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Molecular imprinted polymer with positively charged assistant recognition polymer chains for adsorption/enrichment of low content target protein

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Here, we introduce a new type of molecular imprinted polymer (MIP) with immobilized assistant recognition polymer chains (ARPCs) to create effective recognition sites and with bacterial cloned protein as template for adsorbing the low content target protein from cell extract. In this work, cloned pig cyclophilin 18 (pCyP18), a peptidyl-prolyl cis/trans-isomerase, was used as template. The template protein was selectively assembled with ARPCs from their library, which consists of numerous limited length polymer chains with randomly distributed recognition sites of positively charged amino groups and immobilizing sites. These assemblies were adsorbed by porous microsphers and immobilized on them. After removing the template, binding sites complementary to the target protein in size, shape and the position of recognition groups were exposed, and their confirmation was preserved by the cross-linked structure. The synthesized MIP was used to adsorb the cellular pCyP18, and its proportional content was enriched more than hundred times. The extended experiment on imprinting bovine serum albumin (BSA) with ARPCs shows that this method is also suitable for large protein.

protein-imprinted polymer (PIP), assistant recognition polymer chains (ARPCs) with positively charged recognition sites, template of cloned bacterial protein, enrichment of low content cellular protein

General methods for the selective recognition/adsorption of specific proteins remain a significant challenge. The molecular-imprinting technique, which creates specific recognition sites using template molecules[1−5], provides the possibility for actively recognizing the target proteins by using proteins as templates[6−9]. Until now, nearly only abundant proteins have been used as templates[10−13] due to a request of large amount of the templates. However, there are thousands of types of proteins within a cell and most of them are present at relatively low level. Indeed, some proteins can exist in just a few copies, even though they perform important biological functions in the cell. For recognition and capture of these low content target proteins from cell extract via molecular imprinting technique, sufficient amount of templates is a critical point. Instead of full protein, the synthetic peptide in exposed domain (epitope) of the target protein was used to imprint the capture sites[14,15] and this method can be used for recognition and capture of the low content proteins. PIP synthesized by using cloned bacterial protein template can successfully adsorb and enrich the low content target authentic protein from cell extract[16]. For capturing the low content target protein from thousands of proteins in cell extract, it is important to enhance the recognition specificity of the PIP. Haupt suggested creating molecular memory using a synthetic polymer by assembling the recognition

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monomers and templates into a complex[17]. Introduction of assistant recognition polymer chains (ARPCs) as extender of monomer enhances the recognition specificity obviously[18]. In our former work, we had employed limited length ARPCs with negatively charged carboxyl as recognition sites to synthesize the PIP[16]. In this work, we developed a simple method to introduce the amino groups in ARPCs as recognition sites. Pig cyclophilin 18 (pCyP18) was used as a template[19,20]. This protein is ellipsoid in shape, with axes lengths of 4.30×5.26×8.92 nm and a molecular mass of 18 kD. It makes up around 0.08% of the total cytosolic protein in pig's liver. A protein of this size and abundance was suitable for our pilot investigation of this novel method. Figure 1 shows the strategy of synthesis of this new type of PIP. The cloned pCyP18 was selectively assembled with ARPCs from their library, which consists of numerous limited length polymer chains with randomly distributed recognition sites and immobilizing sites. The assemblies of proteins and ARPCs were adsorbed by the porous polymeric beads, and immobilized by cross-linking polymerization. After removing the template, binding sites that were complementary to the target protein in size, shape and the position of recognition groups were exposed, and their confirmation was preserved by the cross-linked structure. The synthesized imprinted polymer was used to adsorb authentic pCyP18 from cell extract, and its proportional content was enriched more than one hundred times. As a comprehensive technology, this imprinting method with ARPCs must be applied to proteins with a wide range of molecular weight. Thus, the experiment on imprinting BSA with molecular weight of 66 kD was performed. BSA was enriched from the mixture of lysozyme (14.4 kD), trypsin inhibitor (20.1 kD), bovin carbonic anhydrase (31.0 kD), rabbit actin (43.0 kD), rabbit phosphorylase b (97.4 kD) and BSA.

1 Materials and methods

1.1 Materials and reagents

Vinyl acetate (VAc), 2,2′-azobisisobutyronitrile (AIBN) dimethylsulfoxide (DMSO) and piperidine were purchased from KRS Chemical Reagent Ltd. (Tianjin, China) which were all of analytical grade. *N*,*N*-diisopropylcarbodiimide (DIC), Hydroxybenzotrizole (HOBt) and 9-fluorenylmethoxycarbonyl-protected glycine (Fmoc-Gly) were purchased from BMJ Science & Technology Ltd. (Beijing, China). Polyvinylalcohol macropore micro-spheres (80—100 mesh) were produced by Hecheng Corporation (Tianjin, China). Acry-

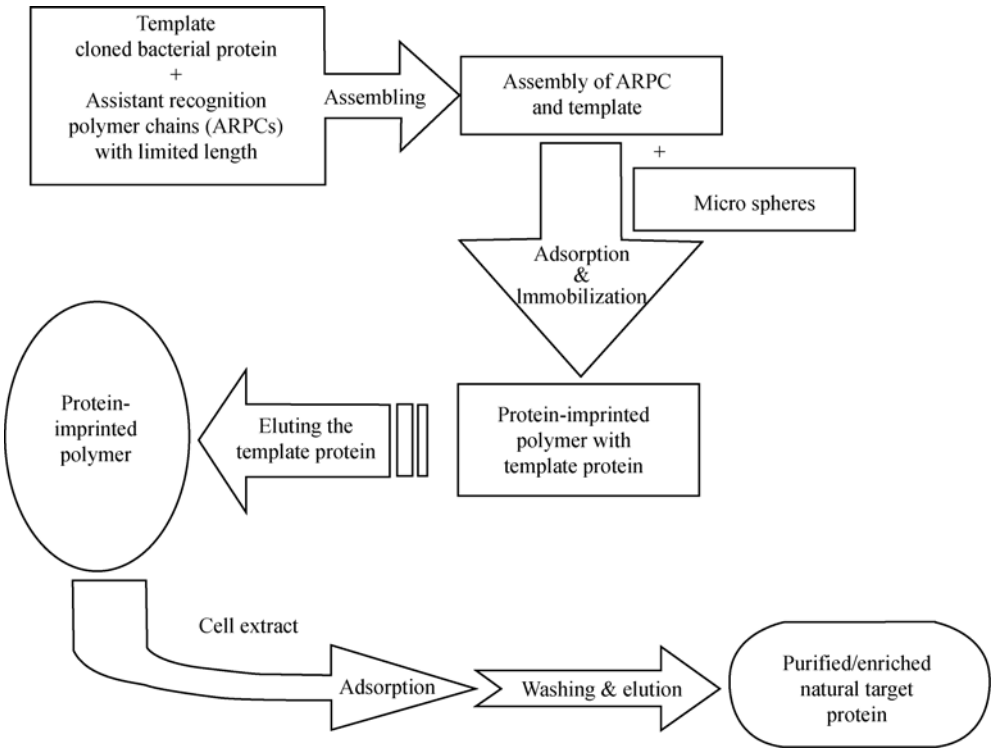

Figure 1 Strategy for synthesis of PIP.

loyl chloride, acrylamide (AM), *N*,*N*′-methylene bisacrylamide (MBA), sodium dodecyl sulfate (SDS) N-[2-hydroxyerhyl]piperazine-N′-ethanesulfonic acid (HEPES) and alkaline phosphatase goat anti-rabbit immunoglobulin (IgG) were produced by Sigma-Aldrich Corporation (USA). Glutathione-sepharose 4B was produced by Amersham Biosciences UK Ltd. (UK). Factor Xa was purchased from Merck Corporation Ltd. (Germany). Nitrocellulose membrane was produced by Millipore Corporation (USA). Coomassie brilliant blue G250, R250 and protein marker were produced by Sino-American Biotechnology Corporation (Beijing, China). Vac was vacuum-distilled before use. AIBN was purified by recrystallization in methanol and water, respectively. DMSO was dried using molecular sieves. All other chemicals and solvents were obtained from commercial sources and used as received.

1.2 Synthesis of ARPCs

The route of synthesis of ARPCs is described in Figure 2. Limited length Polyvinylalcohol (PVA) is the backbone of ARPCs. It was synthesized with vinyl acetate (VAc) as monomer by means of free radical polymerization. As described by Guo et al.[18], the degree of the polymerization was controlled to approximately 45 by regulating the proportion of MeOH to VAc. The polyvinylacetate was hydrolyzed with NaOH in methanolic solution. Then, the amino-groups, which acted as recognition group to target protein, were grafted onto the backbone polymer chain via 9-fluorenylmethoxycarbonyl-protected glycine (Fmoc-Gly)[21]. purified PVA 4 g, Fmoc-Gly 8 g and hydroxybenzotrizole (HOBt) 8 g were dissolved in 50 mL DMSO solvent. *N*,*N*-diisopropylcarbodiimide (DIC) 1 mL was added to the mixture and the reaction was carried on for 16 h at 30℃ under stirring. Afterwards, 4 mL acryloyl chloride and 4 mL pyridine were added to the reaction mixture at 20℃, and the reaction was carried on for 4 h at 40℃ under stirring. In this step, the residual hydroxyl groups on the backbone were partly substituted by acryloyl groups for immobilizing the ARPCs onto the carriers[22]. After precipitation and washing, the brown product named PVA-(Gly-Fmoc&AC) was dried in vacuum at room temperature up to constant weight. The product was added to 20% (*v*/*v*) DMSO solution of piperidine and stirred for 30 min at room temperature for removing Fmoc protection group. After precipitation and washing, the product (ARPCs) was dried in vacuum at room temperature up to constant weight. The ARPCs were soluble in water.

1.3 Surface modification of carrier

Polyvinylalcohol macropore micro-spheres (PVA spheres) were used as carrier. For immobilizing ARPCs, the hydroxyl groups on the spheres were partly substituted by acryloyl groups. swollen PVA spheres 10 g were reacted with 10 mL acryloyl chloride in DMSO solution of pyridine to modify the acryloyl groups. The procedure was the same as grafting acryloyl groups onto ARPCs. This substitution could also improve the hydrophobic property of carriers to benefit the adsorption of complex of template and ARPCs.

1.4 Preparation of the template protein

We used reverse transcription polymerase chain reaction (RT-PCR) to amplify pig Cyclophilin 18 (pCyP18) mRNA for cloning and expressing the template protein

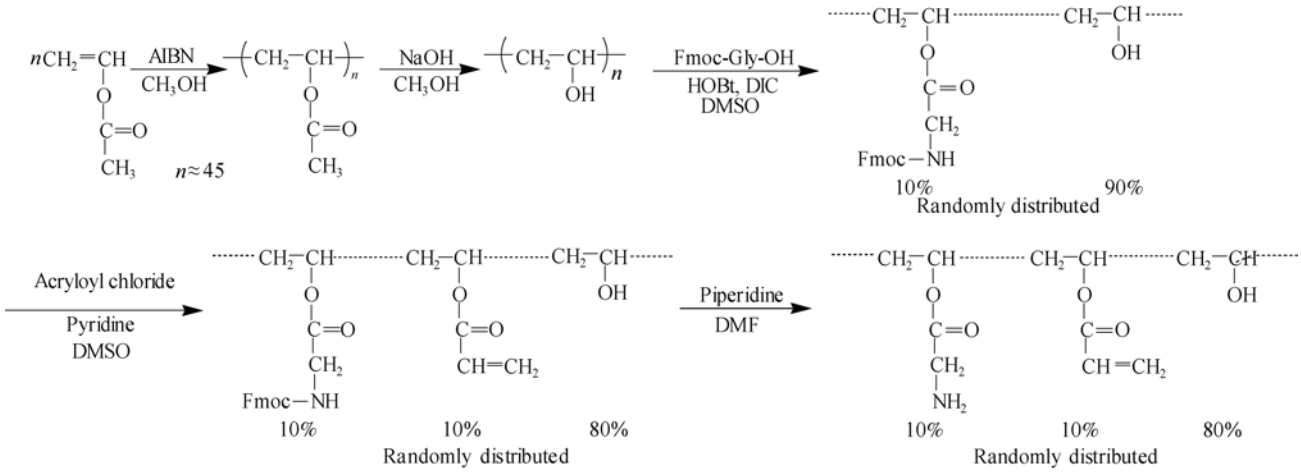

Figure 2 The route of synthesis of ARPCs.

in *Escherichia coli*. The upstream primer (pCyP18f) included an *Eco*R I restriction enzyme site (5′-CCGGCCGAATTCATGGTTAACCCCACCGT-3′), and a downstream primer included an *Xho* I restriction enzyme site (5′-GGCCGGCTCGAGTTAGATTTGTCCACAGTCAG-3′). After being digested by restriction enzymes, the amplified RT-PCR product was ligated into a vector pGEX5X I. The recombinant plasmid DNA was transformed into competent cells of *Escherichia coli* strain DH5α, and the positive clones were amplified. After inducing, purifying, digesting the GST-tag, the template protein was obtained[23].

1.5 Synthesis of the protein-imprinted polymers (PIPs)

PIP I was synthesized by the following routine: 21 mg bacterial cloned pCyP18 and 1.12 g ARPCs (the molar ratio between the template and ARPCs reached to 1:300) were added to 35 mL P-B (phosphate buffer 12 mmol/L Na_2HPO_4, 8 mmol/L NaH_2PO_4) and incubated at 4℃ for 8 h under over-head rotation for selective assembling. Then, 7 g wet modified PVA spheres were added to this solution and incubated at 4℃ for 16 h also under over-head rotation for adsorbing the complex of template protein and ARPCs to the carrier. Then, 1.4 mL water solution of acrylamide (AM) and *N*,*N*′-methylene bisacrylamide (MBA) (29% *w*/*v* AM, 1% *w*/*v* MBA) and 350 μL 10% (*w*/*v*) ammonium persulfate water solution were added and the mixture was rotated for 4 h. The mixture was stirred for 1 h with N_2 ventilation to remove O_2. After addition of 4 μL *N*, *N*, *N*′, *N*′-tetramethylethylendiamine, the reaction was carried out at room temperature for 2 h under stirring and N_2 protection. The template proteins were washed away with P-K_{500} buffer (14 mmol/L Na_2HPO_4, 6 mmol/L NaH_2PO_4, 500 mmol/L KCl) until no pCyP18 could be detected by silver staining[24] in the washing buffer. PIP II was synthesized by the same method to synthesize the PIP I by using BSA as template.

1.6 Characterization

The molecular weight of the limited length PVA synthesized was analyzed using a Waters 410 GPC system. The contents of amino-group and C-C double bonds were determined by element analysis using Vario EL element analyzer. After grafting of side chains onto the backbone, the structure of ARPCs in DMSO solution was analyzed by ^{1}H NMR using a UNITY-plus-400 NMR instrument.

1.7 Preparation of cell extract from pig liver

This was performed as described by Borgeson and Bowman[25], and Chen et al.[26]. All steps of this procedure were performed at 4℃and all buffers and the equipment were precooled at 4℃. 200 g pig liver was suspended in 200 mL 2× Buffer E (2 mol/L sorbitol, 10 mmol/L HEPES, pH 7.4 and 2 mmol/L EDTA) with a homogenator. To break the cells, 1/10 volume of NP-40 (3% solution) was added and shaken on ice for 5 min. The suspension was centrifuged for 20 min at 1000 *g* and the supernatant was collected. The pellet was suspended with 10 mL buffer E and centrifuged for 20 min at 1000 *g* once more. The two supernatants were collected together and then centrifuged sequentially at 12000 and 40000 *g* for 1 h. The supernatant, comprising cell extract (cytosol), was subjected to Western blot analysis[27]. The staining strength with anti-pCyP18 antiserum was determined through scanning in comparison with the standard of bacterial cloned pCyP18, and the relevant amount of pCyP18 was calculated.

1.8 Adsorption of target of protein from cytosol using PIP I

The cytosol was adsorbed by 2 g wet PIP I at 4℃ for 1 h under over-head rotation in P-B (pH 7.0). Then, the spheres were washed by P-B, P-K_{100} (14 mmol/L Na_2HPO_4, 6 mmol/L NaH_2PO_4, 100 mmol/L KCl), P-K_{150} (14 mmol/L Na_2HPO_4, 6 mmol/L NaH_2PO_4, 150 mmol/L KCl) and P-K_{200} (14 mmol/L Na_2HPO_4, 6 mmol/L NaH_2PO_4, 200 mmol/L KCl) to remove non-specific adsorbates. The target proteins were obtained by eluting the PIP with 2 mL P-K_{300} (14 mmol/L Na_2HPO_4, 6 mmol/L NaH_2PO_4, 300 mmol/L KCl), and 2 mL P-K_{500} (14 mmol/L Na_2HPO_4, 6 mmol/L NaH_2PO_4, 500 mmol/L KCl). Before use, the PIP was tested with a blank control to ensure that no template remained under the same elution conditions. The eluent was analysed by SDS-PAGE with silver staining and immuno-staining using anti-pCyP18 antiserum. The total protein was determined by the Bradford method[28].

1.9 Isolation of the target protein by chromatographic method using PIP II

PIP II 7 g was filled into a chromatography column (100 mm×9.0 mm). 500 μL water solution of protein marker (120 mg) was supplied to the column. HPLC analysis was performed. Samples were eluted with gradient of P-B and P-K_{500} buffer (0—10 mL, 100% P-B; 10—20

mL, P-K_{500} increased from 0 to 100%; 20－24 mL, 100% P-K_{500}). The eluent was collected in 2 mL fractions. 100 μL aliquots from each fraction were subjected to SDS-PAGE analysis with silver-staining.

2 Results and discussion

2.1 Characterization of the ARPCs.

The molecular weight of the synthesized PVA was analyzed with GPC. As shown in Figure 3, the molecular weight of the ARPC (M_p) is 1999 and M_w/M_n=1.43.

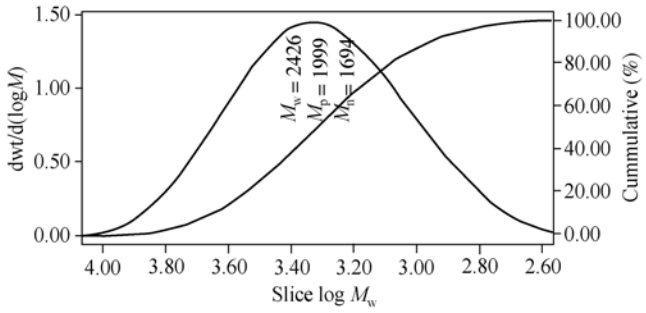

Figure 3 GPC analysis of the synthesized PVA.

After grafting of side chains with acryloyl and glycine groups onto the backbone, the structure of the polymer changed, so did the ^{1}H NMR spectra. Comparison of the ^{1}H NMR spectra for ARPC and PVA shows a peak at 2.12 ppm, attributed to hydrogen in methylene adjoining the amino group in grafting side chain, and reducing the triplet peaks from 4.23 to 4.67 ppm which are attributed to hydrogen in hydroxyl group on backbone (Figure 4). Thus, it can be proved that the grafting is successful and hydroxyl groups decrease. By calculating, we can know that 6.5% side chains were substituted by glycine groups.

This proportion can also be calculated by element analysis which revealed that 7.1% side chains were substituted by glycine groups and 9.1% side chains were substituted by acryloyl groups.

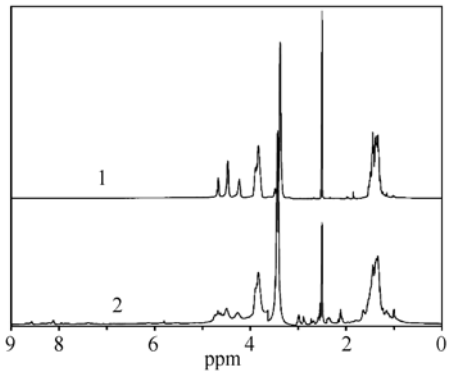

Figure 4 ^{1}H NMR spectrum of ARPC (2) compared with PVA (1).

2.2 Influencing factors of target protein adsorption

The pH value plays an important role in recognition of positively charged amino groups and the negatively charged site chains of acidic amino acid residues in target protein. The experiments of adsorption of target protein from cytosol were performed in different pH buffers, respectively. As shown in Figure 5, pH value of adsorption buffer could influence adsorption of target protein by PIP greatly[29]. When pH value of adsorption buffer was 7.0, the content of pCyP18 in eluent was the highest.

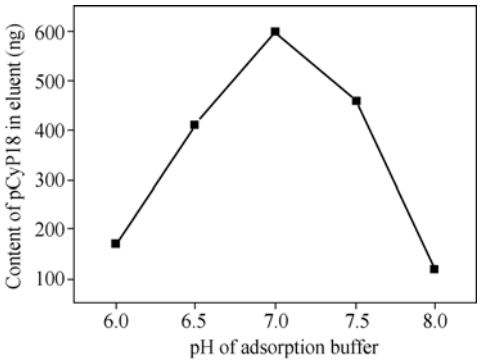

Figure 5 Influence of pH value in adsorption buffer on the adsorption amount of target protein by using PIP with ARPCs.

The molar ratio between ARPCs and template could also affect the adsorbing ability of PIP to target protein. Figure 6 shows that the content of pCyP18 in eluent increased following the increasing of the molar ratio between ARPCs and template up to 300, and higher ratio could not induce obvious increasing of content of pCyP18 in eluent.

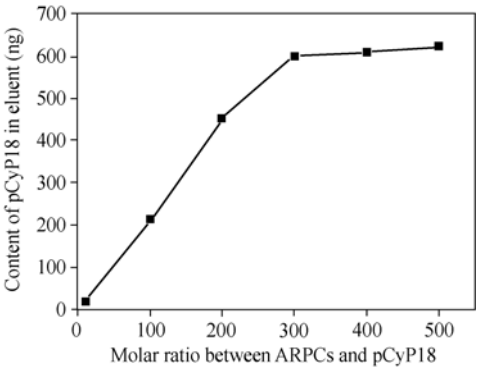

Figure 6 Influence of the molar ratio between ARPCs and template on the adsorption amount of target protein by using PIP with ARPCs.

2.3 Adsorption of target protein from cytosol using PIP I

PIPI was used for direct adsorption of target protein

from cell extract. Every eluent was analyzed by SDS-PAGE with silver staining (Figure 7(a)) and immuno-staining with anti-pCyP18 antiserum (Figure 7(b)). The results showed that the proteins eluted in P-B and in low-salt concentration (100 mmol/L KCl and 150 mmol/L KCl) buffer were nonspecific adsorbents (lanes 1—7), and the protein eluted in high-salt concentration (200 mmol/L, 300 mmol/L and 500 mmol/L KCl) buffer was more specific (lanes 8—10).

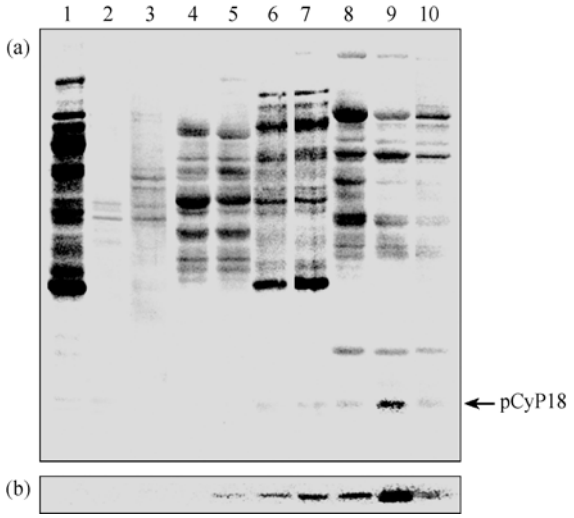

Figure 7 Gel electrophoresis analysis of adsorbed target protein. (a) Silver staining of the gel slide; (b) immune staining of the blotted gel slide using anti-pCyP18 antiserum: Lanes 1—10, 100 μL eluent. The concentration of KCl in eluting buffer: lanes 1 and 2, 0 mmol/L; lanes 3—5, 100 mmol/L; lanes 6 and 7, 150 mmol/L; lane 8, 200 mmol/L; lane 9, 300 mmol/L; lane 10, 500 mmol/L.

As shown in Figure 7, most of the target proteins were in eluent 9 (300 mmol/L KCl), but most of non-target proteins were not in this elution. Thus, the proportion of pCyP18 to total protein reached to 600 ng/7.1 μg (8.5%), whereas in 800 μL cytosol, this figure was 51 μg/64 mg (0.08%). Thus, the proportional content of pCyP18 was enriched 107 times.

As shown in lane 1 in Figure 8, 100 μL probe of the eluent from the PIP contained 355 ng total protein, which included 30 ng pCyP18. The same amount of pCyP18 was found in 0.45 μL cytosol containing 36 μg total proteins (lane 2).

The control-PIP Ⅰ was synthesized by the same method to sythesize PIP I but without ARPCs. No pCyP18 can be detected in the eluent by using this control-PIP I after adsorption of 800 μL cytosol, which shows that the ARPCs play a very important role in specific recognition.

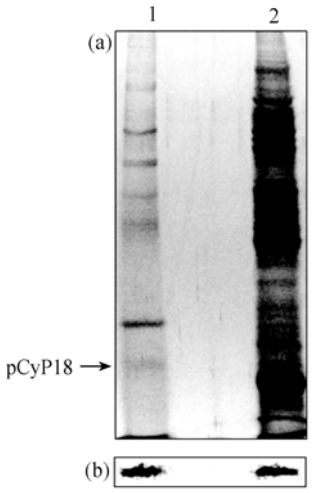

Figure 8 Gel electrophoresis analysis of the eluent from the PIP (fraction 9) and the control. (a) Silver staining of the gel slide; (b) immune staining of the blotted gel slide using anti-pCyP18 antiserum. Lane 1, 800 μL cytosol adsorbed using 2 g wet PIP I. After washing with low concentration saline buffer to remove the non-specific adsorbate, the spheres were eluted with 2 mL high-concentration saline (300 mmol/L KCl) buffer. Elution buffer (100 μL) was the prepared as a probe for the gel-electrophoretic analysis. Lane 2, 0.3 μL cytosol.

2.4 Chromatographic isolation of target protein from protein marker using PIP II

The PIP can be also used for chromatographic isolation of the target protein of high molecular weight. As shown in Figure 9, BSA almost was purified in eluent 12, but its proportional content just was less than 15% in protein marker which was the mixture of 6 proteins.

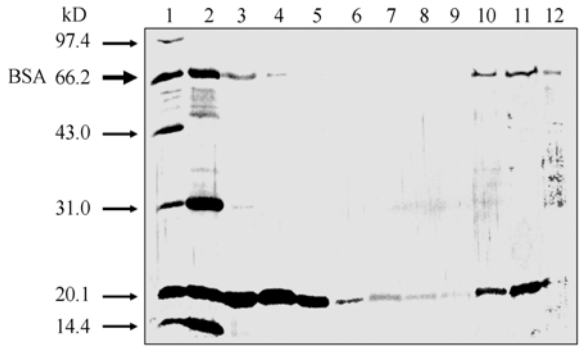

Figure 9 Chromatography for PIP II used as the stationary phase for purification of BSA from the 6-proteins mixture: 700 μL cytosol was subjected to chromatography on a column (10 cm×0.9 cm) of compacted beads of the protein-imprinted polymer with ARPCs. The probe was eluted with P-B (12 mmol/L Na_2HPO_4, 8 mmol/L NaH_2PO_4) with the gradient P-K_{500} buffer (14 mmol/L Na_2HPO_4, 6 mmol/L NaH_2PO_4, 500 mmol/L KCl); the eluate was collected as 2-mL fractions and 100 μL of each fraction was subjected to SDS-PAGE analysis with detection by silver staining.

3 Conclusion

In summary, an improving PIP was synthesized by se-

lective assembly of a cloned bacterial protein with ARPCs from a library, adsorption of the assembled complexes by porous polymeric beads, and immobilizing them onto porous polymeric beads to form a complementary structure to the templates. PVA was used as backbone and modified PVA macropore micro-spheres were used as carrier considering their excellent biocompatibility. A simple method for synthesizing ARPCs with positively charged amino group as recognition group was developed. As an extender of functional monomer, the ARPCs were introduced for synthesizing the PIP. It enhanced the recognition efficiency obviously. This PIP can efficiently recognize, adsorb and enrich authentic target protein with low content from cell extract. The cellular protein was enriched several hundred times up to about eight percent of total protein by using this new type of PIP with ARPCs. This method also can be used for enriching or purifying the protein of high molecular weight. Most non specific adsorbed proteins with this type of PIP are probably the negatively charged acidic proteins. Whereas by using PIP with negative charged acidic recognition sites, one can find that most non specific adsorbed proteins might be basic proteins. Perhaps, a combination of these two types of PIPs can reach the aim of purifying the low content natural proteins.

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