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# Separation/enrichment of active natural low content protein using protein imprinted polymer

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ABSTRACT

We describe a new type of protein-imprinted polymer for separation/enrichment of active natural protein present at a relatively low level in cell extracts, with a cloned bacterial protein as template. In this work, cloned pig cyclophilin 18 (pCyP18) was used as template. The template protein was selectively assembled with assistant recognition polymer chains (ARPCs) from their library, which consists of numerous limited length polymer chains with randomly distributed recognition and immobilizing sites. These assemblies of protein and ARPCs were adsorbed by porous polymeric beads and immobilized by cross-linking polymerization. After removing the template, the synthesized imprinted polymer was used to adsorb authentic pCyP18 from cell extract, and its proportional content was enriched 200 times. The assay of peptidyl-prolyl *cis–trans*-isomerase (PPIase) activity showed that natural pCyP18 is more active than cloned pCyP18 and, in particular, it is much more sensitive to the suppressant cyclosporine A (CsA).



## 1. Introduction

Molecular imprinting technique creates recognition sites for specific substances using template molecules [1–4]. In addition to small molecules, proteins can also be used as templates [5–8]. Until now, only abundant proteins have been used as templates [9–12] due to the request of a large amount of template. However, there are thousand types of proteins within a cell and most of them are present at relatively low levels. Indeed, many cellular proteins are present as just a few copies, even though they perform important biological functions. Proteins can be purified in active form on the basis of their characteristics, such as solubility, size, charge and specific biding affinity, by using centrifugation, gel-filtration chromatography, ion (anion or cation)-exchange chromatography and affinity chromatography [13,14]. For purification of proteins, a combination of above mentioned methods with several other steps is required. Theoretically, all low abundance proteins could be purified if sufficient cell extract was available. However, practically, this is not possible, because there is always a limit on the amount of starting material for separation, and the harvest ratio in every step is quite low. Therefore, a method with high enrichment efficiency is needed. Protein-imprinted polymer (PIP) synthesized using a cloned bacterial protein template, can successfully adsorb and enrich the low-content target protein from cell extract [15]. To capture the low content target protein from thousands of proteins in a cell extract, it is important to enhance the recognition specificity of the PIP. Haupt reported creating molecular memory using a synthetic polymer by assembling the recognition monomers and template into a complex [16]. Introduction of assistant recognition polymer chains (ARPCs) as extenders of monomers clearly enhances the recognition specificity [17].

Fig. 1 shows the synthesis of this type of PIP. First, the template (cloned bacterial protein) is selectively assembled with ARPCs from their library, which consists of numerous limited length polymer chains with randomly distributed recognition sites and immobilizing sites. Then the assemblies of proteins and ARPCs are adsorbed by the porous polymeric beads, and immobilized by cross-linking polymerization. After removing the template, binding sites that are complementary to the target protein in size, shape and position of recognition groups are exposed, and their confirmation is preserved by the cross-linked structure. Considering the varied recognition sites from manifold side chains of protein on its surface, we adopted ARPCs with two types of recognition site, negatively charged acetic sites and aromatic pyridyl sites, to prepare the PIP for separation/enrichment of natural low content proteins from cell extracts. The conventional protein-imprinting method involves polymerization of a functional monomer in the presence of protein template and PIP film on supporting media, such as silica [18,19], chitosan beads [11], hydrogels [20], mica [12], membranes [21,22]

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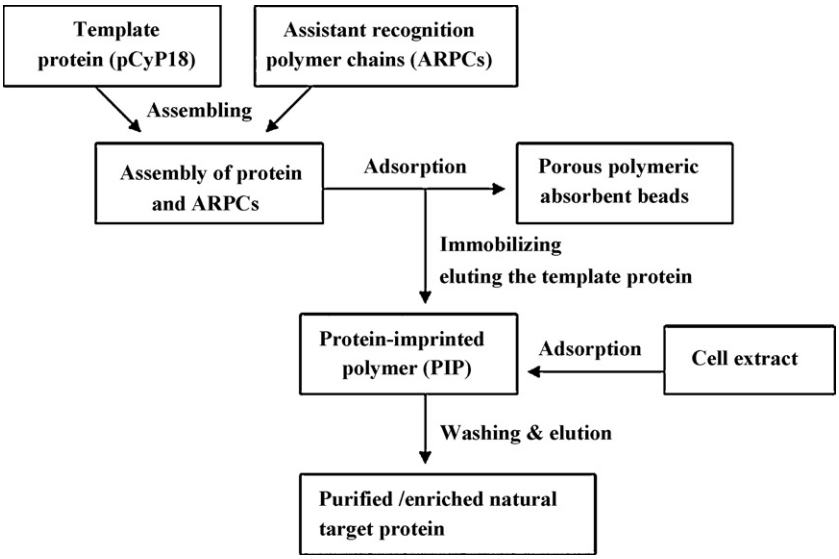


**Fig. 1.** Strategy for synthesis of the PIP.

or microspheres [5,23,24]. In this work, polyvinyl alcohol macroporous microspheres (PVA spheres) were used as carrier on account of their relatively hydrophilic character and biocompatibility.

Pig cyclophilin 18 (pCyP18) was used as template [25,26]. This protein is a peptidyl-prolyl *cis–trans*-isomerase (PPIase). It is ellipsoidal in shape, with axis lengths of $4.30 \times 5.26 \times 8.92$ nm and a molecular mass of 18 kDa. It makes up around 0.08% of the total protein in pig liver extract. The synthesized imprinted polymer was used to adsorb natural pCyP18 from cell extracts, and its proportional content was enriched from 0.08% of total proteins to 16.5% in enriched eluent. The assay of PPIase activity showed that natural pCyP18 is more active than cloned pCyP18 and, in particular, it is much more sensitive to the suppressant cyclosporine A (CsA).

## 2. Experimental

### 2.1. Materials and reagents

Hybond-C nitrocellulose membrane, glutathione-Sepharose 4B and Factor Xa were purchased from Amersham Biosciences UK Limited (UK). *N*,*N*′-Diisopropylcarbodiimide (DIC) and 1-hydroxybenzotrizole (HOBt) were produced by Beijing Bomaijie Technology Led. 4-Vinyl pyridine (4-VP) was purchased from Sigma–Aldrich Corporation and vacuum-distilled before use. Acrylonitrile (AN) was produced by Shanghai Sanaisi Reagent Led. 2,2′-Azobisisobutyronitrile (AIBN) was produced by Tianjin Fuchen Chemical Reagent Led and purified by recrystallization in methanol. Other chemicals were obtained from commercial sources and used as received.

### 2.2. Synthesis of the assistant recognition polymer chains

The backbone of the ARPCs was synthesized with 4-vinyl pyridine (4-VP) and acrylonitrile (AN) as monomers via free radical polymerization and 2,2′-Azobisisobutyronitrile (AIBN) was used as the initiator (Fig. 2). The degree of polymerization was controlled by the proportion of 4-VP, AN and AIBN. After adding 0.5 mL of 4-VP, 30 mL of AN (4-VP/AN, 1:99 m/m) and 2.3 g of AIBN in 200 mL of ethanol, the mixture was stirred for 8 h at 80 °C in a water bath with ventilated $N_2$ for removal of $O_2$. The polymerization degree was approximately 40, according to the yielded of gel permeation chromatography (Mn = 2358 and Mw/Mn = 1.43). Thus, the average extended length of the polymer chains was about 10 nm. This length is relevant to the size of the protein. The polymer chains should be long enough to contain sufficient recognition sites, but they cannot be so long that they will wind around the template proteins and hinder their removal.

After precipitation and washing with ethanol, the product named PANVP was dried in vacuum at 50 °C for 24 h. Pyridyl groups as randomly distributed recognition sites covered approximately 10% of the total side chains (about four randomly distributed pyridyl groups remained on each ARPC) measured by UV spectrophotometry at 242 nm using 4-VP as the standard. Purified PANVP (2 g) was hydrolyzed in 15 mL of 85% (w/w) $H_2SO_4$ for 4 h at 100 °C. Then the product named PAAVP was purified by precipitation and washing with ethanol solution (ethanol/$H_2O$, 1:1 v/v) and dried in vacuum at room temperature for 24 h. To immobilize the ARPCs onto the carrier, the carboxyl groups on PAAVP were partly reacted with allyl alcohol and esterified. Purified PAAVP (2 g), 1.76 mL of *N*,*N*′-diisopropylcarbodiimide (DIC), 2.27 g of 1-hydroxybenzotrizole (HOBt) (DIC/HOBt, 1:1 m/m) and 1.14 mL of allyl alcohol were dissolved in DMSO and the mixture was stirred in a stirred bed reactor in a water bath for 48 h at 30 °C. Then the prod-

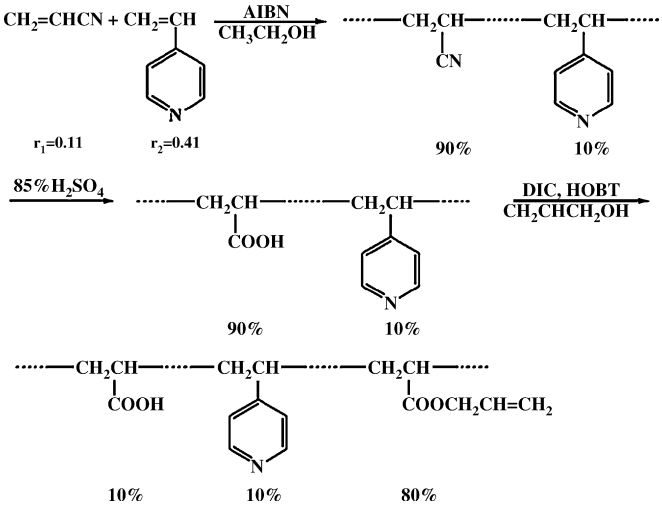


**Fig. 2.** Synthetic route of ARPCs.

uct ARPCs were precipitated and washed with ethanol, and dried in vacuum at room temperature to constant weight. Allyl groups as immobilizing sites covered 80% of the total side chains by measuring with the method of bromine addition and carboxyl groups as recognition sites holded approximately 10% of the total side chains on the ARPCs (about four randomly distributed carboxyl groups remained on each ARPC).

### 2.3. Synthesis of porous polymeric beads

Purified polyvinyl alcohol (PVA, 10 g) was dissolved in DMSO. Pyridine (10 mL) was added to the solution and the mixture was incubated for 15 min at room temperature. Then 10 mL of acryloyl chloride was added and the mixture was stirred electromagnetically for 4 h at 30 °C in a water bath. After precipitation and washing with ethanol, the polymer product was dried in vacuum to constant weight.

### 2.4. Construction of plasmids expressing pCyP18

To clone the template protein pCyP18, pig mRNA was amplified by reverse transcription polymerase chain reaction (RT–PCR) using an upstream primer (pCyP18f) including an *Eco*RI site (5′-CCG GCC GAA TTC ATG GTT AAC CCC ACC GT-3′), and a downstream primer (pCyP18r) including an *Xho*I site (5′-GGC CGG CTC GAG TTA GAT TTG TCC ACA GTC AG-3′). After digestion with *Eco*RI and *Xho*I restriction enzymes, the PCR fragments were ligated into a bacterial expression vector pGEX5X1.

### 2.5. Purification of GST-fusion protein and pure protein

The amplified bacteria containing the recombinant plasmid with the target protein were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The target proteins were expressed as glutathione S-transferase (GST) fusion proteins and purified using glutathione-Sepharose 4B beads. The purified fusion proteins were restriction digested on beads with factor Xa in a digestion buffer (50 mM Tris–HCl, pH 8.0, 5 mM $CaCl_2$ and 100 mM NaCl) at 4 °C for 16 h.

### 2.6. Preparation of cell extract from pig liver

This was carried out according to the method described by Borgeson and Bowman [27]. All steps of this procedure were performed at 4 °C and all buffers and equipment were pre-cooled at 4 °C. About 150 g pig liver was suspended with 150 mL of 2× buffer E (2 M sorbitol, 10 mM Hepes, pH 7.4, and 2 mM EDTA) and applied to the homogenate. After adding 1/10 volume of NP 40 (3% solution), the mixture was stirred electromagnetically on ice for 5 min to break the cells. The suspension was centrifuged for 20 min at 1000 × *g* and the supernatant was collected. The pellet was suspended in 10 mL of buffer E and re-centrifuged for 20 min at 1000 × *g*. The two supernatants were collected together, and then centrifuged sequentially at 12,000 × *g* and 40,000 × *g* for 1 h, respectively. The supernatant was composed of cell extract.

### 2.7. Synthesis of the protein-imprinted polymer

ARPCs (0.24 g) was dissolved in 5 mL of buffer (10 mM $NaH_2PO_4/Na_2HPO_4$, pH 7.5). After adding 4 mg of bacterial cloned pCyP18 (4 mL), the mixture was incubated at 4 °C for 8 h under overhead rotation. Then 2 g of wet grafted PVA was added as absorbent spheres and incubated for 12 h under rotation for adsorption of the protein/ARPCs complex to the porous spheres. Aqueous solution of acrylamide (29% w/v acrylamide, 1% w/v *N,N′*-methylene bisacrylamide, 300 μL) was added and the mixture was stirred for 2 h with ventilated $N_2$ for removal of $O_2$. After adding 90 μL aqueous solution of ammonium persulfate (10% (w/v)) and 3.6 μL of *N,N,N′,N′*-tetramethylethylenediamine, the cross-linking reaction was carried out at room temperature for 2 h under stirring and with $N_2$ protection. The template proteins were removed by elution with high concentration saline buffer (2 M KCl and 10 mM $NaH_2PO_4/Na_2HPO_4$ (pH 7.5)) until no pCyP18 could be detected by SDS–PAGE with silver staining and immuno-staining using anti-pCyP18 antiserum in the wash buffer. The synthesized PIP could be used for adsorbing the target protein from cell extracts.

### 2.8. PPIase activity assays

The PPIase activity of pCyP18 was measured at 5 °C by using the standard chymotrypsin-coupled assay [28–30]. The assay buffer (50 mM Hepes, pH 8.0, 100 mM NaCl) and natural or cloned pCyP18 (40 nM) were pre-cooled in the spectrometer to 5 °C. Then chymotrypsin (30 μM) was added. The reaction was initiated by adding Suc-Val-Pro-Phe-pNA as peptide substrate (45 μM). After mixing, the increase in absorbance at 390 nm was monitored. The inhibition assays were performed in the same manner as mentioned above. CsA dissolved in ethanol was added at different concentrations to the pCyP18 solution pre-incubated for 10 min.

## 3. Results and discussion

### 3.1. Characterization of the ARPCs

In this work, ARPCs with randomly distributed recognition sites were introduced as extenders of monomers in synthesis of the PIP to enhance the recognition specificity. The backbone of the ARPCs was synthesized with 4-vinyl pyridine (4-VP) and acrylonitrile (AN) as monomers via free radical polymerization, as a result pyridyl groups as randomly distributed recognition sites covered approximately 10% of the total side chains (about four randomly distributed pyridyl groups remained on each ARPC). The product named PANVP was hydrolyzed and nitril groups transformed to carboxyl groups. To immobilize the ARPCs onto the carrier, the carboxyl groups on the hydrolyzed product named PAAVP were partly reacted with allyl alcohol and esterified. Allyl groups as randomly distributed immobilizing sites covered 80% of the total side chains and carboxyl groups as randomly distributed recognition sites holded approximately 10% of the total side chains on the ARPCs. As a result, about four randomly distributed carboxyl groups remained on each ARPC. A gravimetric analysis confirmed this structure.

#### 3.1.1. 1H NMR spectra

The polymer products PANVP, PAAVP and ARPCs in DMSO were analyzed by $^1$H NMR using a UNITY-plus-400 NMR instrument (Fig. 3). The spectrum of PANVP shows the peaks at 2.04 and 3.14 ppm, attributed to hydrogens in methylene and methine adjoining acrylonitrile and the peaks at 7.28 and 8.60 ppm attributed to hydrogen in the pyridine ring. In the spectrum of PAAVP, the peak at 10.26 ppm is attributed to hydrogen in carboxyl compared with the spectra of PANVP. Carboxyl group can form intramolecular and intermolecular hydrogen bond. So the peaks from 2.13 ppm to 1.14 ppm attributed to hydrogens in methylene groups in the backbone of the polymer become broad. The spectrum of ARPCs shows the peak at 3.36 ppm, attributable to hydrogen in methylene adjoining ester group, and the peaks at 5.09, 5.22, and 5.89 ppm were attributable to hydrogen in alkene.

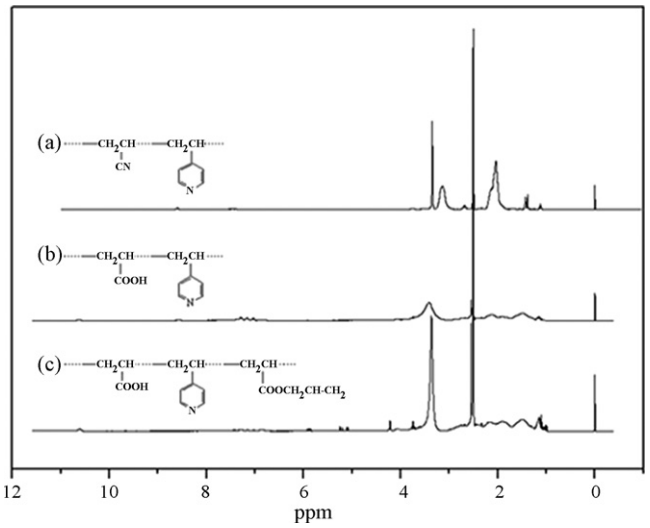


**Fig. 3.** $^1$H NMR spectra of PANVP (a), PAAVP (b), and ARPCs (c).

### 3.1.2. IR spectra

IR spectra of PANVP, PAAVP and ARPCs were recorded with a Bio-Rad FTS 135 FTIR spectrometer using KBr pellets (Fig. 4). The spectrum of PANVP shows a narrow band at 2242.76 $cm^{-1}$, attributed to the stretching vibrations of C≡N. In the spectrum of PAAVP a wide band at about 3181.7 $cm^{-1}$ was attributable to the stretching vibrations of O—H. It shows nitril groups completely transformed to carboxyl groups. In the spectrum of ARPCs, a wide band at about 3194.6 $cm^{-1}$ corresponds to the stretching vibrations of O—H, because some carboxyl groups are still existent, and the band at 3089.96 $cm^{-1}$, attributed to stretching vibrations of =C—H, shows carboxyl groups are partly substituted by ester groups.

### 3.2. Adsorption of target protein from cell extract

The assemblies of template protein and ARPCs were immobilized onto microspheres by cross-linking polymerization. After removing the template, the PIP could then be used for adsorbing the authentic target protein from cell extracts. PIP (2 g) was incubated with 1 mL of cell extract at 4 °C for 15 h under overhead rotation to adsorb the target protein. After adsorption, the PIP was washed five times with 2 mL of buffer (10 mM $NaH_2PO_4/Na_2HPO_4$, pH 7.5) to remove nonspecifically adsorbed proteins, then with 2 mL of saline buffers (10 mM $NaH_2PO_4/Na_2HPO_4$ (pH 7.5) and 100 mM KCl, 300 mM KCl or 500 mM KCl, respectively) to obtain the specifically adsorbed target protein gradually. The eluent was prepared for analysis by SDS–PAGE with silver staining and immuno-staining using anti-pCyP18 antiserum (Fig. 5A). When eluting with high concentration saline buffer including 300 mM KCl, the target protein was largely obtained and adsorbed specifically. As shown in Fig. 5A, lane 4, 100 μL probe of the eluent from the PIP contained 728 ng total protein (determined by the method of Bradford), which includes 120 ng of pCyP18 (determined by Western blot analysis). The proportion of pCyP18 to total protein was 64.7 ng/80.85 μg (~0.08%) in 100 μL of cell extract, whereas the proportion of the target protein from the PIP to total protein in 100 μL of eluent was 120 ng/728 ng (~16.5%). The proportional content of pCyP18 was enriched 200 times. The adsorption character of PIP with and without the template protein was compared. As shown in Fig. 5B, lane 1, 20 μL of cell extract contained 16 μg of total protein with 13 ng of pCyP18. A similar amount of pCyP18 (17 ng) was found in 15 μL of eluent from the PIP containing 110 ng of total protein (lane 2). In 15 μL of elution buffer from the PIP with ARPCs but without the

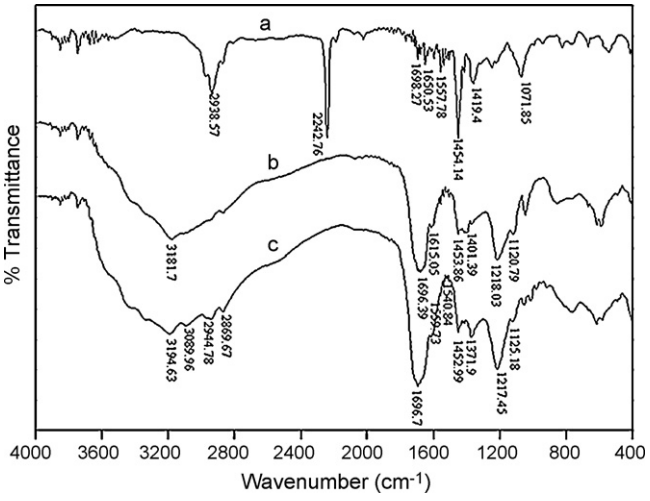


**Fig. 4.** FTIR spectra of PANVP (a), PAAVP (b), and ARPCs (c).

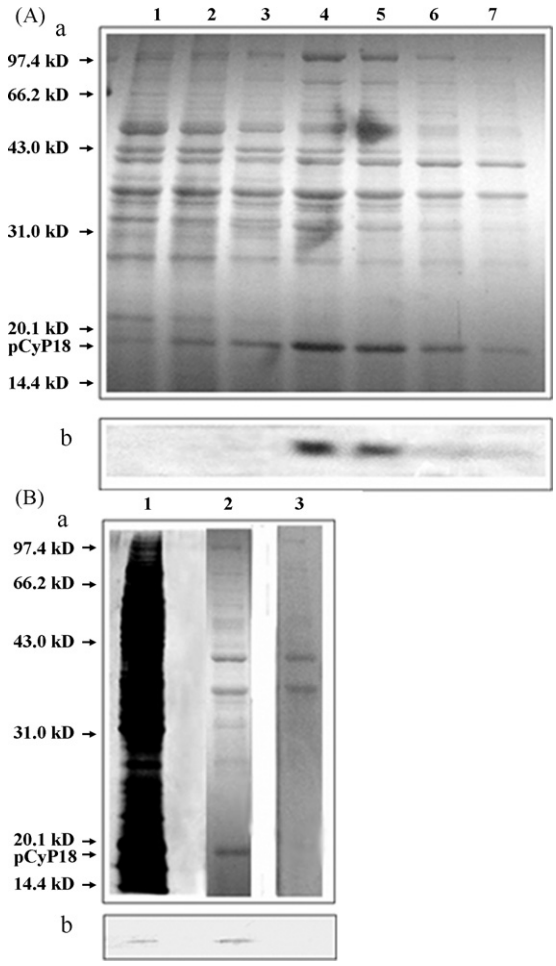


**Fig. 5.** Gel electrophoretic analysis of the adsorbed protein using the PIP: (A) a, Silver staining of the gel slide; b, immune staining of the blotted gel slide using anti-pCyP18 antiserum: lane 1–lane 7, 100 μL of eluent. Concentration of KCl in elution buffer: lane 1–lane 3, 100 mM; lane 4 and lane 5, 300 mM; lane 6 and lane 7, 500 mM; (B) a, silver staining of the gel slide; b, immune staining of the blotted gel slide using anti-pCyP18 antiserum: lane 1, 20 μL of cell extract; lane 2, 15 μL of the elution buffer containing 300 mM KCl from the PIP with ARPCs and the template; lane 3, 15 μL of the elution buffer containing 300 mM KCl from PIP with ARPCs but without the template.

template protein, pCyP18 in elution buffer could not be detected (lane 3). This indicated that the adsorption of pCyP18 by the PIP was not due to the nonspecific ionic effect, but rather to imprinting of the cloned pCyP18. Some proteins other than pCyP18 were also adsorbed by the PIP from the cell extracts. These might be adsorbed nonspecifically onto the surface of the porous polymeric absorbent beads. The PIP was very stable as a chemical material and the adsorption efficiency was repeatable.

### 3.3. Assays of PPIase activity of natural and cloned pCyP18

In eluent the concentration of natural pCyP18 reached to 1.2 μg/mL and its pureness attained 16.5% of total protein. This concentration and purity enabled the assay of PPIase activity. CyP18 belongs to the family of PPIases. The standard chymotrypsin-coupled assay could be used to determine the PPIase activity of pCyP18. Cloned pCyP18 exhibits PPIase activity with a kcat/Km value of $0.85 \times 10^5\ M^{-1}\ s^{-1}$ and natural pCyP18 exhibits PPIase activity with a kcat/Km value of $1.0 \times 10^5\ M^{-1}\ s^{-1}$ (Fig. 6A). The PPIase activity of natural pCyP18 is higher than that of cloned pCyP18, and the ratio of the former to the latter is 1.2. Cyclosporin A (CsA) is an immunosuppressant drug for the prevention of organ rejection after transplant operations. CyP18 has a high binding affinity to CsA and CsA can also inhibit the PPIase activity of CyP18. As shown in Fig. 6B, with the increase in the concentration of CsA, the decrease in PPIase activity of natural pCyP18 is faster than that of cloned pCyP18, and natural pCyP18 is more sensitive than cloned pCyP18 to CsA. So the method using PIP to adsorb and enrich target proteins is highly efficient and applicable.

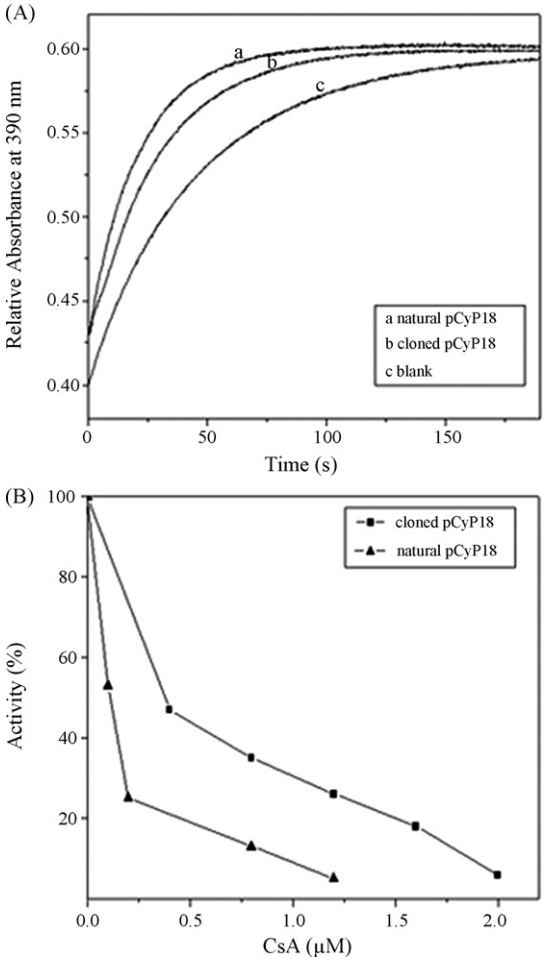


**Fig. 6.** (A) Assays of PPIase activity of pCyP18: a, 40 nM natural pCyP18; b, 40 nM cloned pCyP18; c, blank. Assays were performed in a standard chymotrypsin-coupled assay. (B) Inhibition of the PPIase activity of pCyP18 by CsA. ■ Cloned pCyP18 (40 nM) was incubated with different concentrations (0.4, 0.8, 1.2, 1.6 and 2.0 μM) of CsA, respectively; ▲ natural pCyP18 (40 nM) was incubated with different concentrations (0.1, 0.2, 0.8 and 1.2 μM) of CsA, respectively.

## 4. Conclusion

A new type of PIP was synthesized by selective assembly of the template protein and ARPCs with recognition sites and immobilizing sites, and immobilization of the assemblies onto microspheres by cross-linking polymerization. Pyridyl and carboxyl groups were used as recognition sites. Pyridyl groups involve hydrogen bonding and hydrophobic interactions for protein recognition and carboxyl groups can interact with acidic amino acid with positive charge. The multiple interactions could increase the efficiency of adsorption and enrichment of target protein. Target protein could be adsorbed and enriched from cell extract using the PIP for direct adsorption. The concentration of natural pCyP18 adsorbed in eluent in this work enables the assay of PPIase activity to be performed. The PPIase activity of adsorbed natural pCyP18 is higher than that of cloned pCyP18 and natural pCyP18 is much more sensitive to CsA than cloned pCyP18. Future studies should attempt to synthesize ARPCs with different effective recognition sites, such as positively charged amino groups, to prepare the PIP and sequentially use these PIPs to purify the low content natural proteins.

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