

OBTAINING HIGH PURITY ANTIBODIES WITH THERAPEUTIC POTENTIAL

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Abstract

The aim of this study was to provide a method for obtaining high purity egg yolk antibodies (IgY) with potential use in immunodiagnostic or for the prophylaxis and therapy of infectious diseases. The immunoglobulins were purified by extraction, repeated precipitation and size exclusion dialysis. The purity of the obtained antibodies was assessed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and computerized densitometry. By our optimized method and by using dried egg powder (Globigen® Pig Doser-Ghen Corporation, Japan) as an antibody source we obtained electrophoretically pure antibodies. Results were compared with the amount of IgY extracted from egg yolk.

Rezumat

Scopul acestei cercetări este de a prezenta o metodă de obținere rapidă, economică și cu randament ridicat a anticorpilor vitelini (IgY) de înaltă puritate care pot fi utilizați în imunodiagnostic sau pentru profilaxia și terapia bolilor infecțioase. Imunoglobulinele au fost purificate prin extracție, precipitări repetate și dializă cu cernere moleculară. Puritatea anticorpilor a fost determinată prin SDS-PAGE (*sodium dodecyl sulfate-polyacrylamide gel electrophoresis*) și densitometrie computerizată. Prin metoda de purificare folosită și utilizând pudră de ouă (Globigen® Pig Doser-Ghen Corporation, Japan) ca sursă de anticorpi au fost obținuți anticorpi puri din punct de vedere electroforetic. Rezultatele au fost comparate cu nivelul de imunoglobuline IgY din gălbenușul de ou.

Keywords: IgY, purity, SDS-PAGE, densitometry

Introduction

Hen eggs are an important source of antibodies. Immunoglobulins transferred from the hens' blood into the egg yolk were named yolk

immunoglobulins (IgY). It has been demonstrated that they can contain antibodies against up to 200 different antigens [6]. Avian antibodies are an appealing alternative to mammal antibodies due to economical, ethical and animal welfare reasons [17]. There are various therapeutical and immunological applications of yolk immunoglobulins [5, 24, 27]. IgY is a protein with a molecular weight of 190 kDa [12] and has an isoelectric point at 5.7-7.6 [18]. There are several differences between mammal antibodies and avian IgY: molecular weight [28], structure of the hinge region [26], stability to proteolytic enzymes and temperature [1, 13, 31]. IgY does not bind protein A/G, does not activate mammal complement system and does not interfere with the rheumatoid factor [26].

IgY extraction from egg yolk is difficult because of the high lipid content of egg yolk [29]. Up to date several methods have been described [1,2,3,4,9,11,16,22,23]. High purity antibodies may be used for therapeutic purposes in several drug delivery systems (lyposomes, natural matrix a.s.o.) [14,21,30]. For investigating the organization of the antibody-delivery system complexes, modern techniques were used in the last decade [15,20,28].

The paper presents an optimized method used for extracting the antibodies from the product Globigen® Pig Doser (Ghen Co. Japan). The results were compared with three classic IgY extraction methods from egg yolk in terms of purity and yield. Globigen® Pig Doser is a veterinary pharmaceutical product, an oil suspension of dried egg powder obtained from hens immunized with specific antigens. The egg powder contains both the yolk and the egg white.

The present experiment was aimed to provide a fast, simple, inexpensive method for producing high purity IgY in the presence of ovalbumin.

Materials and methods

Chemicals:

PEG₆₀₀₀, chloroform, sodium chloride, were purchased from Merck (Darmstadt, Germany) and used without further purification. Globigen® Pig Doser product was obtained from Ghen Corporation, Japan. Dialysis membrane (molecular weight cut-off (MWCO) 14,000) was purchased from Spectrum Europe B.V. (Breda, The Netherlands). Phosphate buffer system (PBS), Biuret reagent and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Distilled and deionized water from a Milli-Q water system (Millipore, Bedford, MA, USA) was used for sample solution preparation. For electrophoresis there was used acrylamide, premixed gel-casting buffer,

sample loading buffers, premixed running buffers, Coomassie Brilliant Blue R-250 staining and destaining solution purchased from Bio Rad Laboratories and the Pierce 3-Color Prestained Molecular Weight Marker Mix.

Instruments and equipments:

Analytik Jena AG Specord 210 UV-VIS spectrophotometer, Hettich Mikro 120 centrifuge, BIO-RAD Mini-Protean III electrophoresis cell together with the Bio Rad PowerPac basic power supply unit, BioRad Fluor S MultiImager and Image Quant TL image analysis software (2005) from Amersham Biosciences.

Methods:

The IgY was purified from egg yolk by three extraction methods briefly described below: the polyethylene glycol (PEG), chloroform-PEG and water dilution method. The methods were compared in terms of purity and yield.

In addition we purified the IgY from a commercial product, Globigen[®] Pig Doser (Ghen Corporation, Japan), by a modified chloroform-PEG method.

Extraction of IgY from egg yolk

Yolks were separated from egg white, washed with distilled water and rolled onto paper towels to remove adherent egg white.

IgY extraction by the PEG method is one of the most simple and common. The method was carried out according to the protocol described by Schade et al. (2000) [25]. Samples of yolk were diluted in PBS pH=7.3. Lipids were removed by addition of 3.5% PEG₆₀₀₀ (Merck) followed by centrifugation at 14.000 x g (Hettich Micro 120). The supernatant was filtered to remove residual lipids and then the IgY was precipitated three times with 12% PEG₆₀₀₀.

The chloroform-PEG extraction was made as previously described by Polson [23]. Egg yolks were double diluted in PBS pH=7.3, chilled at 0°C and then double diluted with chloroform. The mixture was kept cold (4°C) for 30 min and then centrifuged at 14.000 x g for 20 minutes (Hettich Micro 120). The resulting supernatant was precipitated three times with 12% PEG₆₀₀₀.

The water dilution method, described in literature [25], consisted of diluting the yolk in chilled distilled water pH 5.0. The mixture was frozen at -20°C for 72 hours and slowly thawed at 4 °C. The solution obtained was centrifuged at 2800 x g for 20 minutes in order to remove the lipids. The IgY was precipitated by solid ammonium sulfate or 2M ammonium sulfate solution followed by incubation at room temperature and centrifugation.

Extraction of IgY from Globigen® Pig Doser

The IgY from Globigen® Pig Doser was purified by a modified chloroform-PEG method. The method is based on the chloroform-PEG method described by Polson with the modification of several parameters (incubation time, precipitate solubilization, concentration of the precipitating agent) with the purpose of maximizing the antibody purity [23].

In order to remove the residual low molecular weight proteins and the precipitating agents all the samples were dialyzed against PBS pH 7.3 using a dialysis membrane, MWCO=14 kDa, purchased from Spectrum Europe B.V.

The IgY extracts were analyzed for total protein content and immunoglobulin purity. Protein levels were determined by either Biuret or UV photometric assay [12]. Antibody purity was assessed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) using a 10% polyacrylamide gel under non-reducing conditions in a Bio-Rad Miniprotein III electrophoresis system. Molecular weight of the protein fractions was determined by using a molecular weight marker (Pierce 3-Color Prestained Molecular Weight Marker Mix). The gels were stained by the Coomassie blue method. The interpretation of the results was performed by computerized densitometry using the software Image Quant TL (2005) from Amersham Biosciences.

Results and discussion

Total proteins from the egg yolk extracts was determined by the Biuret method. The Globigen® Pig Doser extract had protein levels below the Biuret method's sensibility and they were calculated by UV photometric assay, using an UV-spectrophotometer (SPECORD 210).

The total protein content of the samples was:

-egg yolk extract:

- PEG method: 2 mg/mL
- chloroform-PEG method: 2 mg/mL
- water dilution method: 1 mg/mL

-Globigen® Pig Doser extract: 0.3 mg/mL .

The samples obtained from yolk and those from Globigen® Pig Doser migrated on different gels. The final antibody solutions obtained by each extraction method and dialyzed migrated on the first gel: water dilution (line 1), chloroform-PEG (line 4), PEG (line 5) and the protein molecular weight markers on line 8 (figure 1).

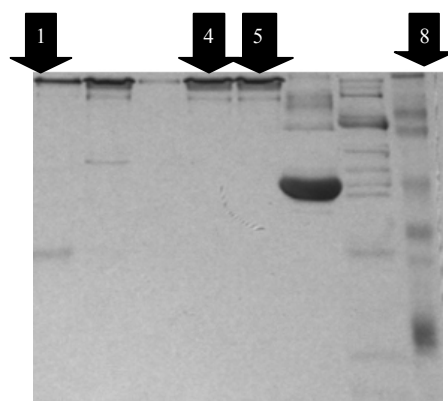


Figure 1

Proteins separated by electrophoresis from egg yolk extracts (line 1 water dilution method, line 4 chloroform-PEG method, line 5 PEG method, line 8 molecular weight markers)

The IgY antibodies, in their native, non-reduced form can be found on the gel in the 190 kDa region. The proportion of the proteins separated in each sample by electrophoresis was calculated by computerized densitometry. The background color intensity of the gel was reduced by the “rolling ball” method.

The IgY is the major glycoprotein in all the samples. The purity of the antibodies is different in the analyzed methods; it was 72.25% for the water dilution method, 89.39% for the PEG method and 91.21% for the chloroform-PEG method. In terms of purity the best extraction method, from those compared, is the chloroform-PEG method.

The Globigen® Pig Doser extracts migrated on a second gel (figure 2). The final IgY extract migrated on line 4, the hydro-soluble fraction obtained after chloroform delipidation on line 5. On line 8 migrated the molecular weight markers.

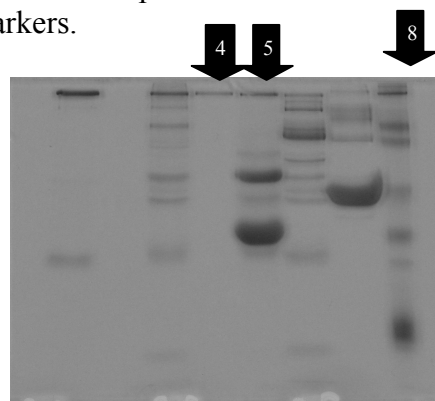


Figure 2

Protein fractions separated from Globigen® Pig Doser (line 4: final IgY Globigen® Pig Doser extract, line 5 hydrosoluble fraction of Globigen® Pig Doser, line 8 molecular weight markers)

The analysis of the gel was carried out in the same conditions as for the previous gel. The IgY purified from Globigen® Pig Doser by the modified chloroform-PEG method is clearly identified as a single band and also the graphical analysis of the gel shows a single peak in the 190 kDa region. IgY purity was 100%. In the water soluble fraction of Globigen® Pig Doser there were present 5 peaks and the IgY purity was 6.79%.

Figures 3 and 4 show the IgY purity and method yield (expressed as milligrams of antibody obtained from one milliliter of egg yolk or Globigen® Pig Doser) for the extraction methods used.

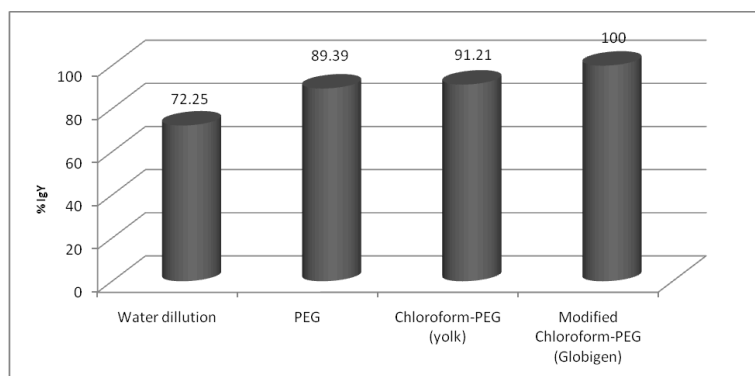


Figure 3
IgY purity

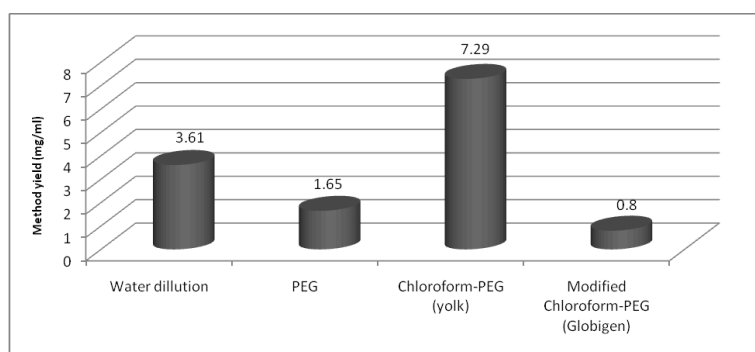


Figure 4
Yield of the extraction methods

Globigen® Pig Doser consists of an oil suspension of dried egg powder, which includes ovalbumin. All the IgY extraction protocols found in literature, in the first step, of separating the egg white from the egg yolk specify to remove the albumen from the yolk membrane as well as possible, by washing it with distilled water and rolling on paper towels to remove

adherent egg white as it may impede the purification. Large scale denaturation of egg white proteins does not occur during drying, but may initiate changes in proteins' structure which can affect the functional properties of rehydrated products [8]. The hydrophobic nature of ovalbumin is also increased by drying [19]. These may be the causes for which the albumin did not interfere the extraction and it was fully removed from the hydrosoluble fraction after repeated precipitation with PEG₆₀₀₀.

Egg yolk also suffers changes during drying, it loses its foaming ability and the solubility of lipovitellin is destroyed[7]. The IgY molecule is not altered during drying and may still be used as a source of antibodies [10]. The solubility changes of the yolk emulsion during drying and its inclusion in an oil suspension may be the reasons for which the antibodies are better separated from Globigen® Pig Doser than from crude yolk.

In the water soluble fraction of Globigen® Pig Doser the ovalbumin (47kDa) is present together with another 32 kDa protein but they were completely removed during the repeated precipitation with PEG.

The yield of the IgY extraction methods analyzed was calculated as the quantity of antibodies that can be obtained from 1 mL of egg yolk or from the product Globigen® Pig Doser. The yield of the chloroform-PEG method was 7.29 mg IgY/mL yolk; for the water dilution method it was 3.61 mg IgY/mL yolk, for the PEG method it was 1.65 mg IgY/mL yolk. The modified chloroform-PEG method using the Globigen® Pig Doser product yielded 0.8 mg IgY/mL Globigen® Pig Doser. The purpose of this work was obtaining high purity antibodies. Since different products were used for extraction (yolk or Globigen® Pig Doser) and the initial IgY concentration was not determined, the method yield reported is only intended for an estimation of the quantity of antibodies that may be produced by each method.

For obtaining high purity antibodies with a good yield we recommend the chloroform-PEG extraction method from egg yolk. If electrophoretically pure antibodies are needed we recommend the use of the modified chloroform-PEG method from the Globigen® Pig Doser product.

Conclusions

The product Globigen® Pig Doser is more suitable than the crude yolk for producing high purity IgY by the modified chloroform-PEG method. The antibodies obtained are electrophoretically pure.

The ovalbumin present in Globigen® Pig Doser is completely removed by repeated precipitation and does not affect the purity of the final product of extraction.

The extraction of IgY from the product Globigen® Pig Doser, by

the modified chloroform-PEG method is a convenient, fast and readily available method for obtaining electrophoretically pure antibodies.

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