Production and Purification of Mouse IgG Subclass Specific Chicken Egg Yolk Antibodies Using a New Indirect Affinity Chromatography Method with Protein G Sepharose

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Summary

Chicken antibodies have been reported to be an excellent alternative to the mammalian antibodies, especially if the antigen is from mammalians. Because chicken immunoglobulin Y (IgY) does not bind to protein G, in this study a indirect method for the purification of murine immunglobulin G (IgG) subclass specific egg yolk antibodies is described. After preincubation of the chicken egg antibodies (e.g. from hens immunised against murine IgG_1) with the non corresponding murine IgG subclasses (e.g. IgG_{2a} and IgG_{2b}) and purification with protein G sepharose, subclass specific chicken antibodies (e.g. against IgG_1) could be isolated from precipitated egg yolk antibodies. Specificities of the egg yolk antibodies were determined in a competitive ELISA and immunoblot. Therefore, the indirect affintity chromatography with protein G provides an efficient method for purifying specific chicken egg antibodies against mammalian IgG subclasses.

Zusammenfassung: Herstellung und Reinigung von Maus IgG subklassenspezifischen Dotterantikörpern unter Verwendung der neuen Methode der indirekten affintitätschromatographischen Reinigung mittels Protein G.
Es wurde vielfach berichtet, daß Hühnerantikörper, insbesondere wenn das Antigen vom Säuger stammt, eine gute Alternative zu Säugerantikörpern darstellen. Da Immunglobulin Y (IgY) vom Huhn nicht an Protein G bindet, wird in dieser Arbeit eine indirekte Methode zur Reinigung von spezifischen Dotterantikörpern, die gegen murine Immunglobulin G (IgG)-Subklassen gerichtet sind, beschrieben. Nach der Vorinkubation der aviären Dotterantikörper (z.B. von Legehennen, die mit murinem IgG_1 immunisiert wurden) mit anderen murinen IgG-Subklassen (z.B. IgG_{2a} und IgG_{2b}) und der anschließenden Reinigung mit Protein G, konnten subklassenspezifische Hühnerantikörper von zuvor gefällten Dotterantikörpern isoliert werden. Die Spezifitäten der Dotterantikörper wurden in einem kompetitiven ELISA und im Immunoblot bestimmt. Die indirekte Affinitätschromatographie mit Protein G stellt deshalb eine effiziente Methode zur Reinigung von spezifischen Dotterantikörpern, die Säuger IgG-Subklassen erkennen, dar.

Keywords: Affinity chromatography; Antibody purification; Egg yolk antibody; IgY; Murine IgG subclasses; Protein G

Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; IgY, Immunoglobulin Y; Mab, monoclonal antibody; PBS, phosphate-buffered saline; POD, horse radish peroxidase; RT, roomtemperature; SDS-page, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, tris buffered saline; TMB, tetramethylbenzidine.

1 Introduction

The species traditionally used for immunisation have been of mammalian origin such as rabbits, sheep, goats, and pigs. But immunisation of chickens results in a plentiful supply of immunoglobulin Y (IgY) in the egg yolk, because IgY antibodies from chicken blood serum are efficiently transferred across the follicular epthilium of the ovary and accumulated in the yolk during oogenesis up to concentrations of 100 to 250 mg IgY per egg yolk (Rose et al., 1974; Kowalczky et al., 1985; Lösch et al., 1986; Erhard, 1995).

Chicken antibodies have several advantages over mammalian antibodies due to the phylogenetical differences between mammalian and avian species. For example, chicken IgY does not react with rheumatoid factor (Larrson and Sjoquist, 1988; Larsson et al., 1991), nor with mammalian complement (Gardner and Kaye, 1982) or Fc receptor (Larsson et al., 1992; Lindahl et al., 1992), nor with staphylococcal protein A or G (Jensenius et al., 1981; Guss et al., 1986). Such reactions lead sometimes to false-positive results in immunological assays. Especially anti mammalian immunoglobulin G (IgG) antibodies from mammalians, but not from chickens (Boscato and Stuart, 1986), often crossreact with different mammalian IgG. Additionally, chicken antibodies often recognise more or conserved epitopes on mammalian or other antigens than antisera raised from mammalian species (Horten et al., 1984; Gassmann et al., 1990; Sturmer et al., 1992). Therefore, Larsson and Lindahl (1993) were able to produce chicken antibodies to protein G.

In the present study, different subclasses of mouse IgG were chosen to produce specific chicken egg yolk antibodies to reduce interferences in im-

munological assays caused by different reasons as discussed above. Because chicken antibodies do not react with protein G, a new methode was investigated to purify specific chicken anti mammalian IgG antibodies by an indirect affinity chromatography with protein G.

2 Animals, materials and methods

2.1 Immunisation of chickens

Laying hens (White Leghorn, 25 weeks old, Lohmann, Dieburg) were immunised with the corresponding antigen (mouse IgG_1, IgG_{2a}, IgG_{2b}, respectively). All antigens were monoclonal antibodies (MAb) and always 2 hens got the same Mab (Mab1: IgG_1; Mab2: IgG_{2a}; Mab3: IgG_{2b}). The initial immunisation was carried out into the pectoral muscle with 1 mg antigen and 0.25 mg of the lipopeptide Pam_3Cys-Ser-$(Lys)_4$ as adjuvant in 0.5 ml physiological NaCl. The adjuvant-mixed antigen was homogenised with a vortex mixer before injection. After 4 weeks booster injections were given like the first immunisation. The hens were submitted to regular light cycles, and food and water was freely available.

2.2 Extraction of chicken IgY from egg yolk

The egg collecting began 2 weeks after the last immunisation. Three egg yolks were pooled after separation from the white. The egg yolks were diluted 1:10 with tris bufferd saline (TBS; 0.1 M; pH 7.3) and mixed for 10 min. After centrifugation for 15 min (2000 g), dextransulfate (0.006 %; w/v) was added to the supernatant. After mixing for 10 min, $CaCl_2$ (0.14 M) was added and mixed again for 10 min. Supernatant was obtained after centrifugation (15 min, 2000 g) and was dialysed against TBS over night. After centrifugation with 26 % (w/v) ammonium sulfate, the precipitate was dissolved in TBS and dialysed against phosphate buffered saline (PBS; 0.1 M; pH 7.8). The protein concentrations were measured spectrophotometrically at 280 nm (Leslie and Clem, 1969).

2.3 Specificity after immunisation

For testing the specificity of chicken egg antibodies, ELISA plates (Immunoplate II, Maxisorb, Nunc, Wiesbaden) were coated with the different Mabs (2 µg/ml; 100 µl/well; over night at 4°C) in 0.1 M $NaCO_3$-buffer (pH 9.6). Nonspecific binding sites were subsequently blocked with 1% bovine serum albumin (BSA) in PBS (200 µl/well) for 1 h at 37°C, after which 50 µl of extracted chicken IgY (25 µg/ml) was applied and incubated for 1 h at 37°C. Binding of specific chicken IgY to the plate coating was detected with the aid of a further monoclonal anti chicken IgY antibody (1:5000; 50 µl/well; 1 h at 37°C)(Erhard et al., 1992) coupled to horse radish peroxidase (POD) and made visible with the aid of 100 µl tetramethylbenzidine (TMB; 0.2 mg/ml 0.1 M acetat-citrate-buffer, pH 5.0; with 0.0005 % H_2O_2). The enzyme reaction was stopped with 1 M H_2SO_4 (50 µl/well) and measured at 450 nm. The ELISA plates were washed with NaCl-Tween between each incubation stage.

2.4 Affinity chromatography with protein G

The isolation of specific chicken IgY from the dialysed solution (see above) was carried out by way of indirect affinity chromatography using protein G sepharose (Pharmacia, Freiburg). 1 mg of chicken IgY (for example anti mouse IgG_1) was mixed with the non corresponding Mabs (here: Mab2 with the subclass IgG_{2a} (0.5 mg) and Mab3 with the subclass IgG_{2b} (0.5 mg)) and incubated in a 1:2 dilution with the protein G sepharose binding-buffer (20 mM phosphate-buffer; pH 6.0) for 30 min at roomtemperature (RT). Then the solution was put on the protein G sepharose. In the binding-buffer subclass specific antibodies could be found (see results). The antibodies affined to protein G (Mabs with the corresponding chicken IgY antibodies) were eluted with 0.1 M glycine-HCl-buffer (pH 2.6).

The procedure was carried out in every combination of the different subclass specific chicken and monoclonal murine antibodies in the same way and concentrations.

2.5 Competitive ELISA

The specificity of each purified chikken antibody for the corresponding antigen was determined with a competitive ELISA. This ELISA for determination of the different subclasses of mouse IgG is based on the competition between immunoplate-immobilised antigen and free antigen.

Microwell titer plates were coated with the antigens Mab1, Mab2 or Mab3 (2 µg/ml; 100 µl/well; over night at 4°C) in 0.1 M $NaCO_3$-buffer (pH 9.6). Nonspecific binding sites were again blocked with 1% bovine serum albumin (BSA) in PBS (200 µl/well) for 1 h at 37°C, after which 25 µl of protein G-sepharose purified chicken IgY was applied with 25 µl of the different Mabs (100 to 0.01 µg/ml) or PBS for control and incubated for 1 h at 37°C. The further procedure was done as described above.

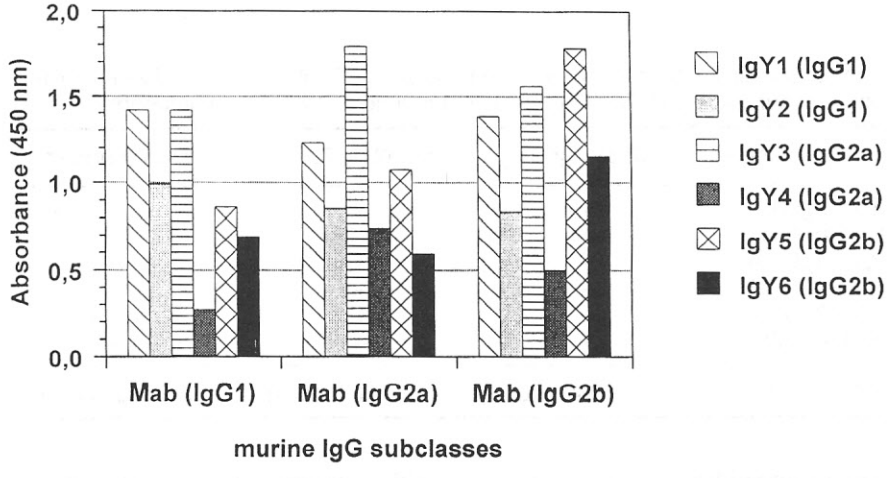

Figure 1: Specificity of chicken egg yolk antibodies (IgY1 – IgY6; 25 µg/ml) after immunisation with murine monoclonal antibodies (Mab) with the subclasses IgG_1 (IgY1; IgY2), IgG_{2a} (IgY3; IgY4) and IgG_{2b} (IgY5; IgY6) determined in a direct ELISA.

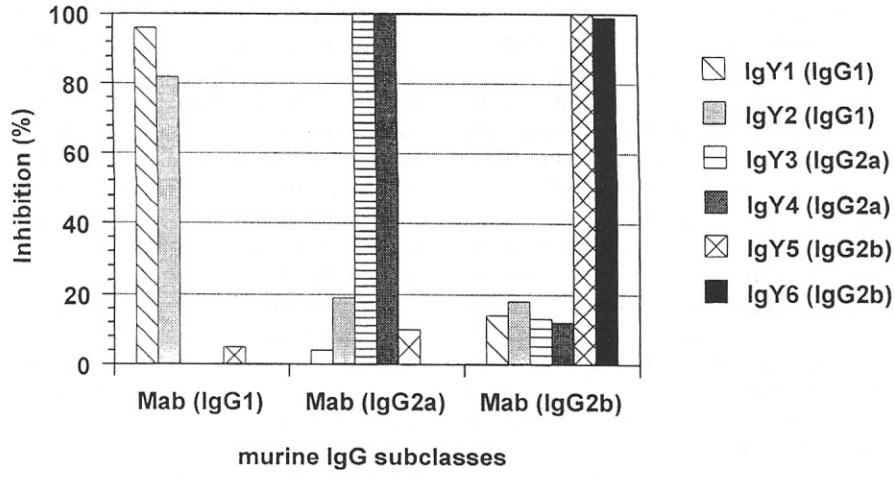

Figure 2: Specificity of chicken egg yolk antibodies (see Figure 1) after indirect affinity chromatography with protein G sepharose using a murine Mab concentration of 100 µg IgG. The specificity was determined in a competitive ELISA.

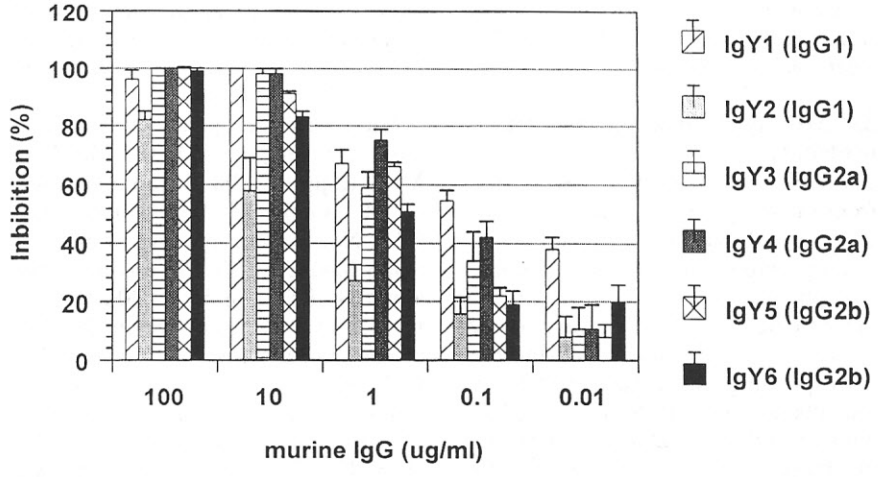

Figure 3: Detection of murine IgG subclasses with the competitive ELISA using specific chicken antibody after indirect affinity chromatography with protein G sepharose (n=6; SD).

Table: Specificity of the egg yolk antibodies (IgY) of different chickens to the different subclasses (IgG_1, IgG_{2a}, IgG_{2b}) of murine IgG (Mab) in immunoblot.

Dotterantikörper	Mab1 (IgG1)	Mab2 (IgG2a)	Mab3 (IgG2b)
IgY1 (IgG1)	+++	+	–
IgY2 (IgG1)	+++	++	–
IgY3 (IgG2a)	–	+++	–
IgY4 (IgG2a)	–	+++	–
IgY5 (IgG2b)	–	+	+++
IgY6 (IgG2b)	–	+	+++
– no bindung	+ low binding	++ middle binding	+++ high binding

2.6 Immunoblot

Additionally, the specificity of each individual chicken antibody was tested with a western blot (Towbin et al., 1979). Murine Mabs with different IgG subclasses were used as samples (2 µg in 20 µl/slot). After electrophoresis (SDS-PAGE; graduent 3–15 %), the ensuing transfer onto the nitrocellulose (Nitrocellulose BA 83, pore size 0.2 µm; Schleicher und Schuell) took 90 min at 0.8 mA/cm² and was made with a semidry electroblotter (SM 17556; Sartorius) as described by Kyhse-Andersen (1984). For this propose, a transfer-buffer according to Bjerrum and Schafner-Nielsen (1986) was used. After blocking the nitrocellulose with 4 % milk powder (in aqua bidest) over night at 4°C, purified chicken antibodies (2 ml of the original sample in binding-buffer) was incubated in each combination to the Mabs for 1 h at RT. Then bound chicken antibodies were detected with the monoclonal anti chikken IgY antibody (1:200; 2 ml)(Erhard et al., 1992) coupled to POD and made visible with 4-chloro-1-naphtol as enzyme substrate.

3 Results

After precipitation with dextran and ammonium sulfate, a mean concentration of 256.5 mg chicken IgY (SD 66.8; n=6) was obtained from 3 egg yolks.

Testing these chicken IgY fractions for specificity against the different mouse IgG subclasses in ELISA, no differences could be found with the different chicken egg yolk antibodies (fig. 1). The indirect affinity chromatography with protein G after mixing the chicken IgY with the non corresponding Mabs leads to a selection of subclass specific egg yolk antibodies (fig. 2). For detection of mouse IgG subclasses, a competitive ELISA was performed. The purified chicken antibodies showed a specific detection of the different mouse IgG subclasses using concentrations from 100 µg murine IgG to 10 ng murine IgG per ml buffer (fig. 3).

Additionally, the specificity of the chicken antibodies was tested in immunoblot. All antibodies showed high binding capacity to the corresponding murine IgG subclasses. Some crossreactions to murine IgG_{2a} could be found with all chicken antibodies (table).

4 Discussion

For passive protection of the neonate, maternal avian IgY antibodies are secreted into the yolk sac from the secretory follicles in the epithelial lining of the oviduct during maturation of the egg. Thus, high levels of IgY could be found in the egg yolk (Lösch et al., 1986). In this study, the amounts of antibodies obtained from one egg yolk was about 85 mg precipitated chicken IgY. In estimating the number of eggs per year from a laying hen with about 300, the use of eggs as a source of antibodies is very easy and collecting eggs represents a non-invasive technique.

Unlike mammalian antibodies, chicken IgY does not bind to protein A or protein G (Jensenius et al., 1981; Guss et al., 1986). Nevertheless, it is possible to use protein G as a purification strategy for mammalian IgG specific egg yolk antibodies with the demonstrated indirect affinity chromatography. It was possible to purify murine IgG subclasses specific egg yolk antibodies by preincubation with the non corresponding murine IgG subclasses before using protein G sepharose. The specific chicken antibodies were found in the binding buffer. The specificity could be demonstrated in a competitive ELISA-system and in immunoblot. With the purified chicken antibodies an ELISA detection system for the different murine IgG subclasses could be developed. This ELISA can be used, for example, for screening monoclonal antibodies for their subclasses. But most important, different murine Mabs can now be used in the same system, for example immunohistochemistry, in combination with the different subclass specific chicken antibodies.

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