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Purification of mCRP35-47 Peptide Related Antibody by Affinity Chromatography on Antigen-Ligand Columns

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Research Article

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ABSTRACT

To purify mCRP35-47 autoantibody from the lupus nephritis for establishing the foundation of the further study on the roles of mCRP35-47 in the clinical course of lupus nephritis. The first step is obtaining the serum of patients with lupus nephritis and purifying of plasma total IgG by affinity chromatograph. The second step is activating the sepharose with CNBr. The most important step is preparing the immobilized ligand by coupling the peptide a.a.35-47 to the gravity column and the total IgG is flow though the gravity column and can be eluted. mCRP35-47 peptide related antibody is harvested and its specificity has been verified by SPR. Human mCRP35-47 peptide related antibody with high purity from the plasma can be obtained by affinity chromatography and can be applied to further functional experiments.

INTRODUCTION

C-reactive protein (CRP) is a serum marker of inflammation and a putative soluble pattern recognition receptor that contributes to efficient removal of dead cells or invading pathogens by activating complement factor H [1,2]. It is, therefore, interesting to note the blunted CRP response in active lupus nephritis and the presence of anti-CRP autoantibodies in a subset of patients [3-6]. Our previous work implied that a.a. 35–47 as the predominant autoimmune epitope on CRP associated with renal injury and prognosis of lupus nephritis. Importantly, this epitope is unique to mCRP conformation and constitutes the major ligand binding site [7].

Investigations of patient-derived antibody repertoires are playing a more and more important role for clinical research, and the purification process got advanced progress since the developments of FPLC. Total-antibody can be easily purified from patient serum samples by Protein A/G affinity purification following with size-exclusion purification. However, the purification of antibodies against specific antigen is still a technical problem since their low abundances and no standard purification procedures. The most obvious targets for human-derived antibody repertoires are antigens associated with infectious agents, such as viruses and bacteria. Thus antibodies against these infectious agents could serve as the source for anti-infective therapy. Besides, many high-value targets of therapeutic interest, such as those associated with cancer or inflammatory disorders, are typically self-antigens. The antibodies against these antigens for this study can be used to study autoimmune diseases. Here, we developed a new method for affinity chromatography purification of antibodies that bind the target antigen to the solid phase agarose for antigen specificity.

Here we have developed a new method of affinity chromatography for purifying the specific antibodies by anchoring the

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target antigen to the solid phase agarose and applying this method to the antibody detection of the clinical autoimmune disease.

Affinity chromatography is a technique based on the specific affinity of bioactive substances and specific ligands to achieve separation purposes. Affinity chromatography is the most effective method of protein separation and purification [8,9]. Affinity chromatography is designed according to the specificity of target proteins, such as enzymes and substrates, antigens and antibodies. Affinity chromatography has the advantages of high purity, high yield, and the ability to maintain the natural activity of biological macromolecules. Therefore, affinity chromatography has good selectivity and is widely used to extract specific target proteins from complex systems. Ahirwar [10] has developed a fast and efficient one step affinity chromatography technique. The fixed phase of this chromatography is agarose carrying the antibody of the bean protein A. The purity of the target protein obtained by one step chromatography can reach 90%, the yield is more than 66%. Plasmid DNA was purified by affinity chromatography with berenil as a ligand. The yield and purity of the plasmid DNA were 87% and 99% respectively, and the operation time and cost were greatly reduced because of their low amount of salt and single step chromatography. The separation efficiency of affinity chromatography has obvious advantages compared with other chromatography techniques. Only one step of separation can achieve the desired effect, that is, the experimental procedure is simplified and the time [11] is saved.

The methods for production of such immobilized ligands and for carrying out affinity-purification of IgG are essentially similar, regardless of which ligand is used. Sepharose 4B is probably the most widely used matrix for affinity chromatography, but other materials are available. Activation of Sepharose 4B is usually carried out by reaction with cyanogen bromide (CNBr); this can be carried out in the laboratory before coupling, or ready-activated lyophilized Sepharose can be purchased. The commercial product is obviously more convenient than “homemade” activated Sepharose, but it is more expensive and may be less active [12].

MATERIALS AND METHODS

Materials

- Sepharose 4B.
- Cyanogen bromide. (Warning: CNBr is toxic and should be handled in a fume hood)
- Sodium hydroxide: 1 M; 10 M.
- Ethanolamine buffer: 2 M ethanolamine.
- Agarose Resin Wash Buffer: 1 mM HCl pH 3.0.
- Coupling Buffer: 0.1 M NaHCO3/0.5M NaCl pH 8.3.
- Polypeptide Coupling Solution: 6 mM Polypeptide Soluble in Coupling Buffer.
- Blocking solution: 0.1 M Tris-HCl pH 8.0.
- Wash Solution 1: 0.1 M Acetate Buffer/0.5M NaCl pH 4.0.
- Wash Solution 2: 0.1 M Tris-HCl/0.5M NaCl pH 8.0.
- Balance Solution: 0.15 M PBS.
- Eluent 1/2/3: 0.1 M acetate buffer/0.5 M NaCl pH 5/4/3.

Solution preparation

- Solution A (pH 7.0): K_2HPO_4: 3.4 g; EDTA: 1.86 g; distilled water: 1000 ml; Adjust pH to 7 with 10 M NaOH solution.
- Solution B (pH 7.0): K_2HPO_4: 3.4 g; EDTA: 1.86 g; NaCl: 29.22 g; distilled water: 1000 ml; Adjust pH to 7 with 10 M NaOH. Solution
- 0.01 M PBS(pH 7.4): NaCl: 8.0 g; Na_2HPO_4*12H_2O: 2.9 g; KCl: 0.2 g; KH_2PO_4: 0.26 g; distilled water: 1000 ml; Adjust pH to 7 with 10 M NaOH solution.

Equipment

- Fast protein liquid chromatograph (FPLC), GE, USA
- Cylindrical shell, GE, USA
- HiPrepTM 16/60 SephacrlTM S300, GE Health Care, UK
- Protein G affinity chromatography column, GE, USA
- 0.22 μm Filter, Millipore S.A. Company, France

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Methods

A. Purification of plasma total IgG by affinity chromatography[13]:

- Sera of plasma exchange from 3 patients with lupus nephritis whose intact mCRP antibody, anti-mCRP35-47 antibody, and anti-mCRP199-206 antibody positive were collected and centrifuged at 10,000 rpm for 20 min at 4°C, and the supernatant was taken.
- AKTA-FPLC was debugged, all buffers must be degassed by ultrasound and filtered with 0.22 μm filter membrane. Solution A and B were used to flush pressure pumps. Solution A is used to equilibrate the Protein G affinity column (5 ml).
- Solution A 10 ml sample was taken and the flow rate was set at 1 ml/min. 5 column volumes of solution A was used to elute the unbound material. The bound IgG was eluted with 5 column volumes of solution B, and the eluted protein was collected by peak and neutralized with 2 M Tris/HCl (pH 9.0) to ensure that the liquid in the tube was pH 7.4. After elution, the protein G affinity column was equilibrated with 5 column volumes of solution A, 20% ethanol, and stored at 4°C.
- The extracted IgG was dialyzed against 0.01 M PBS+0.5 M NaCl buffer overnight at 4°C. After dialysis, the supernatant was taken and the OD280 was measured with a UV spectrophotometer. The protein content was A280 nm/1.43 (mg/ml).

B. The following steps were taken based on the above extraction of total IgG at 4°C:

Lysine Sepharose 4B is supplied freeze-dried in the presence of additives. These additives must be washed away at neutral pH.

B.1 Activation of sepharose with CNBr

- Wash 10 mL (settled volume) of Sepharose 4B with 1 L of water by vacuum filtration. Resuspend in 18 mL of water (do not allow the Sepharose to dry out).
- Add 2 mL of 0.5 M sodium carbonate buffer, pH 10.5, and stir slowly. Place in a fume hood and immerse the glass pH electrode in the solution.
- Weigh 1.5 g of CNBr carefully into an air-tight container (Note: weigh in a fume hood; wear gloves)—remember to decontaminate equipment that has contacted CNBr in 1 M NaOH overnight.
- Add the CNBr to the stirred Sepharose. Maintain the pH between 10.5 and 11.0 by dropwise addition of 4 M NaOH until the pH stabilizes and all the CNBr has dissolved. If the pH rises above 11.5, activation will be inefficient, and the Sepharose should be discarded.
- Filter the slurry using a sintered glass or Buchner funnel, and wash the Sepharose with 2 L of cold 0.1 M sodium citrate buffer, pH 6.5—do not allow the Sepharose to dry out. Carefully discard the filtrate (Note: this contains CNBr).

B.2 Preparation of immobilized ligand

- 1 g of CNBr-activated Sepharose 4B was weighd and diluted in 15 ml 1 mM HCl with a 50 ml centrifuge tube and was poured into the column after the column is completely swelled. The column volume was approximately 3 ml and then rinse with 1 mM HCl for 15-20 minutes.
- 5 ml of coupling buffer was added in, resuspend and transfer to a 15 ml centrifuge tube. The 15 mg peptide was dissolved in 800 μl DMSO and added to the resuspension. PH was adjusted to 8.0. About 9 ml of the suspension was incubated at room temperature for 1 h.
- The suspension was transfered to an empty column and the permeate was collected. The remaining liquid was washed 5 times with a coupling buffer (10 ml each time, and the next wash was started each time the liquid was empty). Then add 10 ml blocking solution and incubate at room temperature for 2 h.
- The suspension was transfered to an empty column and the permeate (10 ml of blocking solution) was discarded and wash three times alternately with washing solutions 1 and 2 (10 ml+10 ml each time).
- Pack the Sepharose into a suitable chromatography column wash with 50 mL of PBS. Store at 4°C in PBS containing 0.1% sodium azide.

B.3. Sample application and elution

- Plasma exchange fluid was added and incubated at room temperature for 1 hour or 4 hours overnight.
- The suspension was transferred to an empty column and the permeate was collected. The column was washed with large volumes (50 ml) of PBS until no protein was detected with the assay stain.

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- Wash with 1/2/3 eluent (pH from high to low), 2 ml each time, and stand for 2 minutes each time. After elution, 13 ml of PBS was rapidly added to the eluate. 15 ml neutralize the eluate. Ultrafiltration to 200 µl (4000 g 10 min) using a 50 ml 50/30 kDa ultrafiltration tube was performed and the amount was removed.
- Monitor the A280 and collect the protein peak into tubes containing 1 M Tris-HCl, pH 8.8 to neutralize the acidic dissociating buffer.
- Wash the column with PBS until the eluate is at pH 7.4. Store the column in PBS containing 0.1% azide. Dialyze the peptide specific antibody a suitable buffer (e.g., PBS) to remove glycine/Tris.

B.4. Verification of the function of peptide related antibody after affinity chromatography

B.4.1. SPR verified that mCRP35-47 antibodies purified by affinity chromatography were peptide related antibody

We purified the total IgG from the serum of a patient with lupus nephritis by protein G column and isolated epitope specific mCRP35-47 antibody by gravity column. We need to verify the specificity of the antibody before making further experiments with this antibody.

Experiment 1: the extracted mCRP35-47 antibody was coated with CM5 chip as stationary phase. mCRP35-47 is used as a mobile phase to do a combination of experiments.

Experiment 2: the mCRP35-47 antibody was extracted by affinity chromatography, but the mobile phase was peptide mCRP 174-185.

Experiment 3: mCRP35-47 antibody was extracted by affinity chromatography, and the mobile phase was CFH.

B.4.2. The influence of antibodies purified by affinity chromatography on the binding of CFH to mCRP was detected by ELISA. The antibody of mCRP and its peptide segments and mCRP were incubated with the CFH cladding plate, and the mouse anti mCRP monoclonal antibody 3H12 was used as the first antibody, HRP labeled anti mouse IgG was as the second antibody, and the OD value was detected. The effect of different mCRP and its peptide antibody on the combination of CFH and mCRP was compared.

B.5. Storage

Swollen medium should be stored at 4–8°C in presence of bacteria, e.g. 20% ethanol. The medium must not be frozen.

In order to prevent the protein(antibody) degradation during the entire chromatographic process We take measures are as follows:

- Temperature: Temperature is the most important factor affecting the stability of proteins. The higher the temperature, the lower the stability of proteins. The purification process of proteins is carried out at 4°.
- Buffer composition: Many molecules have stabilizing effects on the structure of proteins. They can be used to protect unstable proteins in the purification process, suitable solvents, and appropriate ionic strength of buffer also help to stabilize proteins.
- Shaking and shearing: Proteins may denaturate under shaking and shearing, so severe stirring and shaking should be avoided when purifying proteins.

RESULTS

Protein purification

The crude product obtained from the mixed plasma of patients is transparent and clarified. The crude PH was adjusted and the sample was released after dialysis. The eluent peak was eluted from the equilibrium solution to the effluent (**Figure 1**).

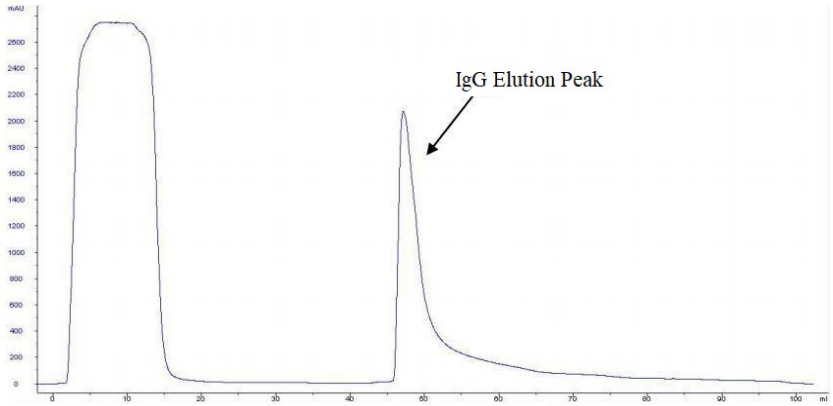

Figure 1: A schematic diagram of the protein G column protein peak.

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Function of peptide related antibody

Subsequent experiments proved the specificity of peptide related antibodies by surface plasmon resonance (SPR) (**Figure 2**).

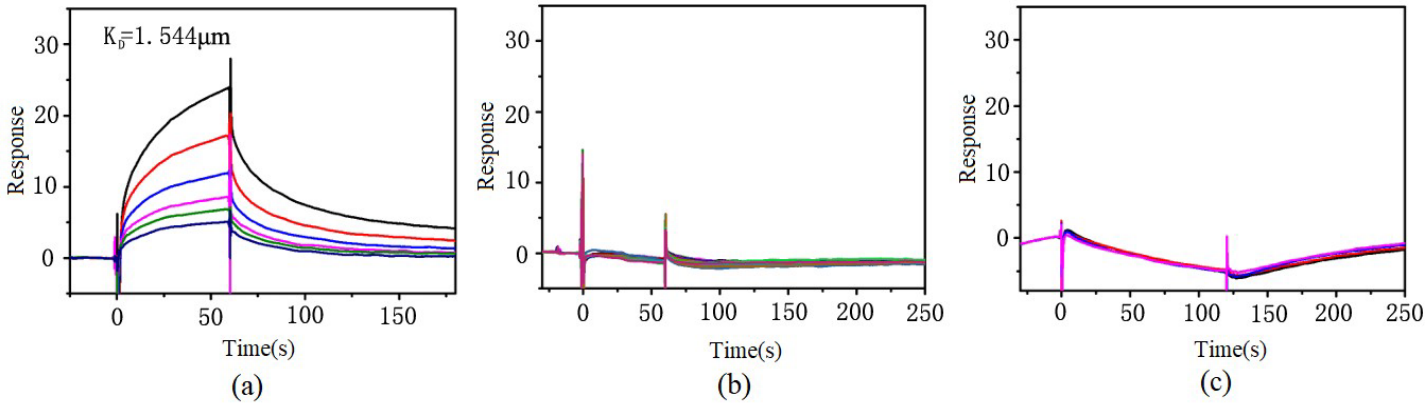

Figure 2: (a)KD=1.544 uM.It suggests that there is a combination of the a.a.35-47 and peptide related antibody, (b)The stationary phase was mCRP35-47 peptide related antibody extracted from affinity chromatography, while the mobile phase was peptide mCRP 174-185. The response value indicates that the two substances had no binding, (c)It showed that the stationary phase was mCRP35-47 peptide related antibody extracted by affinity chromatography, the mobile phase was CFH, and the response value indicates that the two substances had no binding. The above indicated that the mCRP35-47 peptide related antibody extracted from lupus nephritis patients had high relative specificity.

The author find mCRP35-47 peptide related antibody cannot combine peptide mCRP 174-185 while the binding capacity of itself is strong and KD is 1.544 uM.

In addition, mCRP35-47 peptide related antibody can restrain the combination of complement factor H and mCRP and it is in a dose-dependent manner (**Figures 3 and 4**).

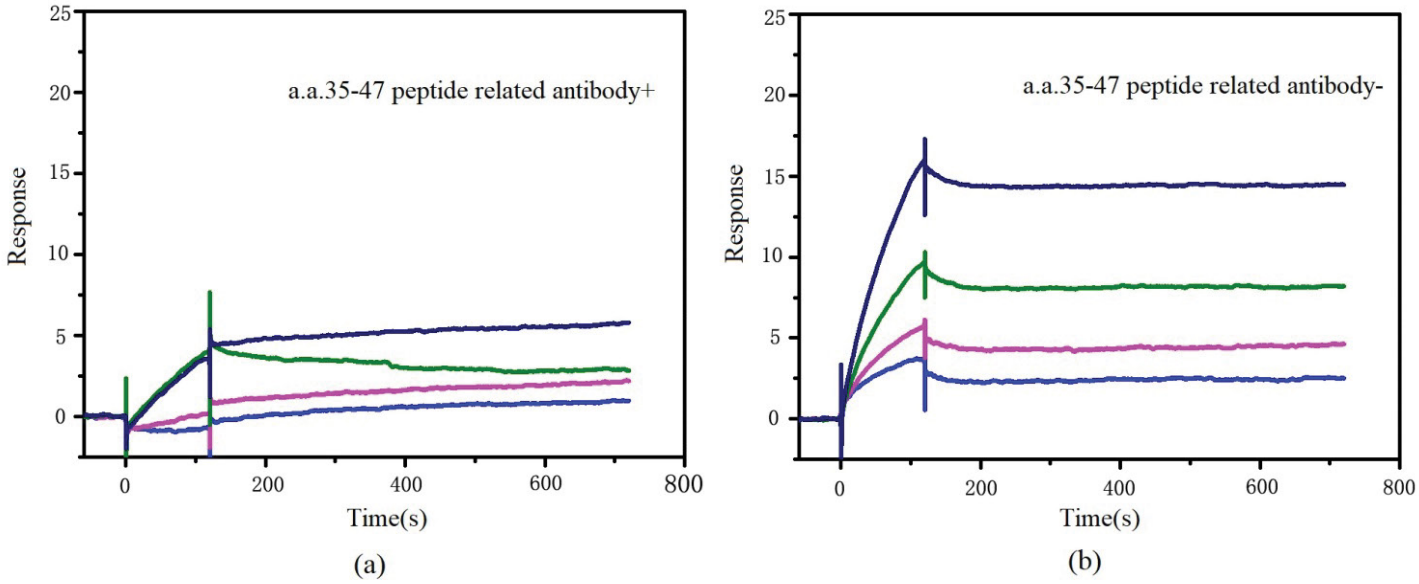

Figure 3: The stationary phase is CFH and the 26.5 nM anti mCRP35-47 peptide related antibody exists (**Figure 3a**) or does not exist (**Figure 3b**), when the mobile phase passes through 6.25 nM (blue), 12.5 nM (pink), 25 nM (green) or 50 nM mCRP (purple), the mCRP35-47 peptide related antibody can significantly inhibit the combination of mCRP and CFH, leading to a decline in the response value.

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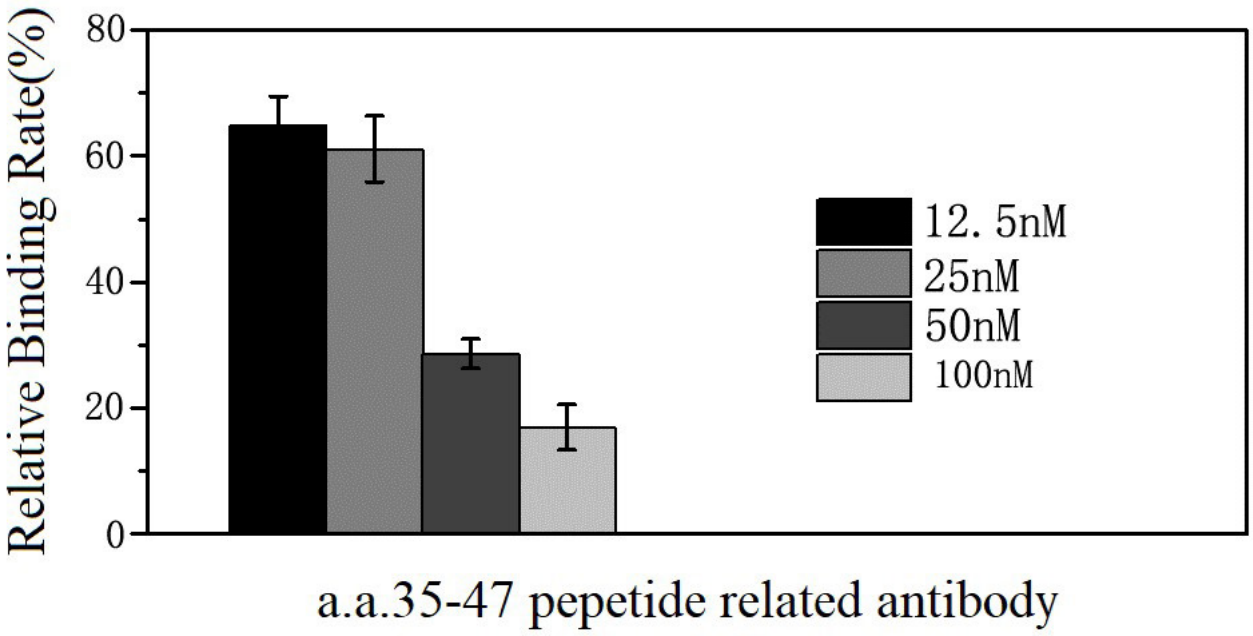

Figure 4: For different concentrations of mCRP35-47 peptide related antibody, the inhibition degree of mCRP and CFH binding was dose-dependent, that is, the higher the concentration of mCRP35-47 peptide related antibody, the poorer the binding capacity.

DISCUSSION AND CONCLUSION

The affinity filler is a polypeptide ligand which is coupled to agarose and polyethers through chemical bonds. These ligands are easily degraded by microorganism, poor in alkali resistance and need high concentration of guanidine hydrochloride with higher price. The cleaning costs are higher, in the process of purification, in the acid buffer and the high concentration denaturant. Under the severe conditions, the ligands are easy to fall off and so on. Therefore, in the production of peptide related antibody, the consumption of the affinity fillers is large, which hinders the scale of the antibody production and becomes an important factor to increase the production cost of antibody.

In addition, the structure of protein molecules is complex and easy to be inactivated, and the crude protein contains a variety of protein impurities. It is often necessary to combine various separation techniques to separate and purify the target protein, which is bound to make the protein molecules in the process of separation and purification, which are different from physiological conditions, chemical and mechanical factors. It leads to irreversible change in the spatial structure of protein, thereby losing its activity. For example, the changes in temperature, PH value and ionic strength during the chromatography process, and the surfactant added during the electrophoretic process may lead to the spatial structure change of the protein. The inactivated protein loses its significance to the researchers, so it is an urgent problem to keep the natural activity of the protein in the process of separation and purification.

The preparation of monoclonal antibody in this experiment is suitable for basic and clinical trials. The author uses this method to find that renal damage is more serious in lupus nephritis patients with mCRP35-47 peptide related antibody positive and the long-term survival of the kidneys is worse. In addition, in view of the fact that there is no uniform standard for the detection of resistance at present, the results of clinical detection do not have good comparability, and it also affects the promotion of clinical research. Therefore, the ELISA detection kit of purified mCRP monoclonal peptide related antibody can be considered, and the standardization of monoclonal antibodies can be explored to provide a basis for further clinical research and diagnosis.

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COMPLIANCE WITH ETHICAL STANDARDS

All the authors declare that they have no conflict of interest.

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HUMAN AND ANIMAL RIGHTS AND INFORMED CONSENT

Informed consent was obtained for blood sampling and renal biopsy from each patient. The research was in compliance of the Declaration of Helsinki. The design of this work was approved by the local ethical committees.

REFERENCES

1. Clos TWD. Pentraxins: Structure, Function, and Role in Inflammation. Isrn Inflamm. 2013; 379040.
2. Singh SK, et al. The connection between C-reactive protein and atherosclerosis. Ann Med. 2008;40:110-120.
3. Bell SA, et al. Autoantibodies to C-reactive protein and other acute phase proteins in systemic autoimmune diseases. Clin Exp Immunol. 1998;113:327-332.
4. Robey FA, et al. C-reactive protein mediates the solubilization of nuclear DNA by complement *in vitro*. J Exp Med. 1985;161:1344-1356.
5. Page M, et al. Purification of IgG using affinity chromatography on antigen-ligand columns. The Protein Protocols Handbook. 2002;739-741
6. Tan Y, et al. Autoantibodies against monomeric C-reactive protein in sera from patients with lupus nephritis are associated with disease activity and renal tubulointerstitial lesions. Hum Immunol. 2008;69:840-844.
7. Li QY, et al. Autoantibodies against C-Reactive Protein Influence Complement Activation and Clinical Course in Lupus Nephritis. JASN. 2017;28: 3044-3054.
8. Ayyar BV, et al. Affinity chromatography as a tool for antibody purification. Methods. 2012;56:116-129.
9. Wang Z, et al. Antibody purification using affinity chromatography: a case study with a monoclonal antibody to ractopamine. J Chromatogr B Analyt Technol Biomed Life Sci. 2014;971:10-13.
10. Ahirwar R, et al. Development of an aptamer-affinity chromatography for efficient single step purification of Concanavalin A from Canavalia ensiformis. J Chromatogr B Analyt Technol Biomed Life Sci. 2015;997:105-109.
11. Behar G, et al. Affitins as robust tailored reagents for affinity chromatography purification of antibodies and non-immunoglobulin proteins. J Chromatogr A. 2016; 1441:44-51.
12. SJÖWALL C, et al. Autoantibodies to C-reactive Protein is a Common Finding in SLE, but not in Primary Sjögren's Syndrome, Rheumatoid Arthritis or Inflammatory Bowel Disease. J Autoimmun. 2002;19:155-160.
13. Yang XW, et al. Interference of antimodified C-reactive protein autoantibodies from lupus nephritis in the biofunctions of modified C-reactive protein. Hum Immunol. 2012;73:156-163.