Molecular imprinted polymer with cloned bacterial protein template enriches authentic target in cell extract

Zhuo Zhao\textsuperscript{a,1}, Chunhong Wang\textsuperscript{a,1}, Minjie Guo\textsuperscript{a}, Linqi Shi\textsuperscript{a}, Yunge Fan\textsuperscript{a}, Yi Long\textsuperscript{b}, Huai'feng Mi\textsuperscript{a,b,}\textsuperscript{*}

\textsuperscript{a} Biochemical Section of Key Laboratory of Functional Polymer Materials, The Ministry of Education of China, Institute of Polymer Chemistry, Chemical School of Nankai University, 300071 Tianjin, PR China
\textsuperscript{b} Medical School of Nankai University, 300071 Tianjin, PR China

Received 31 January 2006; revised 20 February 2006; accepted 13 April 2006
Available online 24 April 2006

Edited by Felix Wieland

Abstract

Here we describe a new method for preparing a protein-imprinted polymer with a cloned bacterial protein template, which recognizes/adsorbs authentic target protein present at a relatively low level in cell extract. In this work, cloned pig cyclophilin 18 (pCyP18) was used as a template. The template protein was selectively adsorbed with memory molecules from their library, which consists of numerous limited length polymer chains with randomly distributed recognition sites and immobilizing sites. These assemblies of protein and memory molecules were adsorbed by 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 300 times.

© 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Protein-imprinted polymer; Memory polymer chain; Cloned bacterial protein as template; Enrichment of authentic aim protein

1. Introduction

The molecular-imprinting technique creates specific recognition sites 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]. However, there are thousands of types of protein within a cell (the more highly developed the organism, the more protein types it contains) and most are present at relatively low levels. Indeed, some proteins can exist in just a few copies, even though they perform important biological functions within a cell. Molecular-cloning techniques allow scientists to study these low abundance proteins by producing them in large quantities from cloned genes. However, cloned proteins are not identical to natural ones in vivo. Therefore, researchers are interested in purifying natural proteins from cell extracts. 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. Therefore, a method with high enrichment efficiency is needed. In the current work, we introduce a new method that can enrich authentic target protein from cell extracts using a protein imprinted polymer prepared with a cloned bacterial protein template in a water system.

Pig cyclophilin 18 (pCyP18) was used as a template [13,14]. This protein is ellipsoid in shape, with axes lengths of $4.30 \times 5.26 \times 8.92$ nm and a molecular mass of 18 kDa. It makes up around 0.02–0.03% of the total cytosolic protein. A protein of this size and abundance was suitable for our pilot investigation of this novel method. The cloned pCyP18 was selectively assembled with memory molecules from their library, which consists of numerous limited length polymer chains with randomly distributed recognition sites and immobilizing sites. The assemblies of proteins and memory molecules 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 extracts, and its proportional content was enriched 300 times.

The conventional protein-imprinting method involves polymerization of a functional monomer in the presence of protein template and protein-imprinted polymer (PIP) film on supporting media, such as silica [15,16], chitosan beads [11], hydrogels [17], mica [12], membranes [18,19] or microspheres [5,20,21]. In this case, the recognition between the imprinted polymer and the protein occurred on the imprinted nanostructured surfaces and the homo-recognition sites of the monomer. Haupt suggested creating molecular memory using a synthetic polymer by assembling the recognition monomers and template into a complex [22]. To achieve our aim of recognizing/adsorbing authentic target protein from cell extracts using an imprinted polymer and a cloned bacterial protein template, two aspects need to be considered. Firstly, precise recognition is more important than the amount adsorbed for the imprinted polymer. For typical biochemical research purposes, the requirement for purified protein is only in the nanogram to microgram range, but the protein must be specifically adsorbed despite its low content among the thousands of types of pro-

\textsuperscript{1} These authors contributed equally to this work.

\textsuperscript{*} Corresponding author. Fax: +86 22 23502749.
E-mail address: hfmi@nankai.edu.cn (H. Mi).
tein within the cell extract. Secondly, the amount of cloned protein used for the template is relatively small. Using the conventional method, the amount of template protein required is in the gram range, but the general harvest of cloned protein is in the milligram range using the molecular-cloning technique. In view of these issues, we employed limited length memory polymer chains with randomly distributed recognition side chains (acid side chains in this case) to synthesize the PIP, in order to create effective recognition sites from a relatively small amount of template.

2. Materials and methods

2.1. Materials and reagents

Glutathione-sepharose 4B, Factor Xa and Hybond-C nitrocellulose membrane were produced by Amersham Biosciences UK Limited (UK) and Coomassie brilliant blue G250 was purchased from Sino-American Biotechnology Corporation (Beijing, China). Bovine serum albumin (BSA-V) was produced by Worthington Biochemical Corporation (USA). Ficol was purchased from Unitedstars Biotechnology Co. Ltd. (Tianjin, China). Acrylamide (AM), N,N'-methylene bis-acrylamide, sodium dodecyl sulfate (SDS) and alkaline phosphatase-labeled goat anti-rabbit immunoglobulin (Ig)G (1:2000 diluted in Tris-buffered saline (TBS)) were produced by Sigma-Aldrich Corporation (USA). Butyl acrylate and allyl chloride were purchased from KRS Chemical Reagent Ltd. (Tianjin, China). Methacrylate (MA) and divinylbenzene (DVB) were purchased from the Research Institute of Chemical Reagents (Tianjin, China). All the above-mentioned reagents were analytical grade. All other chemicals and solvents were obtained from commercial sources and used as received.

2.2. Synthesis of memory polymer chain

The backbone of the memory polymer chain, comprising limited length polyacrylic acids, was synthesized with a butyl acrylate monomer by means of atom-transfer radical polymerization (ATRP) [23] to form poly-butyl acrylate, and was subsequently hydrolyzed. The degree of polymerization was about 28. Thus, the average extended length of the polymer chains was about 7 nm (under alkaline conditions, the length of the C–C bond was 1.54 Å) and the angle was 109.5°. This length is related to the size of the protein; it should be long enough to contain sufficient recognition sites, but not so long as to allow the polymer chains to wind around the template proteins and hinder their removal. To partly block the acid sites on the main chains and immobilize the memory polymer chains, the allyl groups were partly (~80%) linked to the carboxylic groups by a reaction with allyl chloride, and were randomly distributed on the main chains. As a result, five or six (~20%) randomly distributed carboxylic groups re-appeared on the backbone of each memory polymer chain (Fig. 1) as recognition sites. These memory polymer chains contained a wide distribution of recognition sites (see Supplementary Data On Line).

2.3. Synthesis of porous polymeric adsorbent beads

Porous adsorbent beads polymerized with MA and DVB were prepared as described by M-C Xu [24]. The swollen porous polymeric beads were reacted with an excess of allyl chloride to modify the groups with double bonds.

\[ \text{COOH} \rightarrow \text{COO}^- + \text{Bu}^- \]
\[ \text{H}_2\text{C} = \text{CH} \rightarrow \text{H}_2\text{C} - \text{CH}_2 \text{Bu}^- \]

ATRP

[Formyl]

\[ \text{COOH} \rightarrow \text{COO}^- + \text{Bu}^- \]
\[ \text{H}_2\text{C} = \text{CH} \rightarrow \text{H}_2\text{C} - \text{CH}_2 \text{Bu}^- \]

Fig. 1. Synthetic route of memory molecules.

2.4. Construction of plasmids expressing pCyP18

To clone the template protein, pig mRNA was amplified by reverse transcription polymerase chain reaction (PCR) using an upstream primer (pCyP18f) including an EcoRI site (5'-CCC GCCG CCGA ATCCA GGC TTA CCGCT-3'), and a downstream primer (pCyP18r) including an XhoI site (5'-GGGCC GCGC GATC TTA CGA C-3'). The amplified PCR product was digested with EcoRI and XhoI restriction enzymes, and ligated into a bacterial expression vector pGEX5X1 (see Supplementary Data On Line).

The recombinant plasmid DNAs were transformed into competent cells of *Escherichia coli* strain DH5α, and the positive clones were amplified. The purified recombinant plasmids DNAs were identified with sequencing.

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 as described previously [25]. The purified fusion proteins were restriction digested on beads with factor Xa in a digestion buffer (50 mM Tris–HCl (pH 8.0), 1 mM CaCl₂ and 100 mM NaCl) overnight at 25 °C (see Supplementary Data On Line).

2.6. Preparation of cell extract from pig leukocytes

This was performed according to the method described by Borge son and Bowman [26]. All steps in the preparation were performed at 4 °C, and all buffers and equipment were pre-cooled at 4 °C. The leukocytes were separated from the pig blood with Ficol. The pig leukocytes were suspended in buffer E (1 M sorbit, 10 mM HEPES (pH 7.4), 1 mM ethylenediamine tetraacetic acid (EDTA) and 3% NP-40) and shaken for 5 min to break up the cells. 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 re-centrifuged for 20 min at 1000 × g. The two supernatants were collected together, and then centrifuged sequentially at 12000 × g and 40000 × g for 1 h, respectively. The supernatant, comprising cell extract, was subjected to western blot analysis with a series of concentrations of cloned pCyP18. The staining strength with anti-pCyP18 antiseraum was determined through scanning, and the relevant amount of pCyP18 was calculated (see Supplementary Data On Line).

2.7. Synthesis of the PIP

Our strategy for the synthesis of the PIP is illustrated in Fig. 2. Firstly, 400 μg bacterial cloned pCyP18 was incubated with 10 mg memory polymer chains at 4 °C for 8 h for selective adsorbing. The adsorbed complexes of memory molecules and template protein were dissolved in 5 ml buffer (10 mM Tris-Cl, pH 7.5). The molar ratio between the memory molecules and the cloned pCyP18 was 200, in order to ensure that every template molecule could be adsorbed with memory polymer chains with a suitable distribution of recognition sites. Secondly, 1.0 g wet porous spheres were added to this solution and incubated at 4 °C overnight under over-head rotation for adsorbing the complexes to the porous spheres with a pore diameter of about 20 nm. Thirdly, 38.8 mg acryl amide and 1.2 mg N,N’-meth ylene bisacrylamide was added and the mixture was stirred for 2 h with ventilating N₂ for removing O₂. After addition of 1.28 mg ammonium persulfate and 0.65 mg N,N,N’,N’-tetramethylethylenedi amine the reaction would carry out at room temperature for 2 h under stirring and in a nitrogen atmosphere. The poly-acrylamide played three roles here: firstly, as a bridge between the double bonds of the memory polymer chains and the surface of the porous beads; secondly, retaining the form of the protein templates, just as in the conventional-imprinting method; and thirdly, occupying the space surrounding the excess memory molecules to avoid non-specific recognition. The template proteins were removed by elution with high concentration saline buffer (2 M KCl and 10 mM Tris–HCl (pH 7.5)) until no pCyP18 could be detected by SDS–poly-acrylamide gel electrophoresis (PAGE) with silver staining and immuno-staining using anti-pCyP18 antiseraum in the wash buffer. The synthesized PIP could then be used for adsorbing the authentic target protein from cell extracts.
2.8. Western blot analysis

The samples were analyzed by SDS–PAGE and transferred onto a Hybond-C nitrocellulose membrane at 0.8 mA/cm² for 2 h. The membrane was blocked with 5% nonfat milk powder in TBS (50 mM Tris–HCl (pH 7.5) and 150 mM NaCl) for 1 h and incubated with rabbit anti-mFKBP23 or anti-mBiP antiserum (1:500 diluted in TBS) overnight. The blots were washed with TBS/Tween (0.1%) and then developed with alkaline phosphatase-labeled goat anti-rabbit IgG. After washing with TBS/Tween, the blots were developed by the nitro blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indoly-phosphate, disodium salt (BCIP) system in alkaline phosphatase buffer [27] (100 mM Tris–HCl (pH 9.5), 100 mM NaCl and 5 mM MgCl₂).

3. Results

3.1. Adsorption of target protein from cell extract

The leukocyte cell extract was adsorbed by the PIP with a 1:1 (ml:gram) ratio of cell extract to wet PIP beads at 4 °C for 15 h under overhead rotation. After adsorption, the PIP was washed with low concentration saline buffer (10 mM Tris–HCl (pH 7.5)) to remove any nonspecific adsorbants. The target proteins were then eluted with an equal volume of high concentration saline buffer (300 mM KCl and 10 mM Tris–HCl (pH 7.5)) to that of the adsorbed cell extract. Before use, the PIP was tested with a blank control to ensure that no template remained under the same elution conditions. Aliquots (200 µl) of adsorbed eluent were prepared for analysis by SDS–PAGE with silver staining and immuno-staining using anti-pCyP18 antiserum (Fig. 3). As shown in Fig. 3 lane 3, a 200-µl probe of the eluent from the PIP contained 0.1 µg total protein (determined by the Bradford method), which included 7 ng pCyP18 (determined as described in Section 2). A similar amount of pCyP18 (6 ng) was found in 20 µl cell extract containing 26 µg total protein (lane 2). By contrast, in 0.08 µl cell extract containing 0.1 µg total protein, which was a similar amount to that in 200 µl eluted probe, pCyP18 could not be detected (lane 1). In 1 ml cell extract, the proportion of pCyP18 to total protein was 300 ng/1.3 ng (ca. 0.023%), whereas in 1 ml eluent of the adsorbate from the PIP this figure was 35 ng/0.5 µg (~7%). Thus, the proportional content of pCyP18 was enriched 300 times.

Some proteins other than pCyP18 were also adsorbed by the PIP from the cell extract (Fig. 2A, lane 3). These might have been basic proteins that were adsorbed nonspecifically onto the surface of the PIP through an anion effect of the negatively charged recognition sites. When porous polymeric beads were synthesized with the PIP and memory molecules but without cloned pCyP18 for imprinting, a gel electrophoretic diagram

![Fig. 2. Strategy for synthesis of the PIP.](image-url)

![Fig. 3. Gel-electrophoretic analysis of adsorbed target protein: (A) silver staining of the gel slide; (B) immune staining of the blotted gel slide using anti-pCyP18 antiserum: Lane 1, 0.08 µl cell extract; lane 2, 20 µl cell extract; lane 3, 1 ml cell extract adsorbed using 1 g wet PIP with memory molecules. After washing with low concentration saline buffer to remove the non-specific adsorbate, the porous polymeric beads were eluted with 1 ml high-concentration saline buffer. Elution buffer (200 µl) was prepared as a probe for the gel electrophoretic analysis. In lane 4, the probe was prepared as described for lane 3 using porous polymeric beads synthesized with the PIP and memory molecules but without the cloned pCyP18 template. In lane 5, the probe was prepared as described for lane 3 using the PIP synthesized by imprinting the cloned pCyP18 but without memory molecules. The numbers below the figure show the amount of protein corresponding to each lane.](image-url)
ory of the specific formation of antibodies, whereas the con-
of imprinted polymers corresponds to the clonal selection the-}

This new type of PIP – synthesized by selective assembly of a cloned bacterial protein using memory polymer chains from a library, adsorption of the assembled complexes by porous polymeric beads, and immobilization of memory polymer chains onto porous polymeric beads to form a complementary structure to the templates – effectively recognized, adsorbed and enriched authentic target protein from cell extract. Our novel method allowed, for the first time, the possibility of adsorbing/enriching an authentic protein with low content from cell extract using a PIP. This technique for the synthesis of imprinted polymers corresponds to the clonal selection theory of the specific formation of antibodies, whereas the conven-tional method for the synthesis of the PIP, which is directed by the template to form a complementary microstructure, is more alike to the instructive story of forming the anti-

Future studies should attempt to synthesize memory poly-
mer chains with positive or neutral recognition sites, and iden-
tify the smallest operable size of the porous polymeric beads with an optimal pore size, in order to further enhance the spec-

Fig. 4. Adsorption of pCyP18 from cell extract using the PIP with memory molecules in triplicate. The probes were analyzed with silver staining of the gel slide (A) and immune blotting with anti-pCyP18 antiserum (B).

Appendix A. Supplementary data

References


Acknowledgements: This work is supported by the National Natural Science Foundation of China (project approval No. 30170870). We thank the Alexander von Humboldt Foundation and World University Service in Wiesbaden (Germany) for their support of laboratorial instrument, which are very useful for this work.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.04.037.


