ORIGINAL ARTICLE

Construction of Antibody Library and Production of Polyclonal Antibodies Specific to Human Protein C

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Background: Thromboembolism can occur at any age, from infancy to adulthood. The genetic risk factors for thromboembolism in the Thai population are protein C and protein S mutations. The diagnosis depends on the activity level of proteins in the plasma. Management of acute thrombotic events in severe protein C deficiency typically requires replacement with protein C in the form of fresh frozen plasma or protein C concentrate. At present, the measurement of protein C and protein S activities level is limited due to the cost and availability of the tests. Therefore, it can take a lengthy time to determine protein C levels, resulting in a delay in treatment, especially in severe cases.

Objective: To create an antibody library on filamentous bacteriophage for generating antigen specific monoclonal antibodies and to produce polyclonal antibodies specific to protein C.

Results: Recombinant protein C was constructed and produced from human embryonic kidney 293 cells. Protein C was used as an antigen to immunize mice and rabbits. The antibody titers after final boosting immunization reached up to 50,000 in mice and 10,000 in rabbits. The phage antibody library with a capacity of approximately 3×10^5 CFU was obtained from mice lymphocytes. Polyclonal antibodies were purified from rabbit sera using affinity chromatography and achieved a yield of 2 mg/mL rabbit sera. The purified polyclonal antibodies were able to detect protein C by immunoblotting and ELISA.

Conclusion: The present study demonstrated that polyclonal antibodies could be produced from immunized rabbits and the antibody library obtained from immunized mice is beneficial for further isolation of monoclonal antibodies against protein C.

Keywords: Protein C; Recombinant protein; Antibody library; Monoclonal antibody; Polyclonal antibody

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Thromboembolism can occur at any age, from infancy to adulthood. Genetic predispositions to thromboembolism have been reported including factor V Leiden, the prothrombin G20210A mutation and protein C, protein S, and antithrombin deficiencies[1,2]. In Asian countries, the predominant genetic defects affecting thrombosis are protein C and protein S mutations[3-5]. Those genes are inherited as autosomal recessive or dominant. The autosomal recessive pattern results in severe presentations during the neonatal period, including purpura fulminans, thrombosis of the retinal and abdominal veins and arteries, and central nervous system thrombosis. The autosomal dominant pattern results in thrombosis in older age groups including the presentation of less severe venous or arterial thrombosis[6-11].

Protein C deficiency is classified in two types, Type 1, characterized by decreased plasma concentrations of both protein C activity and antigen, and Type 2, characterized by decreased protein C activity but normal plasma concentrations of protein C antigen. Type 1 protein C deficiency is the most common, accounting for 80% of patients[9,12].

The laboratory diagnosis of protein C deficiency may require both activity assays and antigen assays. The recommended initial test for protein C deficiency is an activity assay, which may be clotting time based or chromogenic assay, and if the result was low, an antigen assays to measure the level of protein C antigen using immunologic

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Table 1. Characteristics of developed methods to measure protein C

Method	Capture/detection antibody	Sample volume (µL)	Limit of detection (pmol/L)	Assay time (hour)	Author, year [ref.]
ECA	Polyclonal	200	ND	ND	Comp PC, 1984 [19]
ECA	Monoclonal	20	5	>300	Gruber A, et al., 1992 [20]
ECA	Monoclonal	200	27	26	Orthner CL, et al., 1993 [21]
ECA	Monoclonal	ND	ND	75	Katsuura Y, et al., 1996 [22]
ELISA	Monoclonal	10	1.8	5	España F, et al., 1996 [23]
ECA	Monoclonal	2.5	0.6	18	Takazoe K, et al., 1999 [24]
ELISA	Monoclonal	10	1.8	5	España F, et al., 2001 [25]
ECA	Monoclonal	100	5	3 to 19	Liaw PCY, et al. 2003 [26]
ECA	Monoclonal	50	14	28	Li W, et al., 2005 [27]
ELISA	Polyclonal	10	5	8	Fernández JA, et al., 2006 [28]
ELISA	Monoclonal	10	1.7	4	Martos L, et al., 2016 [29]

ECA=enzyme capture assay; ELISA=enzyme-linked immunosorbent assay; ND=no data

methods such as enzyme-linked immunosorbent assays (ELISAs), radioimmunoassays (RIAs), and electroimmunoassays can be considered to determine the deficiency subtype.

Mild protein C deficiency corresponds to plasma levels above 20 IU/dL but below the normal range. Moderately severe deficiencies describe blood concentrations between 1 and 20 IU/dL while severe deficiencies yield levels of protein C that are below 1 IU/dL or are undetectable. Severe congenital protein C deficiency usually presents during the neonatal period with purpura fulminans, retinal vessel thrombosis, cerebral stroke, and extensive venous thromboembolism. On the other hand, patients with protein C levels greater than 1% to 20% may present purpura fulminans in infancy or extensive venous thrombosis when elderly[9].

The prevalence of protein C deficiency in the population studied in Thailand was 1.99%, which was higher than that in the Caucasian population[13]. In addition, the common genetic mutation, reported in the Thai population was R189W, which was identified in 6% of the normal population[14]. Therefore, the estimated incidence of autosomal recessive disease is 1:10,000 in the population. In addition, the physiologic low level of protein C in the neonate during the first six months may contribute to developing thrombosis in a patient with the heterozygous mutation[15,16].

Replacement therapy together with anticoagulants is crucial to treat severe thrombosis among patients with severe protein C deficiency. Therefore, knowing the level of protein C can guide the physician's decision for replacement therapy. The limitation is that the test is not available in every hospital. Therefore, the study aimed to develop monoclonal and polyclonal antibodies specific to protein C to develop an immunological assay to use as a screening test to detect a critically low level of protein C[9,11,17]. Several assays to measure protein C using monoclonal and polyclonal antibodies have been developed. They differ in terms of method design, the volume of sample needed to perform the assay, the limit of detection, and the time required to develop the assay (Table 1)[18].

Materials and Methods

The present study was conducted between October 1, 2021 and September 30, 2022.

Producing recombinant protein C antigen

The human *PROC* gene encoding a 461 amino acid polypeptide chain (Uniprot accession number P04070) was introduced to HindIII restriction sites at 5' end of gene and FLAG tag followed by BstBI restriction sites at the 3' end of the gene. Genetic codons of amino acids coded were optimized by changing to synonymous codons frequently used by human cell line for increasing the expression levels. The optimized sequence was chemically synthesized by Gene Universal (Newark DE, USA). The synthetic gene was digested with HindIII and BstBI and was ligated to the modified plasmid pcDNA™3.4 expression vector (Thermo Fisher Scientific, USA), which was digested by the same restriction enzymes, using T4 DNA ligase (Promega, USA) to generate the recombinant plasmid pcDNA™3.4-*PROC* vector. The *Escherichia coli* TOP10 was used to transform the pcDNA™3.4-*PROC* vector. The transformants were selected by screening the colonies on 2xYT medium containing ampicillin (100 µg/mL). Then colonies were further analyzed by PCR and the

plasmid was extracted using the QIAprep® Miniprep Kit (Qiagen, Germany) according to the manufacturer instruction. The *PROC* gene in the recombinant plasmid vector was verified the sequence by the Sanger method using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) according to manufacturer protocol and analyzed using an ABI PRISM® 3500xL Genetic Analyzer (Thermo Fisher Scientific, USA).

The FreeStyle™ 293-F cells (Thermo Fisher Scientific, USA), which were derived from human embryonic kidney 293 (HEK293) cell line, were used to express recombinant protein C by transiently transfection. Briefly, the cells were cultured with HyClone™ SFMTransfx-293 medium (Cytiva, USA) at 37°C with an orbital shaking at 125 rpm in 5% CO_2 incubator. The culture at cell density of 1×10^6 cell/mL was transfected with recombinant plasmid pcDNA™3.4-*PROC* vector using FectoPRO® transfection reagent (Polyplus®-transfection, France) at a ratio of 1 µg plasmid DNA to 1.5 µL of transfection reagent according to the manufacturer protocol. The transfected cells were harvested three days post-transfection by centrifugation at 1,800 × g for 10 minutes and passed through a Microfluidizer® M-110P (Microfluidics, USA) at 4,000 psi.

The expressed protein was purified from cell lysate using ANTI-FLAG® M2 Affinity Gel (Sigma-Aldrich, USA), washed with TBS and eluted with 0.1 M Glycine-HCL pH 3.5, then neutralized with 1 M Tris-HCl. The purified fractions were dialyzed against PBS and concentrated using an Amicon® Ultra Centrifugal Filter with 10 kDa MWCO (Merck Millipore, USA) and the concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA) according to the manufacturer instruction. The purified protein was analyzed by 12% SDS-PAGE on Mini-PROTEAN® 3 Cell (Biorad, USA) and subsequently stained with PageBlue™ protein staining solution (Thermo Fisher Scientific, USA). Western blot analysis was performed after transferring the protein on to a nitrocellulose membrane by Mini-Trans-Blot® Eletrophoretic Transfer Cell (Biorad, USA). The membrane was reacted with monoclonal ANTI-FLAG® M2-Peroxidase (HRP) antibody following to the manufacturer protocol (Sigma-Aldrich, USA) and the signal was detected using color development with TMB substrates (Seracare Life Sciences, USA).

Generating antibody library specific to protein C antigen

Mice immunization was performed at the Laboratory Animal Center, National Institute of Health. The animal protocol was reviewed and approved by the Institute Animal Care and Use Committee at the Department of Medical Sciences (approval number 65-035). Two BALB/c mice of six weeks of age, were immunized six times by alternating subcutaneous and intraperitoneal injections at two-week intervals with 20 ug of protein C antigen emulsified with equal volume of Adju-Phos® aluminum phosphate adjuvant (InvivoGen, Hong Kong). Blood samples were collected before the primary injection and one week after the fourth injection to monitor the antibody titers by ELISA. Two weeks after the sixth injection, each mouse was intraperitoneally injected with 20 µg of protein C antigen without adjuvant. Three days later, blood and spleen cells were collected after this final injection and a total RNA extraction was performed from spleen cells using TRIzol™ Reagent (Thermo Fisher Scientific, USA).

First-strand cDNA was synthesized from total RNA using a SuperScrip®III First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) with random hexamers. Immunoglobulin variable heavy chain (VH) and variable light chain (VL) genes were amplified from cDNA by polymerase chain reaction (PCR) using a library primer set which were designed based on several publications and the IMGT® database. All primary PCR reactions were carried out using 16 separate VH primers and 13 separate VL primers. PCR was performed in 50 µL volumes containing 2 µL of cDNA reaction mixture, 0.5 µM of each primer, 200 µM of dNTPs, 1X Q5 reaction buffer, and one unit of Q5 polymerase (New England Biolabs, USA). Amplifications were carried out using a ProFlex PCR System (Thermo Fisher Scientific, USA) by the following protocol: initial denaturation at 98°C for 30 seconds, followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 20 seconds. The program followed by a final extension at 72°C for 2 minutes. The PCR products were analyzed in 1.5% agarose gel in 1X TAE buffer and purified from gel by QIAquick® Gel Extraction Kit (Qiagen, Germany) according to the manufacturer instructions. The scFv genes were assembled by overlap PCR using VH and VL fragments as templates. PCR assembly was performed in 100 µL volumes containing 10 ng each of VH and VL. An initial denaturation at 98°C

for 30 seconds, followed by 30 cycles of 98°C for 10 seconds, annealing plus extension at 72°C for 40 seconds and followed by a final extension at 72°C for 2 minutes were performed.

The scFv genes were digested with SfiI and NotI restriction enzymes (Thermo Fisher Scientific, USA), agarose gel-purified, and ligated into the phagemid vector pCANTAB-5E (GE Healthcare, Sweden) that had been cut with the same restriction enzyme. The ligated products were transformed into *E. coli* TG1. After transformation, cells were plated on 2xYT agar containing 1% glucose, 100 µg/mL ampicillin and incubated overnight at 30°C. All bacterial colonies were scraped off the plates into 2xYT medium and infected with bacteriophage M13 to a multiplicity of infection (MOI) of 20. The culture was grown overnight at 30°C in 2xYT medium containing 100 µg/mL ampicillin and 50 µg/mL kanamycin. A Phage library was prepared using PEG/NaCl precipitation and the concentration of bacteriophage virion was measured by NanoDrop™ Spectrophotometers (Thermo Fisher Scientific, USA).

Producing polyclonal antibody specific to protein C

New Zealand white rabbits of seven weeks of age were used in the immunization and handled in the Laboratory Animal Center at Thammasat University. The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Thammasat University (approval number 028/2021). Two rabbits were given a subcutaneous injection with protein C antigen emulsified with equal volumes of Adju-Phos® aluminum phosphate adjuvant. The primary immunization was injected with the emulsion containing 200 µg of antigen, followed by 100 µg for five booster injection at two-week intervals. Test bleeds were drawn before primary injection and one week after the third injection to check the antibody titers by ELISA. Terminal bleeds were collected from cardiac puncture and the sera were separated from blood by centrifugation at 2,000 × g for 10 minutes. The polyclonal antibodies were purified from rabbit sera by Protein G Sepharose® Fast Flow (GE Healthcare, Sweden). The Poly-Prep® Chromatography column (Biorad, USA) was packed with Protein G resin and equilibrated with binding buffer containing 20 mM $NaHPO_4$ pH 7.0. The rabbit serum was applied through the column and washed with binding buffer. The bounded antibody was eluted using 0.1 M citric acid pH 3.0, neutralized immediately using 1.0 M Tris pH 9.0 and then dialyzed against phosphate buffered saline (PBS). The purified antibodies were analyzed for purity on 12% SDS-PAGE and subsequently stained with PageBlue™ protein staining solution.

Determining specific antibody titer by ELISA

The specific antibody titers in all sera samples were determined by indirect ELISA. The microplates were coated with 100 µL of protein C antigen at a concentration of 1 µg/mL in coating buffer (0.05 M bicarbonate/carbonate buffer pH 9.6) and incubated overnight at 4°C. The plates were washed three times with PBST (PBS containing 0.05% Tween-20), and then blocked with 200 µL of 3% skim milk (Merck, USA) in PBS for one hour at room temperature. The plates were washed again three times with PBST and incubated with 100 µL serum sample at five different dilutions between 1:100 and 1:100,000 for one hour at room temperature. Non-immune serum was used as a negative control. Following three washes with PBST, plates were incubated with 100 µL diluted rabbit anti-mouse/HRP conjugated or goat anti-rabbit IgG/HRP conjugated at 1:2,000 dilution (Agilent Dako, USA) for one hour at room temperature. The reaction was developed by adding 100 µL TMB substrate (Seracare Life Sciences, USA) for 30 minutes at room temperature. Finally, 2N sulfuric acid was added to stop the reaction, and the absorbance was determined at 450 nm using an absorbance microplate reader (Tecan's Sunrise, Switzerland).

Statistical analysis

Microsoft Excel was used for statistical analysis. The indirect ELISA method was used for the titration of antibody then the mean of absorbance values for positive and negative results were analyzed.

Results

The recombinant human protein C was expressed as a soluble protein and slightly secreted in the culture supernatant. Mainly, protein was purified from cell lysate, and the yield obtained from anti-FLAG affinity chromatography was 21.56 mg/L of culture. The SDS-PAGE of protein obtained from the chromatography showed a single protein band of approximately 62 kDa, corresponding to the human protein C precursor (Figure 1).

The immunization of BALB/c mice with protein C antigen elicited antibody titers up to 50,000 after the fourth injection and maintained this level until final immunization at the seventh injection (Figure 2), and the spleens were collected after the final injection. RNA extracted from B-lymphocytes

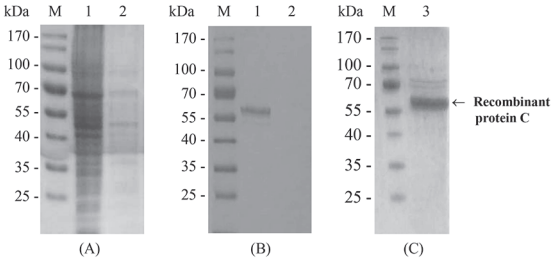

Figure 1. SDS-PAGE and Western blot analysis of recombinant protein C, (A) The expressed protein from cell pellet and cell culture medium was separated on 12% acrylamide gel under reduced condition. (B) Western-blot analysis with monoclonal antibody specific to FLAG tag of the protein, (C) The purified protein was separated on 12% acrylamide gel under reduced conditions. M: PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, USA), Lane 1: cell pellet, Lane 2: cell culture medium, Lane 3: purified protein.

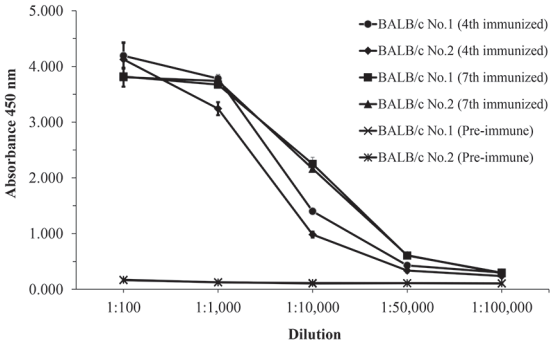

Figure 2. Determination of antibody titer from mice sera by ELISA. The sera samples at different dilutions were reacted with 0.1 µg of the purified recombinant human protein C. Antibody titer is defined as the highest dilution of sera corresponding to an absorbance equal to at least two-fold the value of pre-immune serum. Values in the figure were the means±SD (n=2).

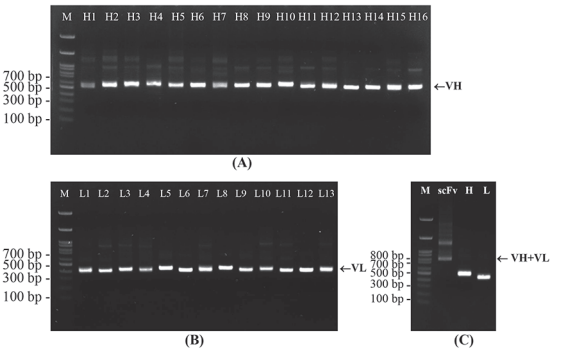

Figure 3. Construction of the scFv antibody library genes. (A) PCR amplification products of immunoglobulin VH genes from cDNA of mouse lymphocytes. M: 100 bp DNA ladder (Bio-Helix, Taiwan), H1-H16: PCR products from each VH family. (B) PCR amplification products of immunoglobulin VL genes from cDNA of mouse lymphocytes. M: OneMARK 100 DNA ladder (Bio-Helix, Taiwan), L1-L16: PCR products from each VL family. (C) PCR amplification product of scFv genes by PCR assembly. M: OneMARK 100 DNA ladder (Bio-Helix, Taiwan), scFv: assembled scFv genes, H: pooled VH genes, L: pooled VL genes.

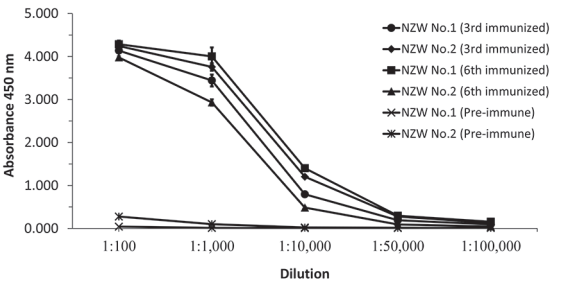

Figure 4. Determination of antibody titer from rabbit sera by ELISA. The sera samples at different dilutions were reacted with 0.1 µg of the purified recombinant human protein C. Antibody titer is defined as the highest dilution of sera corresponding to an absorbance equal to at least two-fold the value of pre-immune serum. Values in the figure were the means±SD (n=2).

was converted to cDNA and used as a template to amplify the immunoglobulin VH and VL genes. The PCR products showed that all 16 VH families and 13 VL families were successfully amplified and linked together in the scFv antibody genes (Figure 3). The library of scFv antibody genes was cloned in the pCANTAB-5E phagemid vector and then transformed to *E. coli* TG1. A total of 3.1×10^5 CFU was obtained, and phage rescue of all transformed cells was performed simultaneously. The number of phages rescued was estimated to be 1×10^{13} PFU, and the library size was calculated to possess 3.1×10^5 members.

The polyclonal antibody was produced in New Zealand white rabbits by immunization with protein C antigen. The highest antibody titers against protein C after final booster immunization were 10,000 (Figure 4). Whole blood from two rabbits was collected after the final booster immunization. A total 34 mL of serum was separated from the blood sample by centrifugation. The polyclonal antibody was purified using protein G affinity chromatography with a yield of 2 mg total IgG antibody from a starting volume of 1 mL rabbit serum. The purified polyclonal antibody appeared molecular mass of 55 kDa and 25 kDa on SDS-PAGE corresponding to the heavy and light chains of IgG antibody, respectively (Figure 5).

Discussion

Protein C, a vitamin K-dependent glycoprotein, is synthesized by the liver as a single-chain precursor

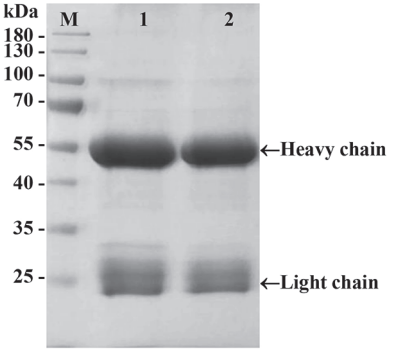

Figure 5. SDS-PAGE of purified total IgG polyclonal antibody from New Zealand white rabbit (NZW) sera. M: PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, USA), Lane 1 and 2: purified total IgG polyclonal antibody of NZW No.1 and 2 respectively.

molecule. Protein C consists of 419 amino acids in multiple domains containing one Gla domain, a helical aromatic segment, two epidermal growth factor (EGF)-like domains, an activation peptide, and a trypsin-like serine protease domain. The precursor molecule is an inactive protein becoming an active protein when a dipeptide of Lys198 and Arg199 is removed and transformed to a heterodimer with N-linked glycosylation. Then the protein has one molecule of light chain (21 kDa) and one molecule of heavy chain (41 kDa), connected by a disulfide bond between Cys183 and Cys319. The inactivated form circulates in the plasma in amounts of about 85% to 90%; activation of protein C occurs when a thrombin molecule cleaves away the activation peptide[12,30].

Based on the results of the present research study, the engineered *PROC* gene can be designed and synthesized to express recombinant protein C in human cell lines. The authors selected HEK293 cell lines for protein expression[31]. When the recombinant vector containing the *PROC* gene was introduced in HEK293 cell lines using transient transfection, the cells could produce recombinant protein C and secrete it in the cell culture medium. However, recombinant protein C also remained inside the cell. To obtain more protein for the experiment, the cells were lysed to retrieve more protein to purify. Although the intracellular process is essential for the glycosylation of proteins, the main structure of proteins could still be preserved. The antigenic specificity of the protein was confirmed in the present study using a commercial monoclonal antibody specific to human protein C before animal immunization.

The experiment successfully induced an antibody response in mice and rabbits, similar to the related reports[32,33]. The highest antibody titer was 50,000 in mice and lower in rabbits at a titer of 10,000. The additional booster immunization could not increase the antibody titer in mice and rabbits. As a result, immunization should be stopped when the highest antibody titer is obtained. The immunoglobulin genes isolated from B-lymphocytes of mice covered all of the immunoglobulin VH and VL families, resulting in the diversity of the antibody library. The generated antibody library could be used to further isolate monoclonal antibodies specific to protein C.

To produce polyclonal antibodies, considering antigen quality and quantity is important. The specificity and high titer of the obtained antibody against protein C depend on the purity of the injected antigen and the immunization protocol with a suitable adjuvant. The polyclonal antibody obtained in the present study is applicable for use as a primary antibody in enzyme immunoassays, western blots, and immunostaining.

Conclusion

In summary, the present study was able to produce recombinant protein C from human cell lines and rabbit polyclonal antibodies specific to protein C. Moreover, the antibody library was also generated to facilitate the further isolation of monoclonal antibodies.

What is already known on this topic?

Purified protein C antigen can be used to generate antibodies specific to protein C in animal models.

What this study adds?

The antibody library obtained from B-lymphocytes of mice is useful for isolating of specific monoclonal antibodies and polyclonal antibodies derived from rabbit sera can be used for future development of antigen detection kits.

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Conflicts of interest

The authors declare they have no conflicts of interest.

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