

## Extraction and Purification of Anti-Proteinase 3 (PR<sub>3</sub>) Antibodies from Egg Yolk

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Received 6 September 2003; revised 7 August 2004; accepted 15 August 2004



### ABSTRACT

Recently it has been reported, that immunoglobulin Y (IgY) can be used instead of polyclonal antibodies extracted from mammals (IgG) for the purpose of diagnosis and therapy. These antibodies are found to have better properties in terms of specificity and ease of large-scale production. In addition, IgY binds neither to mammalian complement or Fc-receptors nor does it interfere with rheumatoid factors (RF), which has proven to be advantageous in many immunological tests. Proteinase 3 (PR<sub>3</sub>), a constituent of azurophil granules of neutrophils, is the target antigen for most anti-neutrophil cytoplasmic antibodies (c-ANCA) in Wegener granulomatosis (WG). Capture ELISA was found to be the method of choice in case of c-ANCA determination for the diagnosis and management of WG. However, in this method, the reaction of RF with the Fc portion of IgG in capture ELISA leads to false positive in the assay of c-ANCA and is found to be the most important shortcomings of available diagnostic immunochemical tests using mammalian antibodies. To avoid such unwanted interactions, laying hens were inoculated with PR<sub>3</sub>, and IgY was purified from egg yolk by acidic extraction with chloroform. The aqueous phase was treated with sodium sulphate and the precipitate collected, was dissolved in buffer and was purified using a T-gel chromatography method. The prepared IgY-anti-PR<sub>3</sub> was used to set up a capture ELISA. Our results showed that the prepared IgY-anti-PR<sub>3</sub> had good titer (1 µg/ml in a coating system) and specificity. Hence, IgY based immunoassay would be a useful alternative to mammalian IgG antibody used in PR<sub>3</sub> immunoassays. *Iran. Biomed. J. 9 (1): 41-45, 2005*

**Keywords:** IgY, Egg yolk Immunoglobulin, Proteinase 3 (PR<sub>3</sub>), Wegener granulomatosis, Anti-neutrophil cytoplasmic antibodies (c-ANCA)

### INTRODUCTION

Immunoassay techniques play an important role in clinical diagnosis and medical sciences. The most important component of these methods are immunoglobulins which are mainly produced and raised in mammalian systems. Yolk Immuno-globulin (IgY) is an antibody that is found in egg, and is different in molecular weight and isoelectric pH compared to IgG of mammals [1-3]. Because of the evolution difference between mammals and birds, the antibody that is produced against animal proteins in birds can

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be prepared easier than in animals such as rabbits or goats. The immune system of birds is similar to that of mammals and the immunoglobulins produced in ovine serum are transferred into the egg [1]. Some other advantages of IgY compared to IgG are as follow: 1) Each hen lays 5 to 7 eggs per week and 50 mg of IgY antibody can be purified from each egg. 2) Yolk immunoglobulins can eliminate the bleeding stage [4]. 3) The eggs can be stored for about one year at 4°C [1]. 4) IgY does not cross-react with mammalian antibodies or bind to mammalian complement, Fc receptors, or rheumatoid factors (RF) [1-4]. 5) The tedious use can often reduce unwanted background in immunoassays, since the diagnoses of autoimmune diseases in human are performed by immunoassay.

In patients suffer from autoimmune diseases, antibodies are produced against the body materials which should be detected in order to diagnose and treat the patients. In these diseases, because of the undesirable reactions of IgG (that produced in mammals) with Fc receptor and RF, the immunoassay methods necessarily need a high amount of antigen. In Wegener granulomatosis (WG) body produces classical anti-neutrophil cytoplasmic autoantibodies (c-ANCA) that are anti-PR3, which cause severe implications in kidneys, lungs, eyes and subsequently the whole body [5-7]. Since the presence of c-ANCA in blood can indicate the WG disease, different methods have been introduced to determine classical c-ANCA in blood [5-7]. Nowadays, the proteinase3 (PR3) is extracted from human blood for ELISA [8], therefore, the kit preparation is very expensive and needs high amount of human PR3. Since the antibodies against PR3 for ELISA method are prepared in mammals such as rabbit and goat, and the Fc of these antibodies can bind to RF in patients with positive RF, therefore, if the patient's serum added to the ELISA plate containing RF, it can binds to the IgG and leads to false positive results [9]. To overcome this problem, PR3 is added to the well before adding patient's serum [9]. This method needs a high amount of PR3; therefore if an antibody can be prepared that does not bind to RF then the problem will be solved.

Since, the yolk antibody (IgY) has this specificity, we injected PR3 into the chicken to purify IgY from egg yolk and to determine its activity.

## MATERIALS AND METHODS

Chloroform, sodium sulfate, divinylsulfone, 2-mercaptoethanol (2 ME), and Sepharose CL-6B were purchased from Merck Company (Germany). Complete and incomplete Freund's adjuvant and other reagents were purchased from Biogen Company (Mashhad, Iran). PR3 and Rabbit anti-PR3 and other antibodies used in this work were gifted by Prof. M.R. Daha from Nephrology Department, Leiden University of Medical Sciences, the Netherlands [10].

**Chicken immunization.** Immunization was carried out according to the modified Per Hansen *et al.* [1] method: 1 ml of normal saline and 1 ml of complete Freund's adjuvant were added to 260 µg of PR3 and mixed. The solution was injected into the breast region of two white Leghorn-laying chickens (20 weeks old) subcutaneously (each chicken received 130 µg of PR3). Booster injections were given after 3 and 6 weeks with incomplete Freund's adjuvant. In each booster injection, the chicken received 100 µg of PR3. One week after the last injection, the eggs were collected and IgY antibody was extracted, purified and titrated by ELISA method.

**Purification of IgY.** IgY and other water-soluble proteins were extracted according to Akita and Nakai [11] and Natakumana *et al.* [3] methods with a slight modification. The yolks of 3 eggs (collected from PR3 immunized chickens) separated from the white and washed with deionized water. The yolk sac was disrupted and the contents were allowed to pass through a nylon mesh into a measuring cylinder, and the final volume was determined. 15ml of 3 mM HCl was added to 3 ml of yolk and the pH was adjusted to 5 by adding 10% acetic acid solution.

The suspension was incubated at 4°C for 3 hours. The supernatant containing IgY was collected by centrifugation at room temperature at 2000 X g for 20 minutes. Equal volume of chloroform was added to stirring supernatant, and the mixture was incubated at 4°C for 12hours. The supernatant was collected by centrifugation at room temperature at 2000 × g for 15 min and used for the next step. Solid Sodium sulfate (2.6 gram) was gently added to 13 ml of stirring supernatant. The precipitate was collected by centrifugation (as above), and redissolved in 4 ml of 10 mM TBS buffer, pH 7.3 containing 0.15 M NaCl and 0.05% NaN<sub>3</sub> and then 2 ml of 36% sodium sulfate was added. The mixture was centrifuged and the precipitate was dissolved in 2ml TBS (10 mM, pH 7.3) buffer containing 0.5 M sodium sulfate.

T-gel chromatography was performed according to the Scoble and Scops method [12, 13], briefly: Sepharose CL-6B (1.8 ml) was suspended in 1.8 ml of 0.5 M sodium carbonate, pH 12 and after adding 150 µl divinylsulfone, the mixture was stirred at room temperature for 6 hours. The activated gel was washed with

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0.5 M sodium carbonate, pH 11 and the volume was adjusted to 3.6 ml by sodium carbonate. Suspension was added 250 µl of 2ME and stirred at room temperature for 1 hour. The prepared gel was loaded onto a column (5 × 0.55 cm). T-gel column was equilibrated with TBS buffer containing 0.5 M Na<sub>2</sub>SO<sub>4</sub>. The effluent was monitored at 280 nm on a Jenway UV-Vis 1038 spectrophotometer. The column was washed with equilibration buffer until the absorbance at 280 nm was equal to zero. The solution obtained from the last precipitation step with sodium sulfate was loaded onto T-gel column and was eluted with TBS buffer containing 0.5 M Na<sub>2</sub>SO<sub>4</sub>. The absorbance of the eluted solution was monitored at 280 nm. Purification of PR3 was confirmed by cellulose acetate electrophoresis as described elsewhere [14].

**ELISA development.** ELISA plates (Nunc, Maxisorp, Roskilde, Denmark) were incubated according to Larsoon *et al.* [15] method with slight modification; briefly: the ELISA plates were incubated at 37°C for 2 hours with 100 µl of serially diluted IgY (1/1000, 1/500, 1/100 and 1/10 corresponding to 0.1, 0.2, 1 and 10 µg/ml respectively) in PBS (20 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.15 mM NaCl, and 0.1% NaN<sub>3</sub>, pH 7.2) or 100 µl of a rabbit polyclonal anti-PR3 IgG (0.1 µg/ml in carbonate buffer containing 74 mM NaHCO<sub>3</sub>, and 26mM Na<sub>2</sub>CO<sub>3</sub> pH 9.6). After each step of incubation, the plates were washed 3 times with 10 mM PBS containing 0.05% Tween-20. All solutions were prepared in 10mM PBS buffer containing 0.05% Tween-20 and 2% casein. Following coating, 200 µl of blocking buffer (10 mM PBS containing 0.05% Tween 20 and 2% casein) was added to each well and incubated at 37°C for 30 minutes. Then, 100 µl of 50ng /ml PR3 was added and incubated at 37°C for 2 hours. After washing, anti-PR3 IgG bounded to digoxin (Dig) was added to the wells and incubated at 37°C for 2 hours. The wells were washed and sheep HRP-anti-Dig was added and then incubated for 2 hours. After washing, 100 µl of tetramethylbenzidine substrate was added to the wells. After 45min, 50 µl of 1N HCl was added to each well and the absorbance was monitored at 450 nm using a Behring 41997 ELISA reader.

## RESULTS

The result of IgY purification is shown in Figure 1. Based on the measurement of the absorbance at 280 nm, the amount of purified IgY was found to be 35 mg per egg. The result of ELISA assay is shown in Figure 2. IgY optimal titer for the assay was 1 to 100 (1µg/ml). The same procedure that was described for ELISA was used for standard curve. IgY (1 µg/ml) was coated on the plate and after incubation and washing, different concentrations of PR3 (1.17, 2.34, 4.68, 9.37, 18.75, 37.5, 75 and 150 ng/ml) were added in duplicate.

**Fig. 1.** A typical chromatogram after T-gel chromatography. Peak II contained IgY. (Column dimensions was 5 × 0.55 cm, flow rate = 15 ml/h).

Other steps were followed as described in ELISA procedure. A typical dose response curve is shown in Figure 3.

To determine whether ELISA system is able to recognize c-ANCA, 5 serum samples that have previously been determined to be c-ANCA positive by commercially available ELISA kit (Ortentec, Germany) and 5 control normal sera were obtained from the Iranian Rheumatology Center Laboratory (Tehran, Iran). For c-ANCA testing, serum samples were tested in duplicate at a dilution of 1: 25 (in PBS 10 mM containing 0.02% Tween 20 and 2% casein) in wells coated with IgY-anti-PR3 and then antigen (PR3) was added to each well. In control



**Fig. 2.** Titer assay of anti-PR3 IgY by ELISA. Optimum dilution of IgY was found to be 1/100 (1 µg/ml).

**Fig. 3.** ELISA dose response curve for concentration of PR3 from 1.17 up to 150 ng/ml. Each data point represents the mean  $\pm$  SEM of 5 consecutive experiments.

wells, no antigen was added (background). Net absorbance value was calculated by subtraction of the background value obtained from wells without antigen and wells containing antigen. Finally, the correlation between the commercial ELISA kit and ELISA that was performed by us was calculated. All samples that were positive for c-ANCA by commercial ELISA kit were also positive by ELISA that was set up by us. The sample dilution test was performed in order to validate ELISA using a sera with high titer of c-ANCA. The result is shown in Figure 4.

**Fig. 4.** ELISA dilution curves of c-ANCA positive sera with PR3. HRP labelled anti-human IgG was used as a secondary antibody. Each data point represents the mean  $\pm$  SEM of 5 consecutive experiments.

## DISCUSSION

In this study, we successfully produced antibody against human PR3 in egg and also purified IgY and the biological activity and efficiency of injection and purification procedure. Since IgY differs in molecular weight and isoelectric pH from mammalian IgG and also binds neither to mammalian complement or Fc-receptors, nor to RF, these antibodies are of use in cases where RF interferences are expected. By injecting some mammalian molecules into the chicken, researchers have produced specific IgY against these molecules, and investigated the advantages of IgY compared to immunoglobulins produced in other animals [1, 4].

One of the important factors in specificity of produced antibody against an immunogen is the method of injection. In the present work, we used Hansen *et al.* [1] procedure, because injection is subcutaneous and many regions are involved and the amount of immunogen is low. Therefore, it is expected that the action of produced antibodies to be specific. We also modified the purification of IgY [1] and the efficacy of this procedure improved and IgY purified in an optimal amount and quality.

PR3 is an autoantigen in human body and causes the production of an autoantibody in WG disease. These antibodies termed as c-ANCA [5-7]. The presence of c-ANCA and its concentration in blood can be used in prognoses of WG, so the determination of the amount of these antibodies in an individual is very important [5-7]. Indirect immunofluorescence is used as conventional method for the measurement of c-ANCA concentration [9]. Despite its advantages in some cases, this method produces false positive results [9]. Therefore, researchers suggest the use of ELISA because in this method the exact amount of c-ANCA is measurable and does not have false positive result [9]. Due to the interference of RF (that is produced in sera of some patients) with the IgG produce in mammals, PR3 should bind to wells, then the serum of suspicious WG individuals should be added and the amount of c-ANCA be determined. The problem of this method is consumption of a lot of PR3 antigen that is extracted from human blood [8] and therefore, the kits are very expensive and only a few laboratories can afford to use them. IgY does not have any interference with RF [1-5], and if this antibody is used first in ELISA, and then PR3 antigen is added, there would be no interference and consuming of PR3 will considerably decreased.

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