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Purification of egg yolk immunoglobulins

A two-step procedure using hydrophobic interaction chromatography and gel filtration

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A two-step chromatographic procedure was developed for the isolation and purification of hen IgY antibodies from egg yolk. The antibodies were completely separated from vitellin and lipids by hydrophobic interaction chromatography followed by gel filtration. Almost no residual yolk proteins, no immunoglobulin aggregates, and no antibody fragments could be detected in the final extract. Moreover, the method described, guarantees the recovery of antibodies of undiminished activity. Although the final yield is somewhat lower than that obtained by an isolation method consisting of two precipitation steps with polyethylene glycol and alcohol respectively, the procedure described is particularly recommended when highly purified antibody preparations are needed.

Key words: IgY, yolk; Immunoglobulin extraction; Hydrophobic interaction; Gel filtration

Introduction

The use of hen egg yolk as a source of specific antibodies (IgY) offers considerable advantages such as compatibility with modern animal protection regulations, cheapness, and convenience (Jensenius et al., 1981; Gottstein and Hemmeler, 1985). Moreover, because of the evolutionary distance of this immunoglobulin from mammalian antibodies, IgY antibodies may be used in serological tests which are affected by rheumatoid factors. Nevertheless, IgY antibodies have not been used as frequently as one might expect. This may be due to unsatisfactory isolation procedures. Although several different purification methods have been described, many of them are of limited value since they lead to poor yields of pure, intact, functionally active antibodies. We have used two chromatographic processes, hydrophobic interaction chromatography (HIC) and gel filtration (GF), for IgY isolation. This combination offers a powerful separation selectivity yielding pure egg yolk immunoglobulins.

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Abbreviations: IgY, hen yolk antibodies; HIC, hydrophobic interaction chromatography; GF, gel filtration; PBS, 0.116 M phosphate-buffered saline, pH 7.2; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; a.m.u., atomic mass units.

Materials and methods

Immunization

Two conventionally maintained laying hens (Darco brown) were each immunized i.m. with 1 mg aqueous proteins of *Toxoplasma gondii* (BK

strain) emulsified in 1 ml complete Freund's adjuvant. Three booster injections were given on days 10, 20, and 30. Eggs were stored at 4° C until processing.

Antibody processing

The yolks of ten eggs were separated from the white, carefully washed with PBS, and mixed. The yolk mixture was divided into two equal portions: the first was treated according to Polson et al. (1985); from the second the immunoglobulins were extracted by HIC and GF. That is: 1 vol. of yolk was thoroughly mixed with 4 vols. PBS containing 4.4% (w/v) polyethylene glycol (PEG 6000), incubated [illegible] min at room temperature, and then centrifuged at 4750 × *g* for 60 min. The aqueous supernatant was applied to a phenyl-Sepharose CL-4B column (Pharmacia, Vienna) at a rate of 2 mg protein/ml gel. For the removal of the unbound material the column was washed with

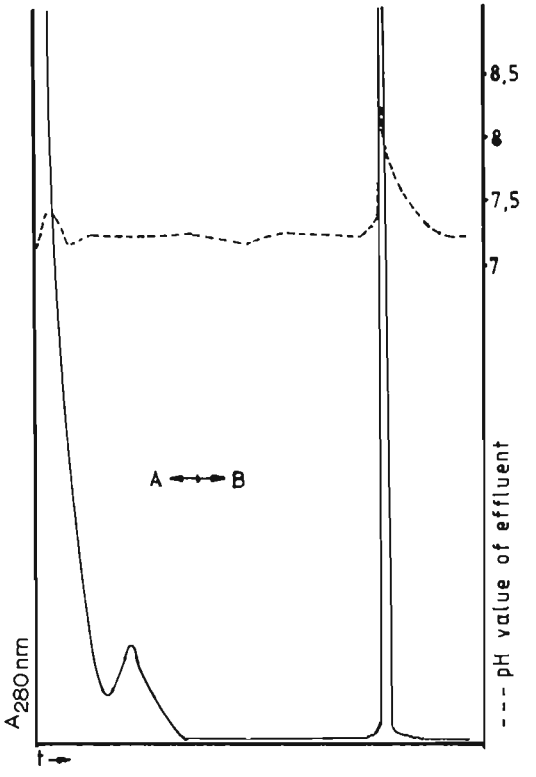

Fig. 1. Chromatograph of yolk proteins on phenyl-Sepharose CL-4B equilibrated with eluant A (PBS diluted with distilled water, 1/10). Elution was performed with eluant B (demineralized water). The protein peak which followed the eluant switch was collected, and shown to contain the yolk IgY.

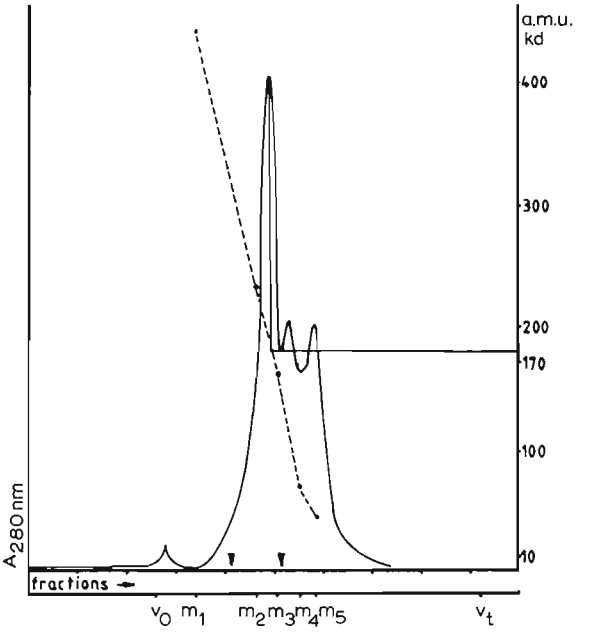

Fig. 2. Chromatograph of the protein peak from HIC on Sephacryl S-300 (80 cm column equilibrated with PBS). The a.m.u. markers were blue dextran (void volume, v_0), ferritin (m_1, 440 kDa), catalase (m_2, 232 kDa), aldolase (m_3, 158 kDa), bovine serum albumin (m_4, 68 kDa), ovalbumin (m_5, 45 kDa), and L-tyrosine ethyl ester (= total volume, v_t). The protein peak between the arrow heads was collected and shown to contain IgY antibodies.

0.0116 M phosphate-buffered saline (PBS diluted 1/10 with distilled water; conductivity: ~ 2000 μS). Elution was performed with demineralized water (conductivity: < 20 μS) (chromatograph: Fig. 1). The eluted proteins were collected, frozen, and lyophilized. After about ten separation steps the column was washed with 75% ethylene glycol in PBS to remove some strongly bound substances.

For the GF procedure the proteins were resuspended in PBS containing 0.02% NaN_3, filtered through a 0.2 μm membrane filter (Flow Laboratorien, Mekkenheim, F.R.G.), and applied to a column containing Sephacryl S-300 Superfine (Pharmacia), equilibrated with PBS + 0.02% NaN_3. GF was performed at a flow rate of 6 ml · cm^{-2} · h^{-1} at 20° C. Details of the procedure are shown in the chromatograph (Fig. 2). The marker proteins used were purchased from Pharmacia and Sigma Chemie, Deisenhofen, F.R.G. The protein fraction with an a.m.u. of 175 kDa was collected.

Quality control

The protein contents of intermediate products and the final extracts were determined by a protein assay (Bio-Rad Laboratories, Vienna, Austria). For comparative purposes the antibody activity of both batches were adjusted to the same protein content (1 mg/ml). Thereafter, the titers of specific antibodies were determined by an indirect hemagglutination assay (Cellognost Toxoplasmosis, Behringwerke, Marburg, F.R.G.). The purities of the antibody fractions were evaluated in an SDS-PAGE system under reducing conditions performed according to Laemmli et al. (1970). Samples, containing 5% 2-mercaptoethanol, were applied to a 12% slab gel (T5/C3). The separation was achieved using a constant current of 8 mA overnight. After the run the gels were stained with Coomassie Blue R-250 (Serva, Heidelberg, F.R.G.) and finally evaluated in a laser densitometer (2202 Ultroscan, LKB Instrument, Vienna) at a wavelength of 633 nm.

Results

The chromatographs of the HIC and the GF procedures are shown in Figs. 1 and 2. At the PEG step of the purification the protein content of 1 ml yolk mass was lowered from 58 mg to 35 mg. In the second step HIC reduced this amount to 4.7 mg and GF to 1.2 mg. Thus, the average yields of IgY antibodies obtained from eggs with mean weights of 60 g were about 14.5 mg each. The purity of the IgY antibodies is demonstrated in Fig. 3. IgY comprised more than 85% of the proteins, as judged from photometric scanning.

In comparison, about 3 mg IgY antibodies could be isolated from 1 ml initial yolk mass by the procedure described by Polson et al. (1985). As shown in Fig. 3 no significant differences were detected between the purities of the IgY obtained by both isolation methods. Comparing the activity of the antibodies isolated by the two methods, similar IHA titers were obtained if the protein

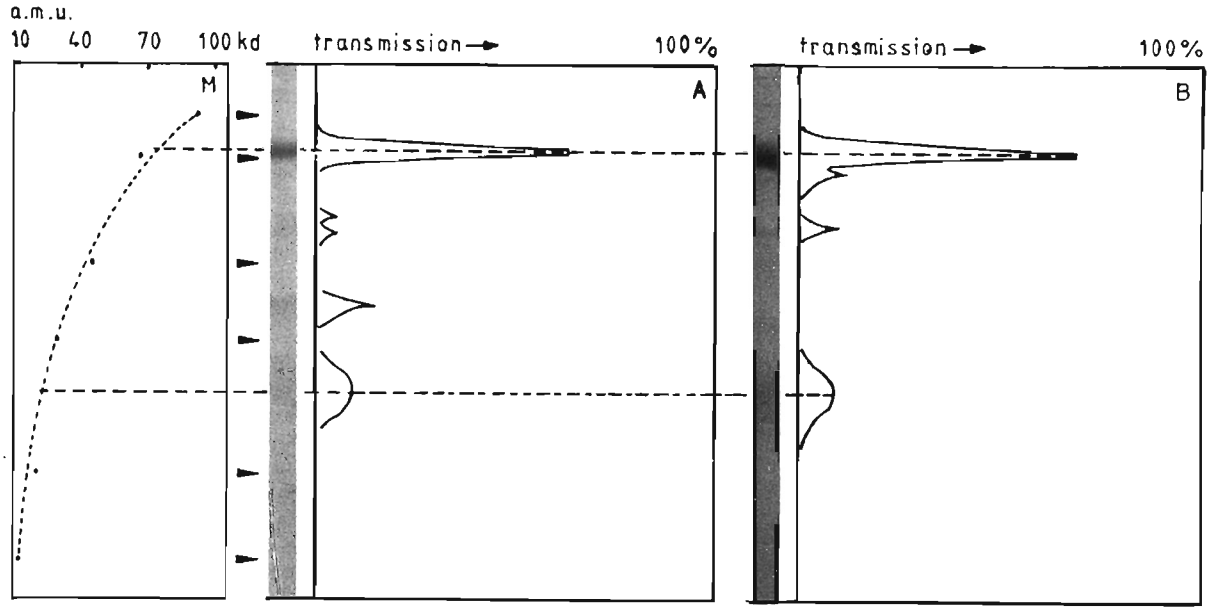

Fig. 3. SDS-PAGE of the yolk immunoglobulins under reducing conditions. Photograph of the gel slices and diagrams of the gel evaluation in a laser densitometer. Left diagram: a.m.u. marker curve. Markers (arrows): phosphorylase *B*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme. Central diagram: IgY isolated by HIC and GF. Right diagram: IgY isolated according to Polson et al. (1985).

contents of both batches were first equalized (1 : 4000 to 1 : 1000). In conclusion, the two methods produced IgY antibodies of almost equal activity and very similar purity, but with markedly different final yields.

Discussion

The use of specific yolk antibodies in serology has many advantages compared to conventional mammalian antisera. The problems of antibody isolation have, however, seriously hindered wider use of IgY reagents. Although several different isolation procedures have been described, none of them is as easy as the purification of most mammalian IgG antibodies by affinity chromatography with protein A. We have carried out a comparative study on various isolation procedures (Hassl et al., 1987) and have confirmed the relatively high efficiency of the method described by Polson et al. (1985). Therefore, in the present study we used this isolation procedure as a reference method.

In the present paper we have described a new isolation procedure which yields pure, intact, and functionally active yolk antibodies more rapidly than the method of Polson et al. (1985). The procedure is based on a combination of hydrophobic interaction chromatography and gel filtration. This combination offers some technical advantages. First, HIC is a convenient separation technique to be used after salt precipitation since samples have to be applied in high salt concentrations. During the IgY purification the salt supply for starting the HIC simultaneously removes most lipids. Secondly, the elution of the IgY antibodies from the HIC column with demineralized water offers the possibility of concentrating the eluant without any increase in salinity. Thirdly, GF is used for a complete buffer exchange, and time-consuming dialysis steps can be avoided. The results of this isolation procedure is an antibody fraction with a purity and an activity equal to these of the reference method (Fig. 3, results of antibody titration).

Characteristic features of the present isolation method are: a good preservation of antibody activity during the procedure, high purity of the final product and the use of simple and convenient chromatographic processes. This combination of characteristics is not offered by any other isolation method known to us. Nevertheless, the yield of the present method is lower than that of the reference method. This may not be a serious disadvantage however since large quantities of yolks are usually available for processing. The losses probably occur during the HIC and may be due to the existence of yolk antibody subclasses with different hydrophobic characteristics.

The isolation method described can be adapted to a chromatographic system equipped with automatic processing and thus offer a time and labor saving procedure for the production of highly purified yolk antibodies.

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