ELSEVIER

Journal of Immunological Methods 297 (2005) 253–257

JIM Journal of Immunological Methods

www.elsevier.com/locate/jim

Technical note

Snake egg immunoglobulins: biochemical characteristics and adjusted isolation procedure

Andreas Hassl*

Department of Medical Parasitology, Clinical Institute of Hygiene and Medical Microbiology, Medical University of Vienna, Kinderspitalgasse 15, A-1095 Vienna, Austria

Received 12 March 2004; received in revised form 24 August 2004; accepted 17 December 2004
Available online 11 January 2005

Abstract

Transmission of specific immunoglobulins from mothers to their offspring via the egg is a common phenomenon in egg-laying vertebrates but the occurrence of this phenomenon in reptiles, especially in colubrid snakes, has not been proven until recently. Thus, the essential biochemical characteristics of antibodies deposited in eggs of *Elaphe guttata* (Colubridae, Serpentes) were studied after isolation of the antibody by precipitation and purification by affinity chromatography and gel filtration, with verification by isoelectric focusing and immunoprecipitation. The immunoglobulin deposited in the eggs of colubrid snakes is a singular, non-truncated IgY antibody in a concentration roughly equal to that in the snake's serum. An efficient method to isolate antibody from snake eggs was developed, based on the PEG precipitation technique of avian yolk immunoglobulins; an unsophisticated protocol for the isolation procedure appropriate for reptile eggs is provided.

Keywords: Egg immunoglobulins; Snake egg antibody; Immunoglobulin Y; Isolation procedure

Although it has been known since 1883 that birds are able to accumulate antibodies in the egg yolk (Klemperer, 1883), and the application of specific chicken yolk antibodies has proliferated in various biomedical areas (Tini et al., 2002), very little information is available about the phenomenon of antibody deposition in reptile eggs. For the first time, Hassl (2005) has stringently demonstrated that specific immunoglobulins are a component of the egg of colubrid snakes, but a definite characterization of these egg antibodies is still lacking. The first aim of this study was to identify the biochemical characteristics of the immunoglobulins deposited in colubrid snake egg and then, as a second goal, to develop a simple but reliable isolation procedure.

Abbreviations: GF, gel filtration; IgY, immunoglobulin of class Y; PBS, phosphate-buffered saline (3 mM potassium di-hydrogen phosphate, 10 mM di-sodium hydrogen phosphate, 116 mM sodium chloride, pH 7.2); PEG, polyethylene glycol with a molecular mass of 6000 kDa; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

* Tel.: +43 1 40490 79438; fax: +43 1 40490 79435.
E-mail address: andreas.hassl@meduniwien.ac.at.

0022-1759/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jim.2004.12.004

An attempt to extract antibodies from snake eggs applying the universal, conventional PEG precipitation method resulted in a poor outcome. Consequently, a small fraction of antigen-binding antibodies was prepared by antigen-antibody adsorption (affinity chromatography). This antibody fraction and fractions from intermediate steps of the purification were biochemically analysed. By means of these data, an isolation procedure adapted to reptile eggs was developed and optimized.

All antibody isolation experiments were carried out using ten unhatched, fertilized eggs of two sibling females of *Elaphe guttata*, previously immunized with highly pure bovine serum albumin (BSA, Merck Darmstadt, Germany) by a conventional adjuvant method (Hassl and Aspöck, 1988). The egg contents were mixed and pooled. First, a crude fraction of immunoglobulins was isolated in triplicate as originally described by Polson et al. (1980) for chicken yolk IgY, in part accommodating the improvements of Jensenius et al. (1981); Polson et al. (1985), and Polson (1990). Briefly, the snake egg immunoglobulins were isolated by precipitation with 3.5% and 12.0% PEG (Merck) and subsequent alcohol or chloroform precipitation, respectively. A poor outcome was indicated by a first purity check using SDS-PAGE (PhastSystem, Pharmacia GesmbH, Vienna, Austria). Thus, four more PEG precipitation experiments were performed stepwise applying the improved procedure specified below. The extracted pellets were frozen at −70 °C for later characterization.

Faced with major problems in isolating a pure antibody fraction by the conventional method, we prepared a fraction of pure, specific egg antibodies by means of affinity chromatography using Blue Sepharose 6 Fast Flow (Pharmacia GesmbH), specifically adsorbing the egg antibodies to the antigen (BSA) fixed to the gel. The preparation was done strictly according to the recommendations of the manufacturer. This antibody fraction was used to determine the essential biochemical characteristics.

Then, we tried to bind the Fc portions of the antibodies to Protein A of *Staphylococcus aureus* applying an affinity chromatography technique with Protein A-Sepharose CL-4B, exactly as described by the manufacturer (Pharmacia GesmbH). A small portion of the total egg antibody preparation did bind to Protein A (Table 1). In addition, three antibody fractions underwent gel filtration in a Superose 6 HR 10/30 column (Pharmacia GesmbH; for details see Fig. 1). Then, the isoelectric points of the egg immunoglobulin and its fragments were determined applying an isoelectric focussing technique in polyacrylamide flat gels (pH 3–10; ECPS 3000/150), using commercially available standard proteins, all done according to the manufacturer's recommendations (Pharmacia GesmbH). The molecular masses of the proteins were determined by SDS-PAGE using PhastGel 12.5%, run on a PhastSystem (Pharmacia

Table 1
Characteristics of corn snake egg immunoglobulins

n=3	Volume/egg (ml)	Protein concentration (mg/ml)	Protein concentration/egg (g)	Protein A-binding capacity	Molecular weight of components in a native SDS-PAGE	p*I* of components
Egg slurry	5.6	1123		Insignificant		
Resolved precipitate	6.2	45	0.28	Insignificant	**173.4**, 57.0, 51.5, 37.3, 22.6	4.30, 4.60, 4.84, 5.20, **5.40, 5.56, 5.75**, 6.90, 7.07, 7.16, 7.40, 7.80, 7.90, 8.19
IgY purified by gel filtration	3.0	67	0.2	Undetectable	**173.8**	**5.40, 5.56, 5.75**
IgY purified by binding to PA	6.0		Very low	Very high	**174.0**	**5.40, 5.56, 5.75**
IgY purified by affinity chromatography	7.0	0.15	0.001	Low	**173.6**, 57.0, 49.9, 46.7, 37.3, 21.7	**5.40, 5.56, 5.75**, 7.07, 7.40, 8.19, 8.34

PA, Protein A; bold, characteristics of the unfragmented snake egg IgY.

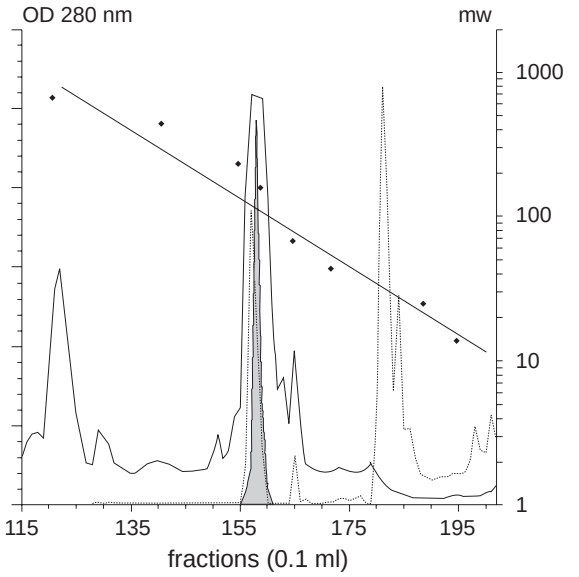

Fig. 1. Graph of absorbance at 280 nm of gel filtration fractions of the PEG-precipitated *Elaphe* egg immunoglobulins (full line), after an antigen-antibody reaction affinity chromatography purification step (dotted line), and re-application of a GF-purified sample (shaded area). (♦) Commercially available molecular weight standards in kDa (right ordinate). Samples were clarified by centrifugation and passage through a 0.22-μm filter. Column, Superose 6 HR 10/30; sample concentration, 2 mg/ml; sample volume, 1 ml; eluent, PBS (pH 7.2) containing 0.05% sodium azide; flow rate, 0.5 ml/min.

GesmbH) under native and reducing conditions, respectively. The proteins were stained with Coomassie Blue R-250 dye applying a customary staining procedure.

The outcome of all the experiments was the design of an unsophisticated isolation procedure, outlined below. All data given in Table 1 refer to this novel isolation procedure; all protein concentrations were measured at 280 nm without any dye using BSA as standard. Moreover, each individual separation step was re-examined for specific antibodies by an immunoprecipitation test in agarose (Ouchterlony test) according to Polson et al. (1980). All antibody fractions precipitated the antigen (BSA), albeit at different strengths. A more elegant isolation method applying hydrophobic interaction chromatography, described by Hassl and Aspöck (1988) for chicken yolk immunoglobulins, could not be adapted successfully; procedures using Protein G or preparative electrophoresis as the core component were also unsuccessful.

The following procedure was used to prepare snake egg immunoglobulins:

Crushed eggs were poured into a measuring cylinder, at least four volumes of PBS (pH 7.2) were added and the mixture stirred slowly but continuously.
For the extraction of lipids and lipoproteins, 3.5% PEG 6000 (w/v) was added dropwise and the mixture stirred for a further 5 min.
After centrifugation at 6000×*g* for 15 min, the liquid portion was transferred to a new container. Liquefied PEG was added dropwise to give a final concentration of 12%, having previously recalculated the amount of PEG needed, i.e. 85 g/l liquid. The mixture was then stirred for 5 min. After centrifugation at 10,000×*g* for 15 min. the clear supernatant was discarded and the pellet dissolved in at least one volume of PBS (pH 7.2).
The sample was then applied to an appropriate gel filtration medium e.g. Superose 6 HR. If Superose 6 HR was used, the fraction with K_{av}=0.56–0.60 was collected ($K_{av]}$ is the fraction of the stationary gel volume which is available for diffusion). Otherwise, the fraction containing proteins with a molecular mass of 173–175 kDa was collected.

Applying this procedure, substantial amounts of egg immunoglobulin could be isolated and immunoglobulins were found to be an essential component of eggs laid by corn snakes. About 3.6% of the soluble egg proteins (average 6.3 g/egg) were immunoglobulins, and about 0.3% of this fraction was specific antibody. This is considerably less than in chicken eggs, where 15–18% of the total yolk immunoglobulins appear to be directed to the antigen (Polson et al., 1980), but this may depend on the quality of the immunization procedure applied.

A comparison of antibody deposition in single eggs between snake and chicken is not meaningful as hens deposit all egg antibodies in the yolk, which can be separated from the white and further processed exclusively. There is no separation of yolk and egg white in snake eggs. Thus, the whole content of snake eggs has to be liquefied in a physiological buffer before removing debris, the embryo, and any precipitation.

According to deSmet (1978), 1 ml of *Elaphe* serum contained about 47.5 mg of proteins. Forty milligrams

(~85% of the serum proteins) were globulins, and the albumin/globulin ratio was 0.1. Comparing the concentration of total globulins in *Elaphe* serum and in egg contents, as much as 36 mg of immunoglobulins per ml of egg contents could be isolated by the adjusted precipitation method (Table 1). This amount is almost equal to the quantity of immunoglobulin in the serum of *Elaphe* snakes. Thus, snake eggs contain roughly as much antibody as the serum, comparing equal volumes.

But, according to the results of the gel filtration experiment and the SDS-PAGE, PEG precipitation produced a considerable amount of fragmented immunoglobulin and agglomerates, although snake IgY is not denatured by precipitation. The high-molecular-weight contamination may be a manifestation of polymer formation and fragment agglomeration, which have been observed repeatedly during purification of chicken yolk IgY (Yazawa et al., 1991; Akita and Nakai, 1993). This high-molecular-weight contamination is definitely not an antibody of the M class, as IgM is not transferred from serum to the egg, either in birds (Polson et al., 1980) or in reptiles, a fact which has been established by the complete lack of any high-molecular-weight component in the affinity-purified antibody fraction (Fig. 1).

Elaphe snakes are assumed to produce two immunoglobulin classes, an ancestral IgM antibody, and an IgY with a molecular mass of about 150–180 kDa (ElRidi et al., 1991; Warr et al., 1995). The IgY is the typical low-molecular-weight serum antibody in birds, reptiles, and amphibians (Warr et al., 1995), and the only one of this type in most reptiles, although accompanied by a truncated form in some reptiles. Summarising our results, corn snake eggs contain an antibody of the Y class with a molecular mass of about 174 kDa; it is a non-truncated immunoglobulin, and it occurs in three isoforms with slightly different isoelectric points of 5.40, 5.56, and 5.75. According to the results of the SDS-PAGE, under reducing conditions (data not shown) the most probable molecular mass of the heavy chain is 57 kDa, that of the light chain is 22 kDa, and that of the Fab′ portions is 37 kDa. ElRidi et al. (1991) proposed that snake serum IgYs consists of two different heavy chains with masses of 63 and 50 kDa, and two light chains with masses of 23 and 20 kDa.

Whereas PEG precipitation of chicken yolk IgY has the advantage of producing pure antibodies as a solid pellet (Polson et al., 1980), the pellet of snake egg antibodies is not homogeneous; it consists of proteins with very different molecular masses. Therefore, an additional purification step is necessary to obtain pure antibodies. Gel filtration seemed to be the best choice, as this technique does not impair the binding capacity, removes all impurities, especially PEG remnants, desalts, and may be scaled up to production proportions. Most other separation techniques tested did not work on an acceptable scale, e.g. immunoglobulin isolation by Protein A adsorption was unproductive. Only a small proportion of all antibodies seemed to bind to this strong mammal antibody capture reagent (Table 1). This observation is in good agreement with that of Barkas and Watson (1979), who found chicken IgY to bind to Protein A only after the formation of immune complexes.

The development and progressive differentiation of adaptive immunity is a characteristic of vertebrates. There seems to be almost no doubt that an immunoglobulin transmission via the egg is not restricted to snakes of the genus *Elaphe*, but is a phenomenon occurring in all egg-laying reptiles. But, studying the phenomenon of vertical immunity transfer in reptiles is nearly impossible without evaluation of the efficiency of immunoglobulin deposition in eggs. Thus, reliable isolation and purification protocols have to be devised as the basis for further serological studies on immunity already present in snakes at the time of hatching.

References

Akita, E.M., Nakai, S., 1993. Production and purification of Fab' fragments from chicken egg yolk immunoglobulin Y (IgY). J. Immunol. Methods 162, 155.

Barkas, T., Watson, M.J., 1979. Induction of a Fc conformational change by binding of antigen, the generation of protein A-reactive sites in chicken immunoglobulin. Immunology 36, 557.

deSmet, W.H.O., 1978. Study of the serum albumin and globulin of the vertebrates. Acta Zool. Pathol. Antverp. 70, 57.

ElRidi, R., Mansour, M., Zada, S., 1991. Immunoglobulins of the snake *Psammophis sibilans.* Studies using a monoclonal antibody. Immunobiology 184, 1.

Hassl, A., 2005. Functional egg immunoglobulins in the snake *Elaphe guttata*. Amphib.-Reptil. 26, in press.

Hassl, A., Aspöck, H., 1988. Purification of egg yolk immunoglobulins. A two-step procedure using hydrophobic interaction

chromatography and gel filtration. J. Immunol. Methods 110, 225.

Jensenius, J.C., Andersen, I., Hau, J., Crone, M., Koch, C., 1981. Eggs, conveniently packaged antibodies. Methods for purification of yolk IgG. J. Immunol. Methods 46, 63.

Klemperer, F., 1883. Über Natürliche Immunität und ihre Verwertung für die Immunisierungstherapie. Arch. Exp. Pathol. Pharm. 31, 356.

Polson, A., 1990. Isolation of IgY from the yolks of eggs by a chloroform polyethylene glycol procedure. Immunol. Invest. 19, 253.

Polson, A., von Wechmar, M.B., van Regenmortel, M.H.V., 1980. Isolation of viral IgY antibodies from yolks of immunised hens. Immunol. Commun. 9, 475.

Polson, A., Coetzer, T., Kruger, J., Maltzahn, E., van der Merwe, K.J., 1985. Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. Immunol. Invest. 14, 323.

Tini, M., Jewell, U.R., Camenisch, G., Chilov, D., Gassman, M., 2002. Generation and application of chicken egg-yolk antibodies. Comp. Biochem. Physiol., Part A Mol. Integr. Physiol. 131, 569.

Warr, G.W., Magor, K.E., Higgins, D.A., 1995. IgY, clues to the origins of modern antibodies. Immunol. Today 16, 392.

Yazawa, S., Hosomi, O., Takeya, A., 1991. Isolation and characterization of anti-H antibody from egg yolk of immunized hens. Immunol. Invest. 20, 569.