgA and IgM from the egg white protect the chicken's gastrointestinal tract against infections.

Mammalian immunoglobulins are classified into five groups: IgA, IgD, IgE, IgG and IgM. On the other hand, birds possess only three classes of immunoglobulins: IgA, IgG and IgM. Moreover, the structures of the corresponding immunoglobulins are significantly different, namely in the number of constant domains within the heavy chains. While avian IgG, like mammalian IgE and IgM, is comprised of four constant domains, mammalian IgG contains only three. Most likely in the case of mammals one constant domain was reduced to a short segment forming the hinge region typical for mammalian IgG (Warr et al. 1995). This region provides both arms of the mammalian IgG with a flexibility that underlines the bifunctional character of IgG. Because of differences between mammalian and avian IgG Leslie and Clem (1969) recommended the term IgY (meaning from yolk) for avian IgG. It is interesting to note that the same difference in the number of constant domains found for mammalian and avian IgG exists for IgE of both species. It is assumed that avian IgY and both mammalian IgG and IgE were developed from a common immunoglobulin similar to IgY. Similar conclusion follows from the analysis of a carbohydrate moiety of mammalian and avian immunoglobulins (Ohta et al. 1991).

### *Properties of IgY*

As mentioned above, IgY is a major avian immunoglobulin, which corresponds closely, from the point of view of its function in the body and immunochemical use, to mammalian IgG. As early as 1962, IgY protein was identified by Williams (1962) as gamma-globulin in a gamma-livetin fraction of yolk. Its concentration in the blood is 5-6 mg/ml, while in egg yolk is 10-25 mg/ml (Leslie & Martin 1973, Rose et al. 1974). However, in both cases the concentration of IgY is lower than that of IgG in the blood of mammals, e.g. for rabbit 35 mg IgG/ml (Hatta et al. 1993).

From the view of application, it is important to note the advantages of chicken IgY: these antibodies do not react with rheumatoid factor, mammalian Fc receptors and do not activate the mammalian complement system. The fact that these factors do not bind with IgY results in e.g. less background due to nonspecific fluorescence as compared to IgG in immunofluorescence assays (Cipolla et al. 2001). These properties of chicken antibodies make them a superior tool for immunodetection techniques on a solid phase when using mammalian sera as samples (Larsson & Sjöquist 1988, Benson et al. 1961). In addition, IgY in contrast to IgG shows very low affinity for binding of protein A and G (Kronvall et al. 1970, Larsson & Lindahl 1993), explaining why sorbents with immobilized protein A and G are not applicable for their purification as it is common for IgG.

Since the heavy chain ($H_c$) of IgY is one constant domain longer than that of IgG, its relative molecular weight increases to $6.4 \times 10^4$ as determined by SDS-polyacrylamide gel electrophoresis. Molecular weight of the light chain ($L_c$) is around $2.8 \times 10^4$ (Hatta et al. 1993). The presence of the forth constant domain affects the ability of IgY to form an immunoprecipitate with antigens. The reason arises from differences in IgG and IgY flexibility reflecting

their different structures. The absence of a hinge region in the case of IgY reduces motion of the arms containing the variable domains resulting in a steric hindrance which affects the cross-linking of a binary complex antigen-antibody necessary for precipitate formation (Gallagher & Voss 1974). Usually, when the molecular weight of antigen is lower than $3 \times 10^4$, precipitation does not occur even though the binding between IgY and antigen is strong enough. On the other hand, in the literature there is an accumulating evidence showing chicken IgY to be precipitating antibodies even without addition of compounds supporting precipitation (e.g. polyethylene glycol 8000) (Polson et al. 1980). The presence of an additional constant domain $H_C2$ of IgY results in a higher (almost 2 times) saccharide content. On this $H_C$ domain there is another site for saccharide chain attachment. Because of high mannose content (110 nmol/mg IgY) IgY strongly interacts with Concanavalin A, a lectin from *Canavalia ensiformis* (Hodek, unpublished results). The saccharide structures found in IgY are unique, since they contain rare glycosylated oligosaccharides of an oligo-mannose type (Ohta et al. 1991).

Moreover, the next difference between IgY and IgG is also in their pI values. IgY has a pI value shifted by about one *p*H unit to the acidic region 5.4-7.6 (Hatta et al. 1993, Hodek 2000). This might be a reason for the differences of IgY and IgG stability in strong acidic conditions. While 2 hrs incubation of IgY at *p*H 2 leads to 90% loss of its activity, IgG retains under these conditions more than 45% of its activity (Hatta et al. 1993). On the other hand, thermo-stability of both, IgY and IgG, is similar. A $T_{max}$ value of 74°C for IgY is 3°C lower than that for IgG. However, these minor differences of chicken immunoglobulins could hardly overshadow all the great advantages of IgY technology.

### *Production and Purification of IgY*

For more than a century the chicken egg has been well known as a rich source of immunoglobulins (Kleperer 1893). An average egg contains about 100 mg IgY per yolk. Although the immunoglobulin concentration in the yolk is lower than that in mammalian serum, the every day production of eggs overcomes this disadvantage. When, for example, the rabbit and the chicken are compared in terms of antibody production, one can draw the surprising conclusion that one chicken produces in a year as many (antigen specific) antibodies as it could be obtained from the blood of 30 rabbits within the same period of time (Hatta et al. 1993). Immunization of the chicken results in formation of specific antibodies which comprise, 0.1-10% of total IgY in dependence on the antigen (Shade et al. 1994). Mostly, the immune response to a common antigen does not increase the total content of IgY in the yolk.

To stimulate the immune response of experimental animals, the desired antigen is applied in combination with various adjuvant compounds (Hilgers et al. 1998). Using these compounds antigen is appropriately presented, e.g. in the form of an emulsion, to the organism and the immune system non-specifically stimulated to produce antibodies by additional components (e.g. adamantyldipeptide, inactivated microorganisms or their parts). Of tested adjuvant preparations the best results were obtained with emulsions of antigens in mineral oil mixed with complete Freund's adjuvant for the first injection and with incomplete Freund's for boosters. Similar immune responses were found when only solutions of antigens in PBS were used for injections (Svendsen-Bollen et al. 1996, Schwarzkopf & Thiele 1996). Just recently, application of lipid nanoparticles causing only minor tissue irritation at the injection sites, appears to be a promising alternative to complete Freund's adjuvant (Olbrich et al. 2002).

In respect to antibody titers it is hard to judge which organism, the rabbit or the chicken, is superior for antibody production. Even when the experimental conditions are kept to be the same for both animal species the titers depend on the antigen immunogenicity for the animal used. For example, the chicken is able to produce antibodies against one serotype of rotavirus with a neutralization titer more than 4 times higher than that derived from rabbit blood. On the other hand, for another serotype chicken antibodies show lower activity than rabbit antisera (Hatta et al. 1993). Thanks to the evolutionary distance between birds and mammals, the chicken is superior for the production of antibodies against conserved mammalian antigens, which are hardly immunogenic for

experimental mammals. Chicken IgY is usually produced against a greater number of antigenic epitopes on a mammalian antigen thus giving an amplified signal and greater test senzitivity.Another advantage lies in the possibility of developing high titer chicken antibodies even though low doses of mammalian antigen (0.001-0.01 mg/dose) are applied (Gassmann et al. 1990, Larsson et al. 1998). In conclusion, when preparation of antibodies against conserved mammalian antigens is considered, chicken IgY technology should be chosen (Knecht et al. 1996).

So far, the major limitation preventing a wide application of IgYs lies most probably in their purification from egg yolks. It is true that a procedure as simple as the preparation of antisera from mammalian blood is not available for chicken antibodies. IgY comprises about 5% of egg yolk proteins dispersed in yolk lipid emulsion together with lipoproteins and glycoproteins (Juneja & Kim 1997). There is a plenty of different procedures developed for IgY purification (Hodek et al. 1998, Stalberg et al. 2001, De Meulenaer & Huyghebaert 2001). The first step of these procedures (after yolk separation) is always based on removal of the lipid fraction by its extraction into organic solvent, precipitation using freezing or precipitation agents or hydrophobic chromatography. Recently, the use of an aqueous two-phase system with phosphate and Triton X-100 separation of lipids and water-soluble proteins (IgY fraction) has been introduced (Stalberg et al. 2001). The resulting water-soluble protein fraction is usually separated by fraction precipitation or chromatography on ion-exchange, thiophilic or size-exclusion columns (Polson et al. 1980, Bade & Stegemann 1984, Hassl & Aspöck 1988, Hatta et al. 1990, Akita & Nakai 1993, Schwarzkopf & Thiele 1996, Cook et al. 2001). Interestingly, chicken antibodies were efficiently captured from crude samples on an affinity column with immobilized synthetic ligand for immunoglobulins. Using this technique in a single purification step the purity of IgY higher than 90% was obtained (Verdoliva et al. 2000). The majority of protocols, however, apply 2-3 purification steps to obtain a final preparation of a high purity (98%), yielding 70-100 mg IgY per yolk. To prepare monospecific antibodies, an affinity chromatography technique on immobilized antigen is usually exploited. Specifically bound IgY is eluted by strong acidic or basic buffers (Ntakarutimana et al. 1992, Kuronen et al. 1997, Tini et al. 2002). Purified IgYs show high stability when they are stored at 4°C. They have retained their activity for more than 10 years (Larsson et al. 1999).

### *Application of IgY*

As described above, chicken eggs are a rich source (25 g IgY/year/chicken) of excellent antibodies, which in several regards surpass mammalian ones. The relatively inexpensive production of large quantities suggests that IgYs could be used for purposes where a high amount of immunoglobulins is necessary to reach the intended therapeutic effect. Prophylaxis and/or acute passive immunization are relevant fields. Common mammalian antibodies were applied for these purposes only exceptionally to treat cases of emergency, because of their cost. That is why, there is a broad area for application of chicken antibodies. IgY are extensively tested to be used for prophylaxis purposes such as against bovine rotavirus causing death of newborn calves (Kuroki et al. 1994), *Salmonella enteritidis* or *typhimurium* infections (Lee et al. 2002), pathogenic strains of *E. coli* of piglets (Jin et al. 1998), for deactivation of urease of *Helicobacter pylori* (Chang et al. 2001) or *Streptococcus mutans* that is considered to be the main oral microorganism responsible for tooth cavity formation (Hamada et al. 1991). In most cases, there is no need for purification of the IgY fraction - it is possible to use dried egg yolks of immunized chicken. On the other hand, for the acute treatment of intoxication caused by microbial or snake toxins, antibodies must be purified as much as possible to reduce side effects (Thalley & Carroll 1990, Devi et al. 2002). Antibodies raised against staphylococcal enterotoxin B were tested in the respect of passive vaccination of nonhuman primates. All rhesus monkeys treated with the IgY specific for enterotoxin B up to 4hr after challenge survived lethal toxin aerosol exposure, suggesting potential therapeutic value of specific IgY (LeClaire et al. 2002). Using of chicken antibodies is absolutely necessary in cases when application of mammalian antisera might result in anaphylactic

shock. Rather new way of application of IgY technology is a medical area of xenotransplantation (Fryer et al. 1999). Antiporcine endothelial cell antibodies effectively block human antiporcine xenoantibody binding that is expected to inhibit xenograft rejection by endogenous antibodies. The purpose of this approach is based on the failure of IgY to activate mammalian complement system (see below) (Tini et al. 2002).

Immunodiagnostics in clinical chemistry is another widespread area of chicken antibody application. In this respect, all the advantages of IgYs, e.g. high titers against conserved mammalian antigens and no reactivity with rheumatoid factor, complement system, Fc receptors, can be fully utilized. Mammalian antibodies used for ELISA frequently give rise to false positive response in tests with mammalian sera because of the interference caused by the afore-mentioned protein systems (Larsson & Sjöquist 1988, Carlander et al. 1999). Since IgYs, in contrary to IgGs, do not bind protein A (produced by *Staphylococcus aureus*) chicken antibodies are well suited also for detection of antigens (pathogens) in stool samples. Another promising application of IgY consists in human haemoclassification (Calzado et al. 2001).

Recently, chicken antibodies have been widely used as primary antibodies for ELISA, Western blotting and immunohistochemistry techniques (Hatta et al. 1997). For example, immunodetection of *Botritis*-specific invertase present in infected grapes is carried out with specific IgYs (Rutz & Ruffner 2002). Since it is possible to conjugate IgY with horseradish peroxidase, FITC or biotin, the resulting conjugates can be used for common immunochemical procedures (Larsson et al. 1998, Kim et al. 1999). IgY, possessing a lower pI value than mammalian IgG, is applicable for rocket electrophoresis to quantify immunoglobulins of mammalian sera (Altschuh et al. 1984). Thus, there is no need for IgY carbamylation to differentiate values of isoelectric point in analyzing and developing antibodies as is common when using rabbit IgG. The only limitation of chicken antibody application consists in the lower ability of IgY to precipitate antigens. However, using optimized reaction conditions, formation of precipitate can be facilitated (e.g. by using a higher ionic strength).

Using of immobilized IgY (bound to gel resin) for immunoaffinity purification of low and high molecular weight compounds has been described in several publications (Shelver et al. 1998, Hatta et al. 1997). Bound antigen is usually eluted with high yields (97%) under milder conditions (*p*H 4) than from columns based on IgG, hence this process is suitable for purification of acid-labile antigens.

## Advantages of Chicken Antibodies

From the point of view of animal welfare and bio-ethics, the production of antibodies using a chicken followed by their purification from eggs is more acceptable than preparation of mammalian antisera from blood. Moreover, immunization of the chicken is usually well-tolerated without the formation of abscesses and/or serious health problems, as is common for rabbits. The amount of injected mammalian antigen is often much lower than it is necessary for immunization of rabbits to assure an adequate immune response. Since the antibody is purified from egg yolks, not blood, the stress of the experimental animal is reduced to only the injection of the immunization doses. Animals do not need to be sacrificed to obtain blood for antisera production. The enormous biosynthesis of immunoglobulins, stored in yolks, makes the chicken a progressive experimental animal for antibody manufacture.

Another advantage of using the chicken results from the evolutionary distance between birds and mammals. Hens are able to produce antibodies with high titers against conserved mammalian proteins. Rabbits used for the same purpose are seldom successful in the formation of antisera with acceptable titers. Moreover, IgY against mammalian antigens usually recognizes several antigen surface regions resulting in a stronger final signal in immunological assays (Tini et al. 2002). As mentioned above, because of lack of reactivity with rheumatoid factor, Fc receptors and complement system, IgYs overcome interference problems known with the use of IgG for immunodetection (e.g. ELISA) in human sera.

Chicken antibodies have been successfully used in our laboratory for more than 10 years for various tasks during the study of cytochromes P450, enzymes playing a major role in the metabolism of

drugs and activation of carcinogens. Since studied cytochroms P450 are of mammalian origin, the chicken was used to produce antibodies against these antigens. Prepared IgYs are used as primary antibodies in ELISA and Western blotting and also as secondary antibodies when conjugated with peroxidase. Antibodies raised against rat recombinant CYP1A1were able to cross-react with human CYP1A1 showing detection limit as low as 0.005 pmol of the enzyme (Stiborova et al. 2002). At present our research is focused on immobilization of IgY in order to prepare an immunosorbent for affinity purification of cytochromes P450.

## Conclusion

Considering all of the mentioned advantages of IgY technology and some drawbacks of IgG, one can conclude that the wider application of chicken antibodies in research, diagnostics and immunotherapy is a matter of time. Avian immunoglobulins will be soon accepted as a viable alternative to mammalian ones, particularly with respect to specific applications such as those discussed in this review. Moreover, a laboratory that is ready to use non-mammalian, e.g. chicken antibodies, will be better able to adhere to stricter rules coming in the near future with regard to experimental animal handling.

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