Analyst

ROYAL SOCIETY OF CHEMISTRY

PAPER

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Cite this: *Analyst*, 2022, **147**, 965

Secretory expression and purification of recombinant PLA2R epitopes for the detection of anti-PLA2R autoantibody in serum

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Most patients with idiopathic membranous nephropathy (IMN) have autoimmune antibodies specifically against the M-type phospholipase A2 receptor (PLA2R). PLA2R acts as a significant biomarker contributing to the clinical diagnosis of IMN. Herein, we performed the secretory expression of the exocellular domain and immune-dominant regions of PLA2R by using mammalian cells. Using ELISA, we confirmed that the purified His-tagged PLA2R variant of CysRC1C2C7, which contained CysRC1C2 (aa 21–510) and CTLD7 (aa 1097–1246), possesses the strongest binding affinity toward serum anti-PLA2R in IMN patients. The signal peptide of interleukin-2 for secretion was selected, and parameters for transient expression were optimized to achieve the highest titer of CysRC1C2C7. Stepwise purification of CysRC1C2C7 using anion-exchange chromatography and gel filtration apparently increased its capability of anti-PLA2R recognition or interaction in ELISA. Under optimal conditions of expression and purification, the yield of CysRC1C2C7 in monomer form was ~14.1 mg L^{-1}, with a recovery rate of ~77%. This recombinant PLA2R variant had decent potential for serological analysis of anti-PLA2R in IMN patients.

Received 15th January 2022,
Accepted 8th February 2022

DOI: 10.1039/d2an00094f

rsc.li/analyst

1 Introduction

Idiopathic membranous nephropathy (IMN), a common form of the nephrotic syndrome, is an autoimmune kidney disease.[1–4] About 70%–80% of patients with IMN are positive for autoantibodies against M-type phospholipase A2 receptor (PLA2R).[5] In contrast, such autoantibodies are usually not associated with secondary membranous nephropathy.[6] Autoantibodies against PLA2R have been considered as a promising diagnostic biomarker for IMN.[7–12] Compared with renal biopsy, a basic examination for IMN diagnosis, serological measurement of anti-PLA2R is safer, more convenient, and more friendly to the patients.

PLA2R is a type I transmembrane glycoprotein with a molecular weight (M_W) of ~185 kDa. It consists of an *N*-terminal cysteine-rich domain (CysR), a fibronectin type II domain (FNII), eight C-type lectin-like domains (CTLDs), a transmembrane domain (TMD), and an intracellular *C*-terminal tail.[13] The detailed epitopes in PLA2R have been identified to be important for the recognition of anti-PLA2R autoantibodies in IMN patient serum. In the absence of either the CysR or CTLD1 domain, the autoantibody cannot bind to the remaining domains. The CysR-FNII-CTLD1 region was examined as it is the immunodominant epitope region.[14] Several linear peptides were tested and were found to be unable to tell the difference between IMN patients and control serum; this suggests that the 3D conformational structure is a critical feature of PLA2R epitopes. It was proven that the conformational β2–β3 strands located in the CysR domain is a dominant epitope that interacts with autoantibodies.[15] Later, Seitz-Polski *et al.* further confirmed that three distinct domains (CysR, CTLD1, and CTLD7) were the dominant epitopes of PLA2R by expanding the number of testing IMN patients.[16]

Recombinant PLA2R protein is an effective tool for detecting autoantibodies in IMN patients. Numerous immunological methods based on antigen–antibody reactions have been established for the initial clinical diagnosis.[17–25] In these studies, either mammalian cells overexpressing full-length PLA2R protein or a soluble form of extracellular PLA2R were capable of recognizing serum anti-PLA2R. Few methods using truncated PLA2R that only contain the dominant epitopes have been reported. Li *et al.* prepared a PLA2R chimera (CysR, CTLD1, and CTLD7) with an *N*-terminal thioredoxin by using the *E. coli* expression system.[26] This PLA2R chimera was successfully applied to serological detection of anti-PLA2R. However, the autoantibody response of PLA2R is restricted to the antigenic epitope, which is dependent on intramolecular disulfide bonds.[1,7] Eukaryotic cells, rather than prokaryotic

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cells, are more suitable for heterogeneous expression of recombinant proteins harboring disulfide bonds.

In the present study, we first constructed and expressed a series of PLA2R variants, including PLA2R exocellular domain (ECD), truncated ones, and chimeras, by using Expi293F cells. We confirmed by indirect ELISA that each purified PLA2R variant was capable of capturing anti-PLA2R in IMN patient serum. We then selected the PLA2R variant of CysRC1C2C7 and optimized for its high efficiency of expression and/or secretion in Expi293F cells. Finally, we analyzed the influences of stepwise purification of CysRC1C2C7 on its binding affinity.

2 Materials and methods

2.1 Materials

All reagents for buffer preparation were supplied by the National Pharmaceutical Group Chemical Reagent (Shanghai, China). The *E. coli* (Top 10) competent cells were purchased from Transgen (Beijing, China). Plasmids with low level of endotoxin were extracted using HiPure Plasmid EF Maxi Kit (Magen Biotech, Guangzhou, China). The microplates were purchased from Thermo Fisher Scientific (Shanghai, China). 3,3′,5,5′-Tetramethylbenzidine (TMB) was bought from Sigma Aldrich (Shanghai, China). Mouse anti-human IgG-horse radish peroxidase (HRP) was purchased from Biodragon Immunotechnologies Co. Ltd (Beijing, China). The empty columns were bought from Biocomma Limited (Shenzhen, China). PNGase F was supplied by Yeasen Biotechnology Co. Ltd (Shanghai, China).

2.2 Buffers and solutions

Buffers and solutions in this study were prepared using ultrapure water. Coating buffer (CBS): 15 mM Na_2CO_3, 34.9 mM $NaHCO_3$, pH 9.6. PBS buffer: 137 mM NaCl, 10 mM NaH_2PO_4, pH 7.4. Blocking buffer: 0.2% (m/v) gelatin in CBS. Antibody diluent buffer: 0.1% (m/v) gelatin in PBS. Washing buffer (PBST): 0.05% Tween-20 (v/v) in PBS. TMB substrate solution: solution A (0.06% TMB (m/v) in glycol) were mixed with solution B (0.018% v/v) of 30% hydrogen peroxide in 0.1 M citrate–phosphate buffer (pH 5.0) at a ratio of 1 : 5. Stop solution: 2 M sulfuric acid.

2.3 Cell culture

The Expi293F cell line originating from HEK293F was purchased from Gibco (Thermo Fisher Scientific, Shanghai, China). Cells were adapted and cultured with SMM 293-TII Expression Medium (Sino Biological, Beijing, China) in suspension. Cells were cultured in a flask at 125 rpm and 37 °C with 8% CO_2. The viable cell density (VCD) and cell viability were measured using Countess II (Thermo Fisher Scientific, Shanghai, China).

2.4 Plasmid construction of PLA2R variants

All plasmids used in this study are listed in Table 1. All PLA2R variants were fused with a His tag at the *C*-terminus. The coding sequence (PLA2R exocellular domain with native signal peptide and *C*-terminal His tag) was synthesized by Tianlin Biotech (Wuxi, China). The fragment was then cloned into the pCMV3 backbone, using the restriction enzyme cutting site of Kpn I and Not I to generate pCMV3-PLA2R-ECD. For the construction of CysRC1 (aa 21–367) and CysRC1C2 (aa 21–510), each corresponding fragment was amplified by PCR and ligated into pCMV3 by using Kpn I and Not I. The PLA2R chimeras including CysRC1C7 and CysRC1C2C7 were constructed using Hieff Clone® Plus Multi One Step Cloning Kit (Yeasen Biotechnology, Shanghai, China), in which the CTLD7 domain (aa 1097–1246) was fused to CysRC1 and CysRC1C2, respectively. The coding sequence of five signal peptides were synthesized and constructed onto pCMV3 separately. The DNA sequences of CysRC1C2C7 variant was amplified by PCR and inserted into plasmids containing different signal peptides.

2.5 Transient transfection in Expi293F cells

Expi293F cells were seeded at a density of 0.3×10^6 cells per mL in a flask and cultured to $3–4 \times 10^6$ cells per mL. Twenty-four hours before transfection, cells were diluted to a density of 1.5×10^6 cells per mL with fresh pre-warmed medium and cultured to $3–4 \times 10^6$ cells per mL. Cells were diluted to a final density of 2×10^6 cells per mL for transient transfection. The transfection reagent was polyethylenimine (PEI) 25K (Polysciences, PA, USA). Total plasmid DNA concentration was 2.0 μg mL^{-1} of culture volume. The plasmid and PEI were mixed by vortex in fresh medium, and incubated for 20 min at room temperature. The DNA–PEI mixture was slowly added to

Table 1 Plasmids used in this study

Plasmids	Description
pCMV3	Backbone vector for protein expression in mammalian cells
pCMV3-PLA2R-ECD	The exocellular domain of PLA2R, *C*-terminal His tag, native signal peptide (SP)
pCMV3-IgK_{sp}-PLA2R-ECD	The exocellular domain of PLA2R, *C*-terminal His tag, mouse immunoglobulin κ light chain (IgK_{sp})
pCMV3-IgK_{sp}-CysRC1	The CysR epitope and CTLD1 of PLA2R (CysRC1), *C*-terminal His tag, IgK_{sp}
pCMV3-IgK_{sp}-CysRC1C2	The CysR epitope and CTLD1–2 of PLA2R (CysRC1C2), *C*-terminal His tag, IgK_{sp}
pCMV3-IgK_{sp}-CysRC1C7	The CysR epitope, CTLD1 and CTLD7 of PLA2R (CysRC1C7), *C*-terminal His tag, IgK_{sp}
pCMV3-IgK_{sp}-CysRC1C2C7	The CysR epitope, CTLD1–2 and CTLD7 of PLA2R (CysRC1C2C7), *C*-terminal His tag, IgK_{sp}
pCMV3-AZU_{sp}-CysRC1C2C7	CysRC1C2C7, *C*-terminal His tag, SP of azurocidin preproprotein
pCMV3-HT_{sp}-CysRC1C2C7	CysRC1C2C7, *C*-terminal His tag, SP of human trypsinogen
pCMV3-GL_{sp}-CysRC1C2C7	CysRC1C2C7, *C*-terminal His tag, SP of gaussia luciferase
pCMV3-$IL2_{sp}$-CysRC1C2C7	CysRC1C2C7, *C*-terminal His tag, SP of interleukin-2

the cells and was gently swirled during addition. The culture flask was immediately transferred to an orbital shaker (125 rpm, 37 °C, 8% CO_2). Twenty-four hours post-transfection, pre-warmed SMS 293-SUPI (Sino Biological, Beijing, China) was added as feed. Fed-batch culture was performed if necessary. More than 5 days post-transfection, the culturing medium was harvested.

2.6 Purification of recombinant proteins

In this study, three steps of protein purification were performed. First, the culturing medium was centrifuged at 12 000*g* for 30 min at 4 °C, and the supernatant was collected. The supernatant was concentrated using Pellicon® Single-Pass TFF (Millipore, Darmstadt, Germany) and buffer-exchanged in binding buffer (300 mM NaCl, 50 mM NaH_2PO_4, 10 mM imidazole, pH 8.0). The concentrated sample was loaded into a gravity column packed with Ni-NTA resin (Qiagen, Hilden, Germany). The column was pre-equilibrated with binding buffer and the PLA2R protein was eluted with elution buffer (300 mM NaCl, 50 mM NaH_2PO_4, 250 mM imidazole, pH 8.0). Secondly, the obtained PLA2R protein was desalted in buffer A (20 mM Tris-HCl, pH 8.0) using AKTA Pure equipped with a HiTrap desalting column (GE Healthcare, Buckinghamshire, UK). After desalting, the sample was subjected to HiTrap Capto Q anion-exchange chromatography (GE Healthcare, Buckinghamshire, UK) pre-equilibrated with buffer A. The purer PLA2R was eluted by a linear gradient of buffer B (1 M NaCl, 20 mM Tris-HCl, pH 8.0) at a flow rate of 1 mL min^{-1}. Thirdly, the protein was concentrated and further loaded to a column of Superdex 200 Increase 10/300 GL (GE Healthcare, Buckinghamshire, UK), pre-equilibrated with buffer C (150 mM NaCl, 10 mM NaH_2PO_4, pH 7.4). The protein was eluted at a flow rate of 0.2 mL min^{-1}.

2.7 PNGase F treatment

The recombinant PLA2R protein was treated with PNGase F under denaturing conditions according to the instruction. In brief, 20 μg protein in PBS was supplemented with 0.5% SDS and 40 mM DTT and then boiled for 10 min. The protein was digested with 0.5 μg of PNGase F to a final concentration of 1% NP-40 and 50 mM NaH_2PO_4 (pH 7.5). After 3 h treatment at 37 °C, the protein was subjected to SDS-PAGE.

2.8 Anti-PLA2R ELISA

Serum samples of biopsy-proven IMN patients and healthy individuals were collected from Wuxi People's Hospital. The performed testing was approved by the local ethics committee. All experiments were performed in accordance with the good clinical practice guidelines, and approved by the ethics committee at Jiangnan university. Informed consent was obtained for any experimentation with human subjects. The anti-PLA2R ELISA was performed as described previously,[16] with slight modification. Typically, the recombinant PLA2R protein diluted in CBS was coated onto 96-well microplates (100 μL per well) at 37 °C for 2 h. The plates were washed with PBST and blocked with blocking buffer (200 μL per well) at 4 °C overnight. Patients' serums were twofold diluted in antibody diluent buffer and added to the plates (50 μL per well). Serums from health individuals were used as negative control. After 30 min incubation at 37 °C, the plates were washed with PBST three times. Mouse anti-human IgG-HRP diluted by 1 : 8000 in antibody diluent buffer was added (100 μL per well) and incubated for 30 min at 37 °C. After three washes, TMB substrate solution (100 μL per well) was added to the microplates and turned to blue after 10 min incubation at 37 °C. The reaction was terminated by the addition of stop solution (50 μL per well), and the absorbance was measured using a microplate reader at 450 nm.

3 Results and discussion

3.1 Preparation of recombinant PLA2R variants in secretory form

The full-length PLA2R contains a TMD (Fig. 1A), making it difficult to be highly expressed and easily purified. The exocellular domain of PLA2R (PLA2R-ECD), in which the TMD and *C*-terminal tail were truncated, was constructed and expressed in Expi293F cells. We failed to obtain any secretory PLA2R proteins from the culturing medium when the endogenous signal peptide of PLA2R was used, probably because of its very low level of expression and/or secretion (data not shown). The signal peptide of mouse immunoglobulin κ light chain (IgK) was introduced to enhance the secretory expression of PLA2R-ECD (Fig. 2A). After affinity chromatography, the Expi293F expression system afforded a yield of ~2.0 mg L^{-1} for PLA2R-ECD transient transfected cells.

To date, many studies have been performed to analyze the immunodominant epitopes of PLA2R recognized by autoantibodies, mainly including CysR domain, CTLD1, CTLD7, and CTLD8.[12,14–16,23,27] The anti-CTLD8 specificity of IMN patient serum remains obscure because of the sequence similarity between CTLD7 and CTLD8.[28] Therefore, we wondered whether the combination of different PLA2R epitopes could be effectively expressed in a soluble form. Subsequently, four PLA2R variants (CysRC1, CysRC1C2, CysRC1C7, and CysRC1C2C7) were constructed as shown in Fig. 1B. All these variants were well expressed in Expi293F cells and successfully purified from the medium using Ni-NTA gravity column (Fig. 2B–E). Compared with PLA2R-ECD, the variants were produced at a higher level in secretory form. After affinity chromatography, the yields of CysRC1, CysRC1C2, CysRC1C7, and CysRC1C2C7 were measured to be ~15.3, 33.0, 23.2, and 13.6 mg L^{-1}, respectively (Fig. 2F).

3.2 Evaluation of anti-PLA2R serum using recombinant PLA2R variants

To confirm whether these recombinant PLA2R variants were effective for anti-PLA2R recognition in serum, indirect ELISA was applied. In this strategy, PLA2R variants were coated on the microplates (Fig. 3A). Subsequently, the anti-PLA2R IgG in IMN patient serum can be captured by PLA2R variants and

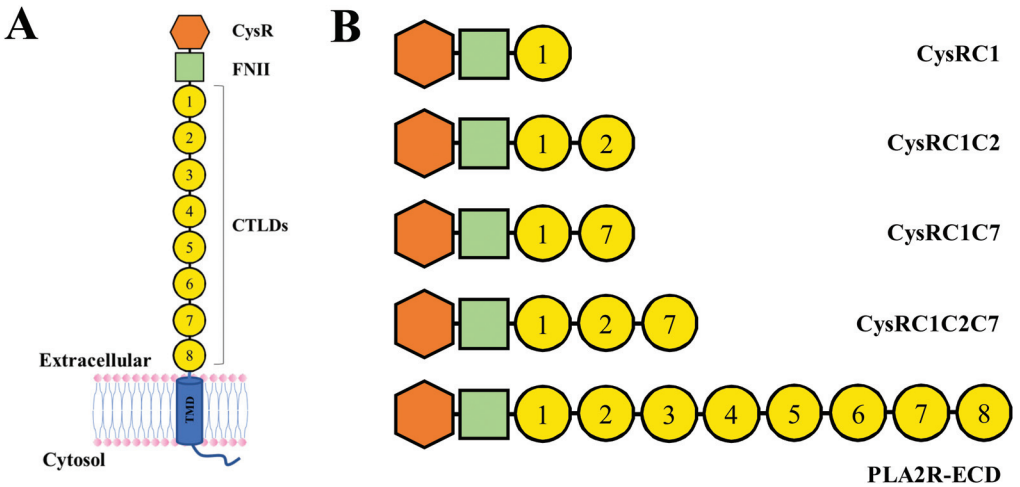

Fig. 1 Schematic diagram of PLA2R and its variants. (A) The full-length wild-type PLA2R associates with cell membrane. It consists of CysR, FNII, CTLDs, TMD, and cytosolic tail. (B) Five mutants of the PLA2R were designated, *i.e.*, CysRC1 that contained the CysR, FNII and CTLD1 domains, CysRC1C2 that contained the CysR, FNII and CTLD1-2 domains. CysRC1C7 in which the region from CTLD2 to tail was deleted and fused with additional CTLD7 domain by a GGGGS flexible linker. CysRC1C2C7 in which the region from CTLD3 to tail was deleted and fused with CTLD7. PLA2R-ECD indicated the TMD and tail were truncated. The signal peptide was not shown. A 10× His tag was fused to the *C*-terminus of these PLA2R variants (not shown).

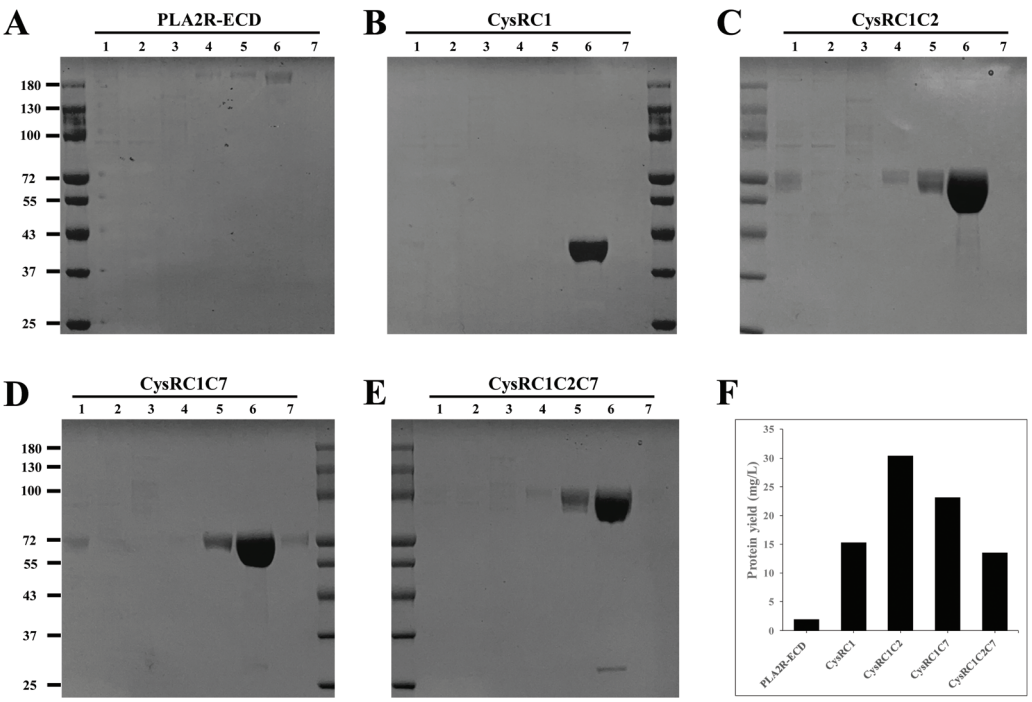

Fig. 2 The expression and purification of secretory PLA2R variants. (A)–(E) SDS-PAGE of PLA2R variants purified by Ni-NTA gravity column. Lane 1: supernatant of cell culture. Lane 2: flow-through. Lanes 3–4 represented eluates of 20 mM and 50 mM imidazole-containing buffer. Lanes 5–7 represented eluates of 250 mM imidazole-containing buffer with three column volumes (CVs). (F) Protein yield of PLA2R variants after a step gradient elution with imidazole.

then recognized by mouse anti-human IgG labeled with HRP. The OD450 was measured after incubation with substrate TMB.

Here, six anti-PLA2R-positive and two anti-PLA2R-negative serum samples were chosen to evaluate the reactivity and specificity toward PLA2R variants. The results show that all of the PLA2R variants were well recognized by the serums from IMN patients. In contrast, signals were barely detectable when negative serums were used (Fig. 3B and C), indicating the specificity of recombinant PLA2R variants. Although the

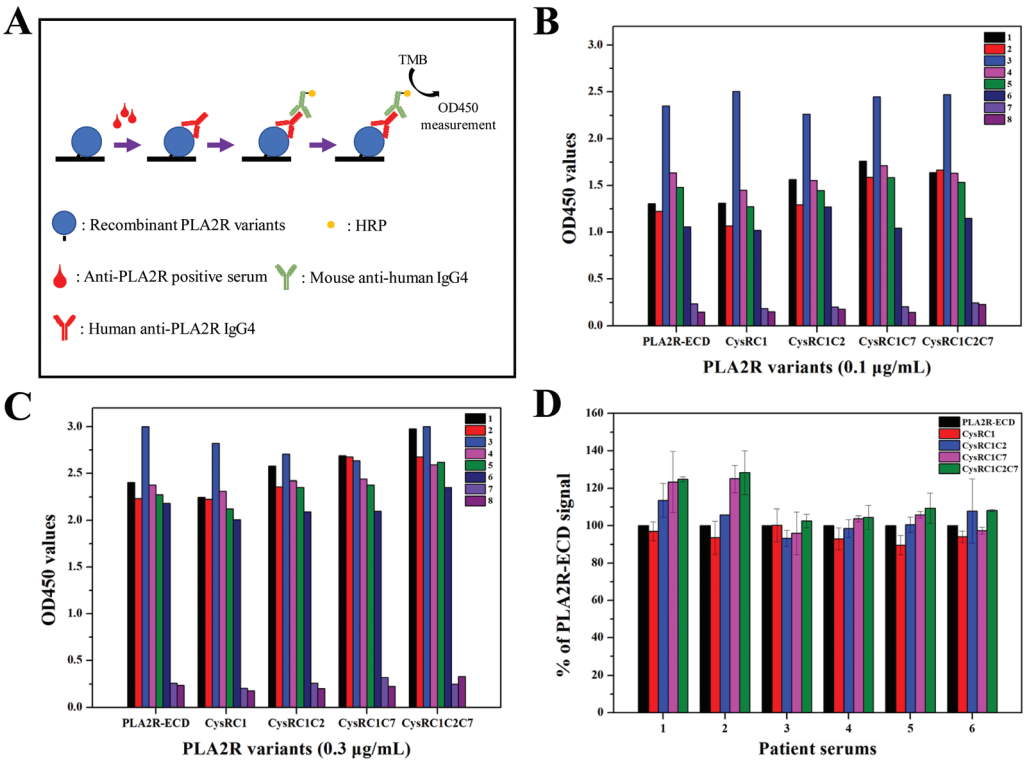

Fig. 3 Evaluation of anti-PLA2R serum. (A) The schematic diagram of anti-PLA2R ELISA. (B) and (C) The OD values of anti-PLA2R ELISA. 0.1 μg mL^{-1} and 0.3 μg mL^{-1} of PLA2R variants were coated as indicated. 1–6 represented six positive anti-PLA2R serums from IMN patients. 7–8 represented two negative serums from healthy individuals. (D) Comparison of PLA2R variants against anti-PLA2R autoantibody in serum samples using ELISA. Serum samples were derived from IMN patients 1–6. The measured OD450 values of PLA2R-ECD were defined as 100%.

variant of PLA2R-ECD maintained the intact and native spatial conformation of wild-type PLA2R, we unexpectedly observed that the variant of CysRC1C2C7 interacted with serum anti-PLA2R at the highest level (Fig. 3D). Among these variants, the ability of anti-PLA2R IgG recruitment presented a tendency that was CysRC1C2C7 > CysRC1C7 > CysRC1C2 > PLA2R-ECD > CysRC1 (Fig. 3D). Taking consideration of protein yield and immunodominant epitope, we selected CysRC1C2C7 as the most suitable variant for serum anti-PLA2R determination in the subsequent experiments.

3.3 Screening of signal peptide for CysRC1C2C7 expression

The signal peptide (SP) affects the expression and secretory efficiency of heterologous proteins in mammalian cells.[29] Therefore, several secretory SPs were introduced to obtain the highest titer of CysRC1C2C7. The DNA sequences of five non-native SPs (IgK_{sp}, AZU_{sp}, HT_{sp}, GL_{sp}, and $IL2_{sp}$) were constructed onto the pCMV3 backbone. The source of signal peptides is listed in Table 2. CysRC1C2C7 with a *C*-terminal 10His tag was then inserted into the plasmids harboring different signal peptides. Each construct was transiently transfected into Expi293F cells. Five days post-transfection, the culturing medium supernatants were harvested and concentrated. After loading into the Ni-NTA gravity column, we directly eluted proteins with buffer containing 250 mM imidazole.

As shown in Fig. 4A, CysRC1C2C7 could be expressed and secreted under almost all SPs except HT_{sp}, whereas the intensity of bands was easily distinguished. Compared with IgK_{sp}, the SPs of AZU and IL2 obviously increased the secretion of target protein at a similar level. The amounts of secretory proteins derived from these constructs were measured using Braford protein assay kit. Among them, $IL2_{sp}$ construct showed

Table 2 Signal peptides used in the study

Signal peptides	Amino acids	Protein	Organism
$Native_{sp}$	MLLSPSLLLLLLLGAPRGCA	PLA2R	Homo sapiens
IgK_{sp}	METDTLLLWVLLLWVPGSTG	Ig kappa chain	Mouse
AZU_{sp}	MTRLTVLALLAGLLASSRA	Azurocidin	Homo sapiens
HT_{sp}	MNLLLILTFVAAAVA	Trypsinogen-2	Homo sapiens
GL_{sp}	MGVKVLFALICIAVAEA	Luciferase	Gaussia princeps
$IL2_{sp}$	MYRMQLLSCIALSLALVTNS	Interleukin-2	Homo sapiens

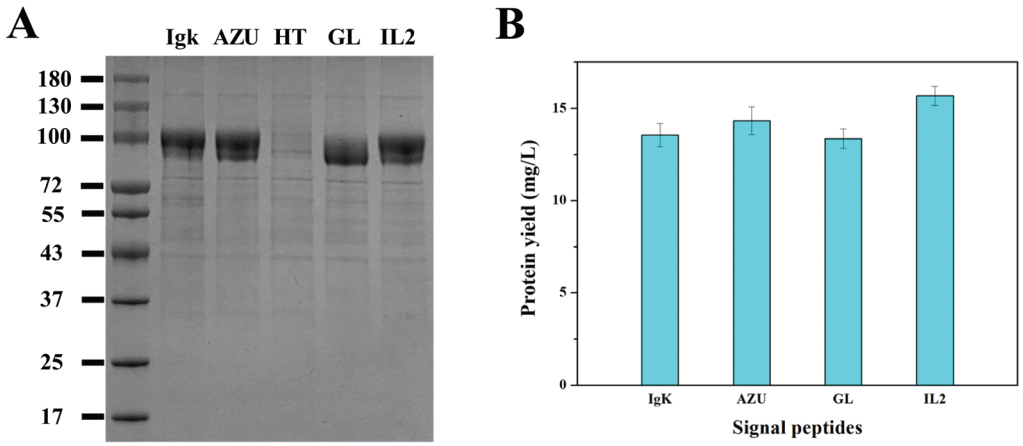

Fig. 4 Screening of signal peptide for CysRC1C2C7 transient expression. (A) SDS-PAGE of the secretory CysRC1C2C7 under different signal peptides of IgK, AZU, HT, GL, and IL2. (B) Quantitative analysis of the protein yield. The transient expression of CysRC1C2C7 was performed in triplicate.

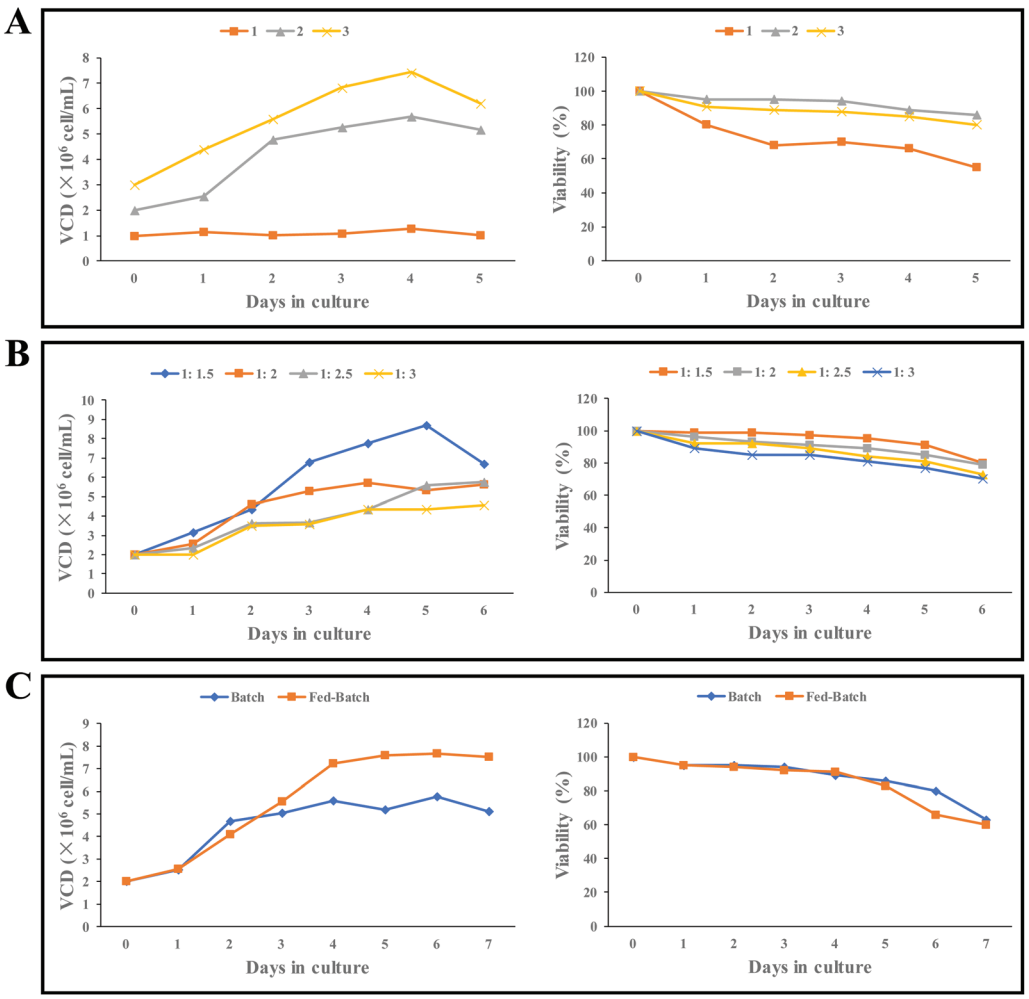

Fig. 5 The viable cell density and viability of Expi293F cells under different culturing condition. (A) Optimization of initial cell density (1 × 10^6, 2 × 10^6 and 3 × 10^6 cells per mL) for transient transfection. (B) Optimization of DNA/PEI ratio (1 : 1.5, 1 : 2, 1 : 2.5, and 1 : 3). (C) Comparison of batch and fed-batch culturing.

the best efficiency for CysRC1C2C7 secretion, which was 1.16-fold of IgK_{sp} (Fig. 4B). Thus, the $IL2_{sp}$ was screened as the optimal signal peptide for CysRC1C2C7 secretory expression.

3.4 Optimization of conditions for CysRC1C2C7 expression

At first, the cell density (1×10^6, 2×10^6 and 3×10^6 cells per mL) of transient transfection was optimized for CysRC1C2C7 expression. The culturing media were harvested on day 5 post-transfection because cell viability of the first group decreased below 55% (Fig. 5A). As shown in Fig. 6A, the highest titer of CysRC1C2C7 was obtained when the cell density of transfection was 2×10^6 cells per mL. The different DNA/PEI ratios were then evaluated (Fig. 5B). According to the results, the optimal ratio was 1 : 3 (Fig. 6B). Theoretically, the cell viability dramatically drops four days post-transfection. In such case, the efficiency of protein expression and secretion is lowered. Protein degradation may happen during long-term incubation with dead cells at 37 °C. Thus, the duration of cultivation must be optimized. On the basis of cell viability and titer, day 6 post-transfection was chosen for harvest. The cell viability was 80%, and the viable cell density was 5.77×10^6 cells per mL (Fig. 5C, Batch). Under the optimal condition of cell density and DNA/PEI ratio, the influence of feed schedule was then tested to achieve better culture performance. For fed-batch cultivation, 5% of initial culture volume of SMS 293-SUPI was supplemented into the shaken flasks on days 1, 3, and 5 post-transfections. Compared with the batch group, the cell density of the fed-batch group during cultivation was obviously higher (Fig. 5C). The media were harvested on each day post-transfection. The proteins were detected by SDS-PAGE and measured using Braford protein assay kit. As shown in Fig. 6C and D, the fed-batch condition increased the secretion of CysRC1C2C7. The protein amount did not increase significantly along with the extension of culturing time (day 6 and 7).

3.5 Stepwise purification of recombinant CysRC1C2C7

To achieve the best performance of CysRC1C2C7 for serum anti-PLA2R detection, the purification strategy was carried out. At first, the secreted CysRC1C2C7 in culturing medium was concentrated and loaded into a gravity-flow column packed with 5 mL of Ni-NTA resin. The yield of CysRC1C2C7 obtained from the step gradient elution was ~18.3 mg L^{-1}. The recovery rate was defined as 100%. Ni-column belongs to immobilized-metal-ion affinity chromatography, which may cause non-specific protein adsorption and nucleic acid residue. Some bands of non-specific protein were observed on the presented

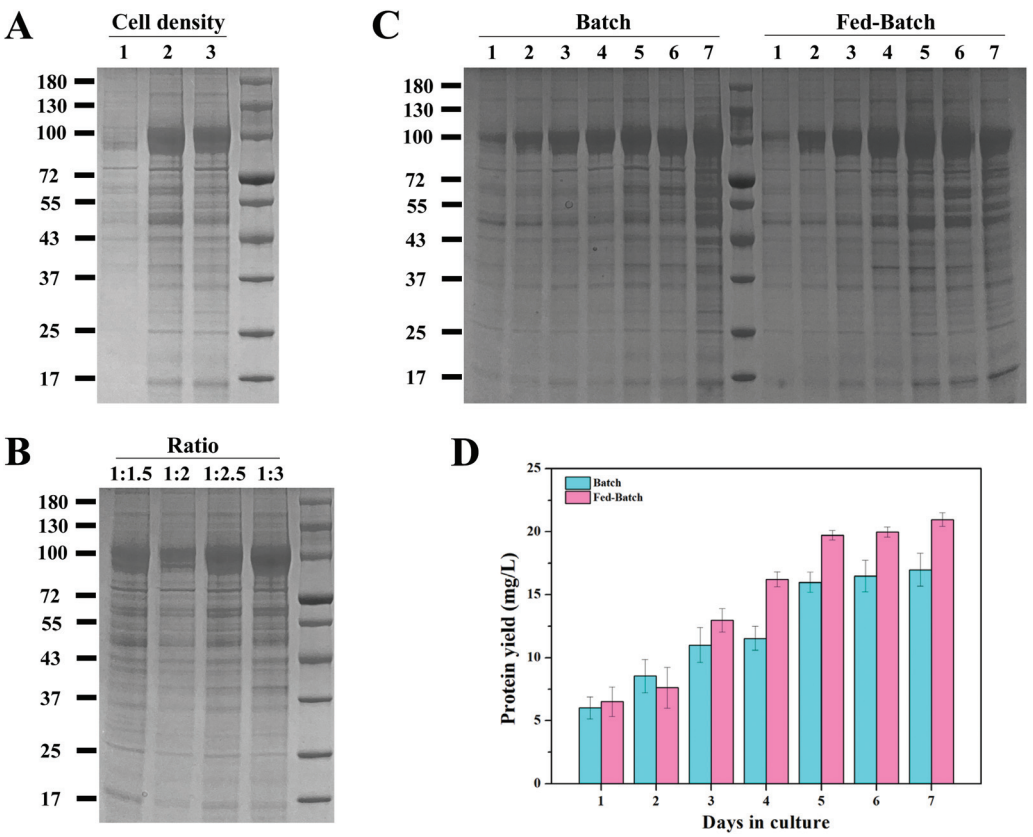

Fig. 6 The optimization for CysRC1C2C7 secretion. (A) The initial cell density (1, 2, and 3×10^6 cells per mL) for transient transfection was optimized. The ratio of DNA/PEI was 1 : 2. (B) The ratio of DNA/PEI (1 : 1.5, 1 : 2, 1 : 2.5, and 1 : 3) was optimized. (C) The secretory proteins from non-fed cells (Batch) and fed cells (Fed-Batch) were compared. Lanes 1–7 indicated days in culture. The harvested mediums were concentrated and subjected to SDS-PAGE. (D) The amounts of secretory proteins from batch and fed-batch groups were measured and compared.

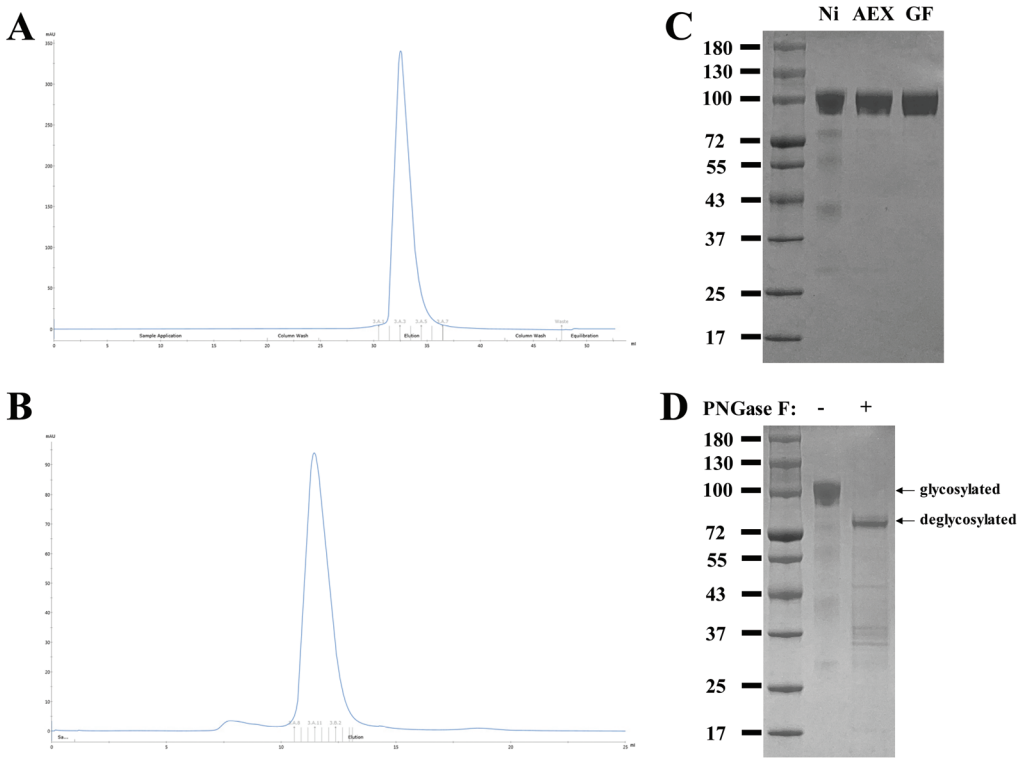

Fig. 7 Stepwise purification and SDS-PAGE analysis of CysRC1C2C7. (A) Anion-exchange chromatography. (B) Gel filtration. (C) Comparison of CysRC1C2C7 derived from stepwise purifications. Ni represented protein purified using Ni resin. AEX meant protein further purified using anion-exchange chromatography. GF indicated protein further purified using gel filtration. (D) PNGase F treatment of CysRC1C2C7.

SDS-PAGE gels. To solve this problem, the Ni-NTA-purified protein was further subjected to anion-exchange chromatography (AEX) and gel filtration (GF) (Fig. 7A and B). The non-specific bands (ranged from 37–100 kDa) were nearly removed through anion-exchange chromatography. Moreover, the remaining band around 30 kDa was eliminated after gel filtration (Fig. 7C). During gel filtration, a small peak was eluted earlier than the main peak (Fig. 7B) possibly because of the homo-oligomerization of CysRC1C2C7. The small peak was discarded, while the main peak was collected. Using BSA-dimer (132 kDa) and BSA-monomer (66 kDa) as controls, we found that CysRC1C2C7 was eluted between BSA-dimer and BSA in gel filtration; this suggests that the native molecular weight of CysRC1C2C7 was lower than 132 kDa (Fig. 8). Therefore, we considered that the finally obtained CysRC1C2C7 was a monomer. After additional two-step purification, recombinant CysRC1C2C7 with higher purity was obtained with a yield of ~14.1 mg L^{-1}. However, the recovery rate was relatively low (~77.1%).

According to the result of SDS-PAGE, the M_W of recombinant CysRC1C2C7 was ~100 kDa, which is not consistent with the predicted M_W (~75 kDa). Using the NetNglyc server

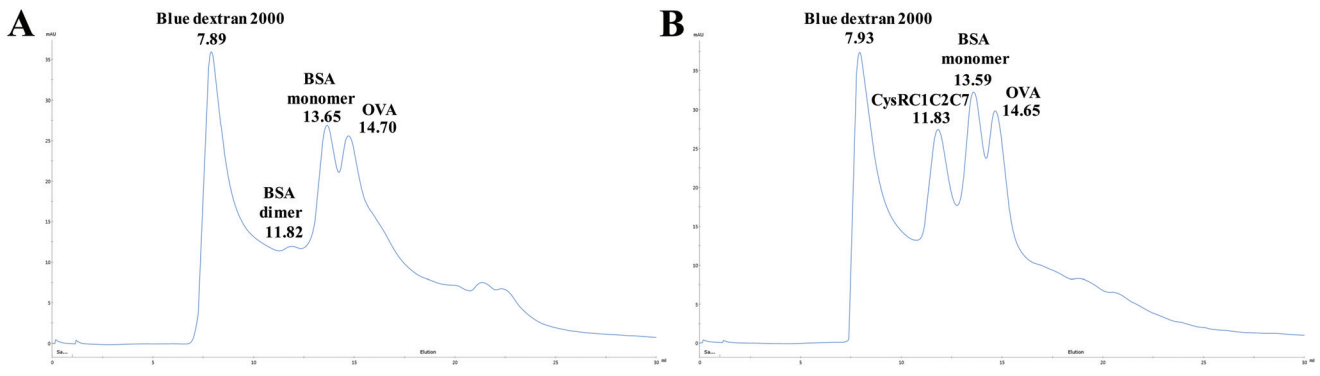

Fig. 8 Determination of the native molecular weight of CysRC1C2C7. (A) Gel filtration of blue dextran 2000, BSA mixture, and OVA. (B) Gel filtration of blue dextran 2000, recombinant CysRC1C2C7, BSA mixture, and OVA. The elution volume of each sample was indicated.

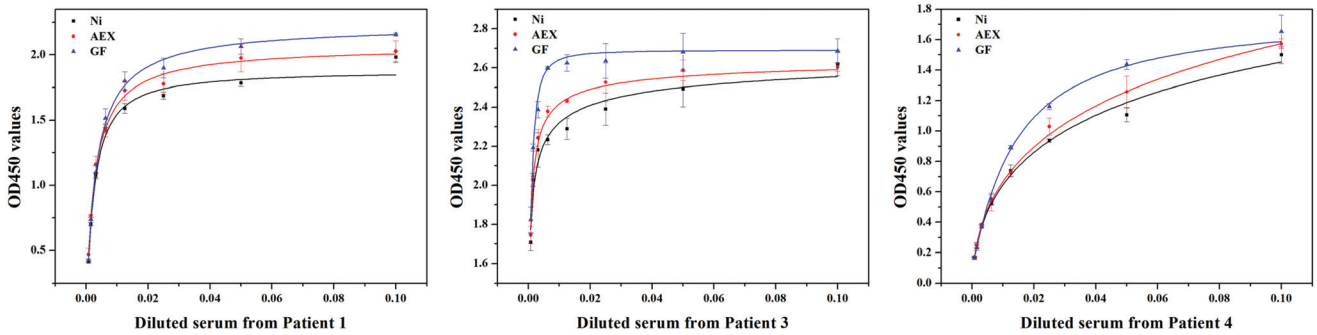

Fig. 9 Anti-PLA2R analysis of stepwise purified CysRC1C2C7 proteins. Ni, AEX, and GF indicated CysRC1C2C7 proteins obtained from one-step, two-step, and three-step purification, respectively.

(https://services.healthtech.dtu.dk/service.php?NetNGlyc-1.0), we found six Asn residues of CysRC1C2C7 that are possibly post-translationally modified by *N*-glycan. The recombinant CysRC1C2C7 was treated with PNGase F to remove attached *N*-glycans under denaturing conditions. After PNGase digestion, the band of CysRC1C2C7 shifted to ~75 kDa (Fig. 7D), suggesting that the recombinant CysRC1C2C7 was an *N*-linked glycoprotein.

3.6 Comparison of each CysRC1C2C7 for anti-PLA2R detection

To ask if stepwise purification will enhance the capability of CysRC1C2C7 for the recognition of anti-PLA2R in serum, anti-PLA2R ELISA was performed as described above. Each serum from three IMN patients was serially diluted (10-, 20-, 40-, 80-, 160-, 320-, 640- and 1280-fold dilution) and tested. As shown in Fig. 9, the group of three-step-purified CysRC1C2C7, indicated as GF, possessed the strongest signal in ELISA. The fitting curves were established. In the case of GF, the regression coefficients related to patients 1, 3 and 4 were calculated to be 0.9998, 0.9887, and 0.9982, respectively. These data suggest that recombinant CysRC1C2C7 obtained by three-step purification was the optimal one for anti-PLA2R analysis in serums. Because of the lack of enough amounts of positive serums, future works should be done to expand the serum sample numbers and to evaluate the clinical relevance of recombinant CysRC1C2C7 by using anti-PLA2R ELISA or other immunologic methods.

4 Conclusion

In this study, we constructed extracellular, truncated, and chimeric forms of PLA2R. All of them can be successfully expressed and secreted into culturing medium by using the Expi293F suspension cell system. They possessed comparable sensitivity and specificity against anti-PLA2R in the serum of IMN patients. The chimera of CysRC1C2C7, in which the CysR domain, FNII, CTLD1, and CTLD2 of PLA2R were *C*-terminally fused with CTLD7, had the best performance in anti-PLA2R ELISA. Under optimal conditions for protein expression, the yield of CysRC1C2C7 could reach 18.3 mg L^{-1}. Higher purity and activity could be achieved by subsequent polishing of AEX and GF. We concluded that the finally obtained CysRC1C2C7 was effective and sensitive for the serologic detection of anti-PLA2R and that it should be compatible for reported immunoassays.

Conflicts of interest

All the authors declare no competing financial interest.

Acknowledgements

This work was partially supported by the National Key Research and Development Program of China (2019YFC1604604).

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