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Full Length Research Paper

Production and purification of polyclonal anti-hamster immunoglobulins in rabbits

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Polyclonal antibodies are mixtures of monoclonal antibodies that were produced against different epitops. The goal of this project is to know the production, purification and horseradish peroxidase (HRP) conjugation of polyclonal antibodies against hamster immunoglobulins in rabbits. 300 µg/300 µl of ten hamster immunoglobulins was mixed with the same volume (300 µl) of adjuvant and injected into three 6-month-old white New Zealand rabbits. Anti hamster rich rabbits serums were isolated from whole blood and precipitated with ammonium sulfate in the final concentration of 50%. The precipitate was dialysed against phosphate buffered saline (PBS) (pH: 7.4) and applied to ion exchange chromatography (IEC) on diethylaminoethyl (DEAE)-sepharose 6B with tris-phosphate (pH: 8.1), and tris-phosphate contain 50 mM NaCl buffer. The purity of produced antibody was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reduced condition. Then purified immunoglobulin G (IgG) was conjugated with HRP. For exact measurement of conjugated IgG titer and evaluating of cross reaction, enzyme linked immunosorbent assay (ELISA) test was designed. Since IEC is a more simple and inexpensive method for the purification of IgG, we obtained a protein with approximate purity of 95%. Produced IgG showed high titer and high specificity in the designed ELISA. Purified antibody and its conjugation with HRP are used in research and diagnosis of hamster disease.

Key words: Production, purification, hamster immunoglobulins.

INTRODUCTION

Polyclonal antibodies represent a group or mixture of antibodies produced by different B-lymphocytes in response to the same antigen; thus, different antibodies in the group recognize different parts of the antigen. The diversity of antibodies provides an advantage by allowing the detection of multiple epitope sites on the protein of interest (Afolabi et al., 2009).

Polyclonal antibody production in mammals is generally associated with multiple injections of antigens, adjuvants and repeated blood sampling procedures (Hau and Hendriksen, 2005), although, the production of these antibodies requires the use of substantial number of animals with considerable animal welfare consequences. In the case of polyclonal antibodies (PAbs), animals are given injections of antigen or antigen/adjuvant mixtures for the induction of effective antibody responses, and it is usually necessary to collect blood to monitor antibody response during the experiment to obtain the antibodies (Leenaars and Hendriksen, 2005).

In biochemical and biological researches, polyclonal antibodies are routinely used as ligands for the preparation

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Abbreviations: PAbs, Polyclonal antibodies; **ELISA,** enzyme linked immonosorbent assay; **HRP,** horseradish peroxidase; **IgG,** immunoglobulin G; **PBS,** phosphate buffered saline; **IM,** intra muscularly; **SDS-PAGE,** sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **CFA,** complete Freund's adjuvant.

of immunoaffinity columns (Shin et al., 2001) and they have application in immunoassay tests such as: Enzyme linked immonosorbent assay (ELISA), western blot, double diffusion (Calabozo et al., 2001; Cheung et al., 2002; Verdoliva et al., 2000), immunohistozchemistry (Smedley et al., 2007) and immunofluorescence (Lipman et al., 2005).

Separation and recovery of proteins from ion exchange chromatographic (IEC) media are affected by factors such as buffer type and pH, length of gradient, flow rate of the mobile phase, ionic strength, nature of counter ion and characteristics of the proteins (Stec et al., 2004).

This study was carried out to know the production, purification and horseradish peroxidase (HRP) conjugation of polyclonal antibodies with the nature of immunoglobulin G (IgG) class against hamster immunoglobulins in rabbits. HRP conjugated IgG against hamster immunoglobulins are used in diagnosis of hamster diseases such as Leishmania by ELISA or western blotting tests.

MATERIALS AND METHODS

Preparation of antigen

For production of polyclonal antibodies against hamster immunoglobulins, ten hamster were bled and related serums were pooled, clarified with centrifugation (1000 g, 15 min) and diluted 1:1 with phosphate-buffered saline (PBS, pH: 7.2) (Stec et al., 2004).

Equal volumes of diluted serum and saturated ammonium sulfate were mixed by slow addition of the ammonium sulfate during gentle stirring. Therefore, immunoglobulins were precipitated with ammonium sulfate at the final concentration of 50%. The suspension was centrifuged at 2000 g for 20 min, and washed twice with 50% saturated ammonium sulfate (Sakhuja et al., 2009). Immunoglobulins rich precipitate was dialyzed against phosphate buffered saline (PBS) (pH: 7.2) overnight with stirring (Abdel et al., 2003). Final protein concentration of solution was adjusted to 1 mg/ml.

Immunization of rabbits with hamster immunoglobulins

300 µg/300 µl of prepared immunoglobulins was emulsified with equal volumes of Complete Freund's adjuvant (Sigma) and inoculated intra muscularly (IM) in to the three 6-month–old New Zealand white rabbits. The second and third inoculations were performed on days 21 and 35 with incomplete Freund's adjuvant (Sigma), and fourth inoculation was done on day 45 without any adjuvant. After final immunization, blood samples were taken from the rabbits (Uwaifo and Winter, 2003).

Purification of rabbit anti-hamster immunoglobulins

Immunized rabbits serum were collected and precipitated by 50% ammonium sulfate after dialysis against PBS and tris-phosphate buffer (pH: 8.1). The concentrated crud extract was loaded onto an ion exchange chromatography column packed with diethylaminoethyl (DEAE)-Sepharose (Javanmard et al., 2005) fast flow (Pharmacia) equilibrated with tris-phosphate buffer (pH: 8.1). The column was eluted with starting buffer and samples of fraction 1 were collected in 5 ml fractions. The second fraction was eluted with the same buffer containing 50 mM NaCl. The flow rate used at all stages was 0.25 ml/min. Protein content of the collected samples was assayed by using UV spectrophotometer on 280 nm absorbance.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Purity of the eluted fractions from the IEC column was checked by SDS-PAGE analysis that was performed under reducing conditions according to the standard Laemmeli method (Mohammadian et al., 2010). The total polyacrilamide concentration was 12% for separating gel and 4% for stacking gel. Detection of the protein bands was performed with the Coomassie Brilliant Blue R-250.

Conjugation of rabbit IgG with peroxidase

The conjugation was performed by the periodate method (Nakane and Kawaoi, 1974). 5 mg of peroxidase (Sigma) was dissolved in 1.23 ml of distilled water in dark glass container. And 0.3 ml of 0.1 M sodium periodate (Merck) was dissolved in 10 ml of sodium phosphate (pH: 7) and placed in room temperature for 20 min. Then, it was dialyzed against 1 mM sodium acetate (pH: 4) with several times exchanges overnight. Peroxidase was added to the solution of 10 mg/ml of IgG dissolved in sodium carbonate (pH: 9.5) and incubated for 2 h in room temperature. 100 µl of sodium bourhidrate (Merck) (4 mg dissolved in 1 ml water) was added and incubated for 2 h at 4°C. Finally, it was dialyzed against PBS overnight at 4°C with several times exchanges.

Enzyme linked immunosorbent assay (ELISA)

Direct ELISA for determining the titer of HRP conjugated IgG and evaluating cross-reaction of produced antibody among rat and Balb/c was designed. 100 µl of prepared hamster, rat and Balb/C immunoglobulins were added to each well of 96 well microtiter plates and incubated at 37°C for 1 h. The wells were washed with PBS-Tween (0.05% Tween 20) three times. The wells were saturated with PBS containing 2% bovine serum albumin (BSA) for 1 h at 37°C, to block nonspecific sites of the wells. After a washing step, 100 µl of 1:400, 1:800, 1:1600 and 1:3200 dilutions of prepared HRP conjugated anti-hamster immunoglobulins were added to each well. The plate were incubated for 0.5 h at 37°C and washed five times with PBS 0. 5% Tween 20. The reaction was developed using 100 µl of 3,3´,5,5'-tetramethylbenzidine (TMB) as substrate. The absorbance was determined at 450 nm after stopping the reaction by 5% sulfuric acid.

RESULTS

For the evaluating of the production of rabbit anti-hamster immunoglobulins double diffusion test was performed.

In double diffusion test interaction among antigen, undiluted and diluted (with 1:2 and 1:4 dilution) antibodies were shown the sharp and weak line of precipitation respectively. The line of precipitation are not formed between antigen and undiluted antibody with 1: 8 dilution.

At the first step of purification, elution of protein with Tris-phosphate buffer resulted in purification of about 37 mg of pure IgG. In this step the elution profile gave one main IgG peak. At the second step of IEC the elution of protein was performed with Tris-phosphate buffer containing 50 mM NaCl at the same pH, over 2 hours, at

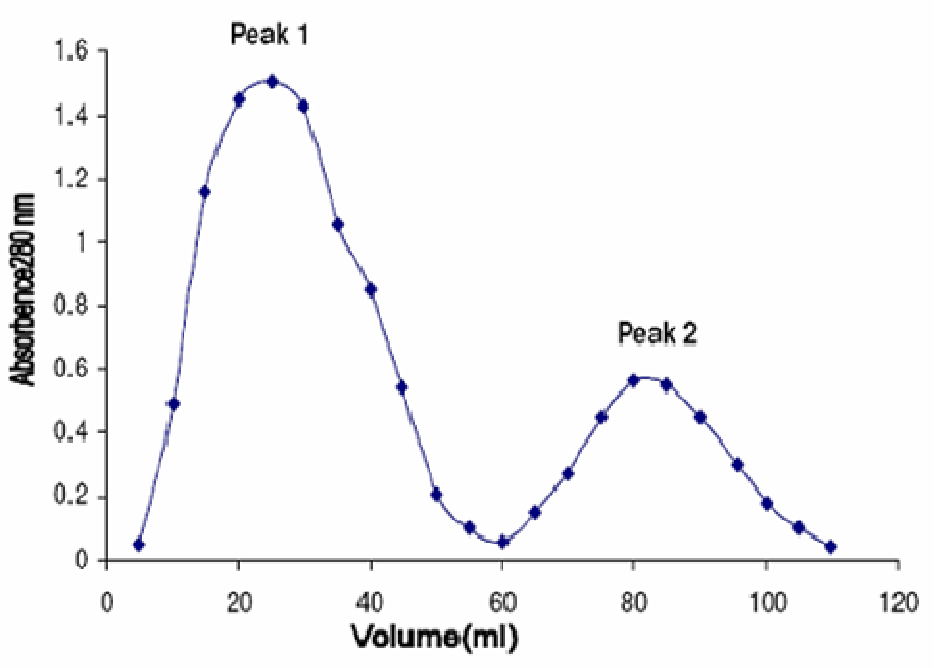

Figure 1. Chromatographic pattern of purified rabbit anti-hamster immunoglobulins by ion-exchange column with tris- phosphate buffer, pH = 8.1 (first peak) and 50 mM NaCl elution (second peak). Sample, Rabbit IgG; Column, DEAE-Sepharose; Flow rate, 0.25 ml/min; Fraction 1, tris-phosphate pH = 8.1; Fraction 2, tris-phosphate + NaCl 50 mM pH = 8.1.

a flow rate of 0.25 ml/min and resulted in isolation of about 8 mg of pure IgG. Finally we obtained approximately 45 mg purified IgG after elution from IEC which was about more than one third of the primary gross protein mixture. The result of chromatography has been shown on figure 1.

In this study we obtained a protein with approximate purity of 95% which its purity was confirmed by SDS-PAGE under reduced condition (figure 2).

Lane 1 and lane 2 contain polled fractions from the first ion exchange step. Lane 3 corresponds to the sample of 2nd fraction obtained from salt step elution. On figure 2 the distinct polypeptide bands show molecular weight about 50 KDa which correspond to rabbit IgG heavy chain. Also diffused bands between molecular weight s of 20-30 KDa correspond to rabbit IgG light chains. Lane 4 contains LMW marker proteins.

The result of titration of HRP conjugated IgG with the disigned ELISA was 1600 Prepared HRP conjugated IgG has no cross reactivity with Rat and Balb/C immunoglobulins at optimized dilution.

DISCUSSION

In this study rabbits were immunized with hamster immunoglobulins was mixed with the same volume (300µl) of complete Freund's adjuvant (CFA), which this adjuvant is a very potent and effective adjuvant with a wide range of antigen (Harold and Stills, 2005).

For evaluating the production of IgG double diffusion test was used that the result was recognized by line of precipitation. These result showed that the polyclonal anti-hamster immunoglobulins could be readily produced in New Zealand rabbits. The ability of diagnostic of this test is 18-30 µg/ml. The traditional purification of IgG involves several steps, including salt precipitation, dialysis, and anion exchange (Akita and Li-Chan, 1998). We used ion exchange chromatography for purification of rabbit IgG polyclonal antibody. Separation and recovery of proteins from ion exchange chromatographic media are affected by factors such as buffer type and pH, length of gradient, flow rate of the mobile phase, ionic strength and nature of counter ion, and characteristic of the proteins. The selec-tion of ideal conditions for protein purification involves changing some or all of these parameters.

Result showed that salt step elution between 0-50 mM may elute a considerable volume of bound protein from the column. Using Tris-phosphate buffer with DEAE-Sepharose gel for obtaining high purity products from purification strategies. Our findings like previous experiments verified that IEC, is an appropriate and useful method for the purification of polyclonal antibodies with the nature of IgG class appropriate method for the purification of rabbit IgG (Javanmard et al., 2005)

We obtained a protein with approximate purity of 95%, which its purity was approved by SDS-PAGE. We have

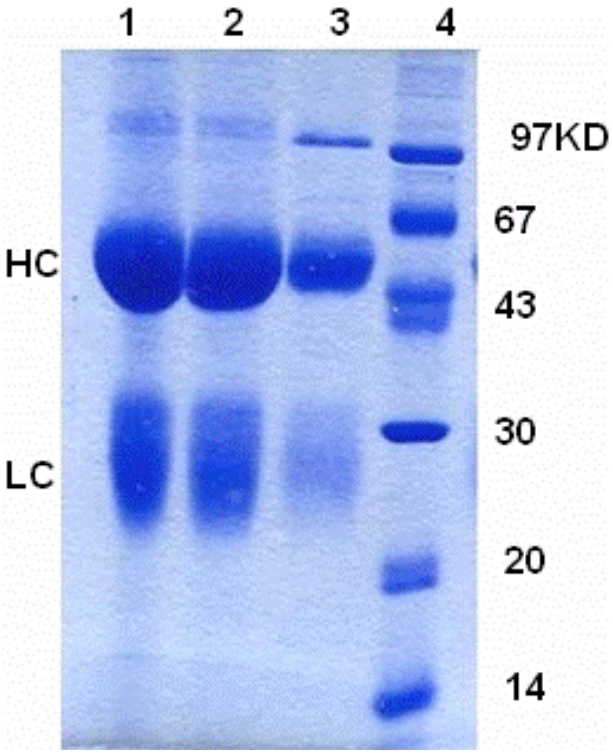

Figure 2. SDS-PAGE analysis of rabbit anti-hamster immunoglobulins purified by ion exchange chromatography in 12% polyacrylamide gel under reduced condition and stained with Coomassie brilliant blue R-250. Lanes 1 and 2, First fraction; Lane 3, second fraction; Lane 4, low molecular weight markers; HC, IgG heavy chains; LC, IgG light chains.

used enzyme horseradish peroxidase (HRP) to prepare enzyme conjugate for enzyme immunoassay through periodate method. HRP is one of the most popular enzymes used in immunoassay for its stability, wide pH range and high turnover (Anupam et al., 2005). HRP widely is used in enzyme based, immunodetection techniques to detect and characterize analytes. We used the periodate method for IgG cojugation becouse this method is an efficien means to conjugate peroxidase and protein. The performance of conjugates prepared with $NaIO_4$-activated HRP is superior by other common conjugation to that of conjugates prepered methods. Following the reduction of the Schiff base bond the conjugates are stable for extended periods of time. This mdification has minimal effect on HRP activity (Nakane and Kawaoi., 1974). Enzyme immunoassays rea-gents are more stable and do not have safety problems associated with radio isotopic labels. In addition, enzyme assays can be at least assensitive as radio-immunoassays. Many enzyme detection methods are visual or use a standard spectrophotometer eliminating the need for expensive, sophisticated equipment (Ali et al., 1981).

The produced antibody showed a high specificity to hamster immunoglobulins in the designed ELISA test in optimized titer because the conjugated IgG did not interact with Rat and Balb/C immunoglobulins.

The purified IgG is applicable in immunoassay tests for detection of hamster disease and instruction these tests to the medical students.

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