Production and Purification of Rabbit's Polyclonal Antibody Against Factor VIII

Simin Sohrabi · Azim Akbarzadeh · Dariush Norouzian · Ali Farhangi · Mehri Mortazavi · Mohammad Reza Mehrabi · Mohsen Chiani · Zahra Saffari · Soheil Ghassemi

Received: 26 April 2011 / Accepted: 30 April 2011 / Published online: 8 June 2011
© Association of Clinical Biochemists of India 2011

Abstract The attempt is made to produce recombinant factor VIII but the first step in producing such product is production and purification of rabbit's polyclonal antibody against factor VIII. The second and third steps involve monoclonal antibody and recombinant factor VIII production. Factor VIII is one of the most important coagulating factor where its deficiency leads to diseases like hemophilia type A or classic. It is an inherited disease. Previously, it was obtained through fractionation of blood plasma of blood donors. After processing, factor VIII could be used to manage such patients. Due to transfer of viral disease like hepatitis and HIV through factor VIII obtained by fractionation, high cost of production, insufficiency of the donors and the process of virus removal, thus production of factor VIII through recombinant technology can be useful and helpful. The reaction between antibody and antigen is one the most specific reaction; therefore, such reaction can be employed to identify factor VIII. Thereby, rabbits were injected several times with adjuvant-linked antigen to produce antibody. The antibody was separated from the blood sample, purified and used to identify factor VIII in the research.

Keywords Factor VIII · Polyclonal antibody · Antibody purification · Recombinant antibody · Quality control · Hemophilia

Introduction

Nowadays, purified antibodies are playing major role in the treatment and diagnosis of biological molecules. With regard to the advances in biotechnology and production of recombinant drugs, they are used to separate and identify manufactured products. Hemophilia is one of the inherited hemorrhagic diseases. Safety and the life of hemophilia patients are dependent on the preparation of safe factor VIII [1]. Previously, treated hemophilia patients with FVIII derived from blood plasma were contaminated with hepatitis and HIV viruses [2]. The aim of production and purification of antibody is to control quality of the produced recombinant factor VIII in order to minimize the side effects of plasma derived factor VIII and on the other hand, to prevent the payment of foreign currency to buy such drugs. The molecule of factor VIII (hFVIII) is secretory glycoprotein with the molecular weight of 330 KDa containing 2332 amino acids which is comprised of three similar domains A, of 2 similar domains C and 1 unit domain B. FVIII is arranged as A1-A2-B-A3-C1-C2 domains [3, 4]. The protein with Von Will Brant factor in the form of complex is circulated in blood to be protected and stabilized [5, 6].

The carbohydrate content of FVIII obtained from plasma is 1% sialic acid, 2.8% hexoseamin and 1–2% hexoses (mannose, galactose and focuses) [6]. It was also analyzed to contain a few molecules of non-polar lipid (include less than 5% protein, this lipid is composed of cholesterol, fatty acid and may contain triglyceride) and no

S. Sohrabi
Payame Noor University, Tehran, Iran

A. Akbarzadeh (✉) · D. Norouzian · A. Farhangi · M. Mortazavi · M. R. Mehrabi · M. Chiani · Z. Saffari · S. Ghassemi
Department of Pilot Biotechnology, Pasteur Institute of Iran,
12 Farvardin Street, Pasteur Avenue, Pasteur Square,
Tehran 13169-43551, Iran
e-mail: azimakbarzadeh@pasteur.ac.ir

phospholipids. Domain B is not needed for clotting activity of factor VIII [7]. But recent studies on its role in protecting the active form of factor VIII from the inactive proteolytic form are described [8]. Breakage of high chain length (HCL) containing A1-A₂ and domain B, low chain (LCH) A3-C₁, domain C₂ are formed [2, 9]. Purification of factor VIII gene created a new chance of treatment in 1984 [10, 11]. Through recombinant technology, recombinant factor VIII (rFVIII) which is a safe molecule and free of viruses can be produced [12–14]. Although, rFVIII is a biologic, and obtained from CHO culture but there is a danger of transferring infectious agents [15]. From the point of viruses transfer, about 25% of first concentrated generation of rFVIII, is positive (due to use of human/calf serum albumin) [16], on the other hand, the second generation of rFVIII product includes two products, one with full length domain (FL-rFVIII) and the other rFVIII without (BDD-rFVIII) by replacing serum with sucrose which may delete the transfer of viral agents, is produced [16]. In recent years, advancement and safety and therapeutic use of factor VIII have been increased [17].

Materials and Methods

Today, polyclonal antibodies are produced against specific antigens and then utilized. Antibodies are reagents widely employed in biological fields to test proteins, carbohydrates, lipids and nucleic acids. In addition, synthetic and non synthetic antigens are considerably produced by biochemical, biosynthetic and recombinant techniques to cause specific immune responses. Most of such new antigens are small molecules and weak immunogens so they need to be adsorbed onto adjuvant. In this research, first a vial of factor VIII by the name of “Antihemophilic factor (human) 570 IU (Talecris)” was taken to weigh out, the powder of factor VIII was added to an adjuvant namely Aluminum hydroxide with the composition of Al(OH)₃ 0.3 mM, NaCl 0.4 mM, Na₂HPO₄ 0.135 mM, NaH₂PO₄·H₂O 0.135 mM, the volume of the vial was made to 20 ml with such adjuvant solution. They were divided into 500 µl in the tubes equally. The empty vial was dried, weighed in order to obtain the weight of factor VIII. The weight of factor VIII was also determined spectrophotometrically at λ 280 nm [12] and the data obtained was compared to that of Bradford method. Four male Newzealand rabbits were selected, weighed (Italian Macchi balance), and blood was taken from the ears before inoculation of antigen. On 1, 5, 14 days, 4000 µg/0.5 ml of factor VIII antigen was injected into 3 parts of the rabbit's body. Before each injection, they were weighed, and their body temperature was controlled. The blood was taken from the rabbits' ears. Blood samples were kept at

refrigerator for 2 h for coagulation. The coagulated blood was separated from the serum by centrifugation at 5000 rpm for 5 min. The serum was kept in Eppndrof tubes and stored at –20°C. The produced antibody was qualitatively and quantitatively evaluated by employing techniques like immunodiffusion, hemagglutination, immunoelectrophoresis. SDS-PAGE, western blot, dot blot and ELISA (19, 20, and 21).

Immunodiffusion

Two wells were made on agarose gel; antigen and antibody were added to each well. It was kept at refrigerator temperature in a moist chamber for 24 h. It was stained with Coomassie blue. The presence of stained zone indicates antigen–antibody reaction [18, 19] (Fig. 1).

Single Radial Immunodiffusion (SRID)

The amount of antibody was determined by single radial immunodiffusion method in a Petri plate (10 cm in diameter). Antibody was diluted as 1/10, 1/100, 1/1000, 1/10000 and 1/100000. The plate was kept at refrigerated temperature in a moist chamber for 24 h. The corona was formed at 1/1000 dilution, therefore the titer of antibody is between 1/1000 and 1/10000, due to high titer of passive hemagglutination [20] (Fig. 2).

Staining

Solutions for staining contains: 0.25 g Coomassie blue, 10 ml acetic acid and 50 ml methanol. After mixing, final volume was made to 100 ml with distilled water. The gel was kept in staining solution for 10 min. The excess of stain was removed by destaining solution (methanol 50 ml, acetic acid 50 ml mixed well, the final volume was made to 400 ml with distilled water) for 2 h (19, 20, and 21).

Heamagglutination

It is based on the reaction between antibody and antigen. Here, passive heamagglutination was used. In this test, soluble antigen is sensitized by reagents like, tannic acid,

Fig. 1 Immunodiffusion: in right hole antibody and in left hole antigen was poured. Presence of stained zone indicates the reaction between factor VIII antibody with its corresponding antigen

Fig. 2 Radial immunodiffusion to estimate antibody titer, in first well 1/10 and the next wells were 10 times diluted respectively. The last corona was formed in 1/1000, therefore the titer is between 1/1000 and 1/10000

trypsin, periodate, glutaraldehyde carbodeamide etc., and it is coated onto sheep RBCs then produced antibody at different concentration is added to it. The formed lattice between antibody and antigen linked to RBCs prevents precipitation of RBCs [2, 15]. In this test, if antibody is in excess with regard to antigen, antibody alone attaches to RBCs and the phenomena is called as prozone and the low ratio of antibody to antigen causes postzone. This method is sensitive to antigen, antibody proportion, concentration of RBCs, pH, ionic strength, temperature and needs time to link RBCs to antigen and altogether to antibody [1]. Sheep red blood cells are maintained in Alsvers solution (glucose 20.50 g, sodium citrate 0.58 g, acetic acid 0.55 g, NaCl 4.20 g). This solution in addition to anticoagulant property has nutritional value. Red blood cell is maintained in this solution for 14 days. The volume is made to 1 l and the pH adjusted to 6.1. The used ratio is 1:1 for blood. The following reagents are prepared in order to make buffers with various pHs for washing and immobilization of RBCs.

A: NaH_2PO_4	0.15 M
B: Na_2HPO_4	0.15 M
C: NaCl	0.15 M

Solution A and B were mixed at the ratio of 2:5 to obtain pH 7.2. The equal volume of C was added to obtain buffer no. 1. They were also mixed at the ratio of 5:2 to obtain pH 6.4. The equal volume of C was added to obtain buffer no. 2. To 15 ml RBC in Alsvers solution, 30 ml of buffer no. 1 was added, mixed and centrifuged for 7–10 min at 800 rpm. This procedure was repeated till to get clear supernatant and to obtain washed RBCs. By using buffer

no. 1, 50 ml of 1:1000 tannic acid solutions was prepared and 10 ml of this solution along with 10 ml of buffer no. 1 were added to washed RBCs; thereby the concentration of tannic acid solution became 1:20000. It was kept at room temperature for 30 min, and then it was washed twice with buffer no. 1 and once with buffer no. 2. The RBCs sensitized with tannic acid were diluted to 2.5% RBCs with buffer no. 2. This was mixed with equal volume of antigens in buffer no. 2 and left at room temperature for 1 h. It was washed with buffers no. 1 and 2 (twice and once respectively) and centrifuged as before. The antibody in 96 wells plate was diluted serially with buffer no. 1 and to each well 100 μl RBCs (2.5%) was added. Positive and negative controls were designed on two rows of the wells. The prepared wells were kept at refrigerator for 24 h and the titer of the antibody was determined [13, 19, 21] (Fig. 3).

Immunoelectrophoresis

On agarose gel prepared on glass slid, two wells of 3 mm on each side were made by Pasteur pipette and a groove in the middle by Gellman LKB. The slide was kept in IEF tank [22].

Tank buffer solution was made of: Tris-barbital buffer 4X, diethyl barbitoric acid 22.4 g, tris base 44.3 g and calcium lactate 0.53 g and volume was made to 1000 ml with distilled water and adjustment of pH was not needed. It was diluted at the ratio of 1:4 before use. Tank buffer was added from two sides of the chamber reaching under the gel where the gel was in the middle of the chamber. Then Whatman paper (12.5 $\times$ 3 cm) was used as bridge between gel and buffer to connect them together. 5 μl of

Fig. 3 Hemagglutination of sheep RBCs plate: In horizontal rows blood of different rabbits [1–3, 5] and in vertical rows various serial dilutions of prepared antibody were added. The first dilution in well no. 1, is 1/20 pertains to rabbit's no. 1 antibody diluted serially. Series 2 and 3 pertains to rabbits no. 2 and 3. Rabbit no. 4 died during study. The last series is negative control i.e. rabbit's serum without antibody. The titer is exactly negative in well 8 which indicate the titer of antibody is 1/1280 which confirms the titer of radial immunodiffusion

antigen was added to a well on top right side and it was marked as anode. The immunoelectrophoresis was carried out at the current of 6 mA or 120 V for 45 min. Then 15 μ l antibody in 50 μ l tris-barbital buffer was added to the stria, it was incubated in moist chamber at refrigerator temperature overnight and stained by Coomassie blue. Deviation of antigen toward antibody could be distinguished (Fig. 4).

SDS-PAGE: It is a qualitative and quantitative method. Different concentrations of antigen ranged from 5 to 30 μ l (at the interval of 5 μ l) were used. The best band was obtained with 5 μ l antigen concentration after staining [23] (Fig. 5).

Western Blot

Western blot or immune blot is employed to detect the target protein [9] and it is meant to check the target protein in the sample. It is a sensitive method and is designed to track and detect macromolecules. Detection is based on the specific reaction between antibody and antigen. The protein band obtained by SDS-PAGE is transferred to nitrocellulose or polyvinylidene difluoride membrane which is then probed by its corresponding antigen/antibody.

Materials, Reagents and Equipments

Preparation of running buffer or tank buffer: glycine 28 g, tris base 6 g, SDS 2 g were dissolved in 1.8 l of distilled water, pH was adjusted to 8.3 and the volume was made to 2 l.

Tris saline tween 20 buffer was prepared by dissolving NaCl 8.8 g, KCl 0.2 g, tris base 3 g and 0.5 ml tween 20 in 800 ml distilled water. The pH was adjusted to 4.7, the volume was made to 1000 ml and then sterilized (Fig. 6).

Purification of Antibody Produced in Rabbits

To purify antibody, different techniques such as Ion-exchange, affinity chromatographies, ammonium sulfate precipitation of IgG or caprylic acid methods have been mentioned in the literatures [21–23].

Fig. 4 Immunoelectrophoresis: deviation precipitated line toward antibody indicates the complement of antigen with produced antibody; lengthy precipitation is due to presence of impurity

Fig. 5 : SDS-PAGE: From right to left: 5, 10, 15, 20, 25 μ l of factor VIII (Buffer system, 14.5 cm * 9 cm gel slab containing 5% polyacrylamide)

Fig. 6 Western blot: The most distinguished band was observed at 170 KDa which is the molecular weight of active factor VIII

Purification of IgG by Caprylic Acid and Ammonium Sulfate

In this method, 2 ml serum was added to 8 ml acetate buffer (sodium acetate 0.147 g, acetic acid 295 μ l, D/W 100 ml). It was then mixed, pH was adjusted to 4.5 and then caprylic acid 250 μ l was introduced drop wise under stirring condition. It was centrifuged at room temperature, 10000g for 30 min. The supernatant was filtered and equilibrated at 4°C then ammonium sulfate was added, stirred and then kept at 4°C for 30 min. The precipitated IgG was separated by centrifugation at 10000g and 4°C for 15 min. The precipitate was dissolved in PBS at the ratio of 1:2 and dialyzed over night. SDS-PAGE was performed to check the purity of the product with the reference literature (Fig. 7).

Results

The aim of this research is to produce recombinant factor VIII and to control the quality of such product; the production and purification of factor VIII polyclonal antibody should be carried out. Our previous experience involves

Fig. 7 The antibody bands formed in SDS-PAGE corresponds to reference showing proper purification procedure

production of morphine antibody [24], extended to production of factor VIII polyclonal antibody, application of polyclonal antibody in production of factor VIII, measurement of produced antibody in patients suffering from hemophilia, various methods to determine antibody, antigen determination, determination of concentration, serology, immunodiffusion, electrophoresis methods, different hemagglutination like passive hemagglutination for serum titer determination, SDS-PAGE to check the purity of purified antibody, dot blot, western blot, antibody purification methods, antibody quality control, protein determination, rabbit immunization, blood sampling of rabbits, serum separation, methods of antibody titer determination, methods of dialysis are the techniques set up and the infra structures available in the department make us capable to produce recombinant factor VIII. We considered safety, quality control, stability, production processes by following WHO and NRA of I. R. Iran guide lines.

Discussion

Factor VIII is one of the most important coagulation factor where its deficiency causes hemophilia A disease. Hemophilia A, one of the most severe bleeding disorders, results from an inherited deficiency of factor VIII (FVIII) function. Factor VIII can be produced either by Cryo or recombinant methods. It is used intravenously. Due to presence of impurities in factor VIII, it is expressed in IU rather than protein concentration. Therefore, in this research, the weight was expressed in terms of protein concentration which was determined spectrophotometrically. Due to low absorbance (purified factor VIII) obtained

by many investigators at λ 280 nm, this could be due to low aromatic amino acid content of factor VIII [25], thereby protein was also measured by Bradford method. The amount of factor VIII in patients is determined by PTT method, in these patients the amount of PT is usually normal and PTT will be high hence, by adding plasma of normal person, the amount of PTT will be normal. Antibody against factor VIII can be observed in patients injected with factor VIII several times. Antibody against factor VIII in old people is seen, of course, in rare cases [19]. Passive hemagglutination test was employed to evaluate the presence of antibody where agglutination with homologue antibody is prevented. Passive hemagglutination is used for many proteins. RBCs coated with antigen agglutinates with proper anti serum. This is a fast method to evaluate the presence of antibody. Factor VIII contains carbohydrate, sialic acid, neutral lipid [25]. Therefore, in coating the RBCs with factor VIII care must be taken. High content of triglyceride in membrane of sheep RBCs as compared to chicken's RBCs, therefore coating on sheep's RBCs is more difficult than chicken. We have used both types of RBCs. The first band corresponds to 220 KDa on SDS-PAGE is as compared to standard molecular weight marker. The MW of factor VIII is found to be 220 KDa. Other bands seen could be either peptide separated from factor VIII or impurities. By transferring such band to nitrocellulose membrane, the prominent band at 170 KDa was observed. This reveals that light chain (80 KDa) is linked to heavy chain (90 KDa) showing factor VIII is active. When antibody produced in rabbits against factor VIII is in contact with antigen, a single precipitated band is observed on the gel upon immunoelectrophoresis. The titer of antibody was determined by hemagglutination and radial immunodiffusion method indicating the potency of antibody, it can distinguish antigen even at low titer. In various papers, antibodies are purified by different procedures but here, we have employed caprylic acid to purify antibody. The bands observed on SDS-PAGE correspond to bands observed in reference papers [21, 23]. In this way, selected method is a proper method to purify antibody. In this article attempts are made to produce antibody in rabbits through proper methods of antibody production and quality control.

References

1. Stavitsky AB, Jarchow CC. Procedure and general applications of hem agglutination and hem agglutination-inhibition reactions with tannic acid and protein-treated red blood cells. *J Immunol.* 1954;72:360–7.
2. Knör S, Khrenov AV, Laufer B, Saenko EL, Hauser CA, Kessler H. Development of a peptidomimetic ligand for efficient isolation

- and purification of factor VIII via affinity chromatography. *J Med Chem.* 2007;50:4329–39.
3. Fay PJ, Chavin SI, Schroeder D, Young FE, Marder VJ. Purification and characterization of a highly purified human factor VIII consisting of a single type of polypeptide chain. *Proc Natl Acad Sci USA.* 1982;79:7200–4.
 4. Fulcher CA, Zimmerman TS. Characterization of the human factor VIII procoagulant protein with a heterologous precipitating antibody. *Proc Natl Acad Sci USA.* 1982;79:1648–52.
 5. Gralnick HR. Factor VIII/von will brand factor protein. *J Clin Invest.* 1978;62:496–9.
 6. Canadian Council on Animal Care Guidelines on: antibody production, 2002.
 7. Robert R, Anja R, Yvonne M, Caroline T, Arnout FG, Mohamed RD. Immunoglobulin M-enriched human intravenous immunoglobulin prevents complement activation in vitro and in vivo in a rat model of acute inflammation. *Blood.* 1999;93:942–51.
 8. Bar-Dayyan Y, Bonnin E, Bloch MF. Neutralization of disease associated auto antibodies by an immunoglobulin M- and immunoglobulin A-enriched human intravenous immunoglobulin preparation. *Scand J Immunol.* 2000;51:408–14.
 9. Huntington JA, Baglin TP. Targeting thrombin-rational drugs design from natural mechanisms. *Trends Pharmacol Sci.* 2003;24:589–95.
 10. Ludlam CA, Das PC. Plasminogen assay by a haemagglutination inhibition technique. *J Clin Pathol.* 1971;24:136–42.
 11. O'Brien DP, Pattinson JK, Tuddenham EG. Purification and characterization of factor VIII 372-Cys: a hypo functional cofactor from a patient with moderately severe hemophilia A. *Blood.* 1990;75:1664–72.
 12. Fulcher CA, Zimmerman TS. Characterization of the human factor VIII procoagulant protein with a heterologous precipitating antibody. *Proc Natl Acad Sci USA.* 1982;79:1648–52.
 13. Hornsey VS, Griffin BD, Pepper DS, Micklem LR, Prowse CV. Immunoaffinity purification of factor VIII complex. *Thromb Haemost.* 1987;57:102–5.
 14. Casey M, Kiok TR, Liisi Linask K, Mohan VP. Production, isolation and purification of plasmids for factor VIII. ChE-554. *Eng Biotechnol.* 1999. <http://www.kiok.com>.
 15. Patent: (WO/1993/022337) factor VIII purification.
 16. Cryoprecipitate Transfusion Guidelines American Red Cross Blood Services—New England Region Name: Cryoprecipitate Major Products Available, First Edition, May 2002.
 17. Antibody Purification Handbook. Amersham Biosciences (Cat. No. 18-1037-46).
 18. DAS PC. Investigations and preparation of fibrinogen coated tanned sheep red cells. *J Clin Path.* 1970;239:149–55.
 19. Goetz DW. Cross-reactivity among edible nuts: double immunodiffusion, crossed immunoelectrophoresis and human specific IgE serologic surveys. *Ann Allerg Asthma Immunol.* 2005;95:45–52.
 20. Carry Cross B. Hemagglutination inhibition assays. *Semin Avian Exot Pet.* 2002;11:15–8.
 21. Gupta RK, et al. The standardization of the reverse indirect haemagglutination test for the assay of the viral antigen of Japanese encephalitis vaccine. *J Biol Stand.* 1987;15:271–9.
 22. Dorothy K, Xing L. Single radial immunodiffusion as a method for the assay of the acellular pertussis vaccine components, pertussis toxoid, filamentous haemagglutinin and pertactin. *Biological.* 1998;26:217–24.
 23. Tai kim Y, Ault J, Wodzinski RJ. Detection of virus-specific antibody-forming cells of mice immunized with Newcastle disease virus. *Infect Immun* 1975;11:991–5.
 24. Akbarzadeh A, Norouzian D, Farhangi A, Mehrabi MR, Bakhtiari M, Afshar P, et al. Study of human therapeutic morphine vaccine: safety and immunogenicity. *Asain J Biochem.* 2007;2:58–65.
 25. Ouyang W, Xue J, Liu J, Jia W, Li Z, Xie X, et al. Establishment of an ELISA system for determining soluble LAIR-1 levels in sera of patients with HFRS and kidney transplant. *J Immunol Methods.* 2004;292:109–17.