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Using Protein-imprinted Polymers as Artificial Antibodies to Isolate Immunoglobulin Binding Protein (BiP) and Study Protein–Protein Interactions

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In this work, protein-imprinted polymers (PIPs) synthesized by introducing assistant recognition polymer chains (ARPCs) could be used as artificial antibodies in a similar manner to study protein–protein interactions by immunoprecipitation by using biological antibodies. Bacterially expressed BiP was used as template. The ARPCs could enhance the recognition specificity of the PIPs for template protein significantly. It was confirmed that FK506-binding protein (FKBP23) could bind to immunoglobulin-binding protein (BiP) in the ER regulated by concentration of Ca^{2+} using artificial antibodies of PIP. The concentration of Ca^{2+} at which the interactions between BiP and FKBP23 were disrupted was in accordance with results obtained by using biological antibodies. Therefore, this new type of PIP provides a feasible alternative to biological antibodies in biochemical research.

Molecular imprinting[1–3] is a technique that can be used to create template-shaped cavities, specific recognition sites, or catalytic sites in polymer matrices with memory of the template molecules. Molecular imprinted polymers[4] (MIPs) could be used as tailor-made separation materials,[5] antibody and receptor binding site mimics,[6,7] enzyme mimics, and recognition elements.[8] However, there have been few reports of the substitution of biological antibodies by protein-imprinted polymers (PIPs) with respect to protein isolation and the study of protein–protein interactions.

The isolation[9] of proteins is crucial in biochemistry to allow the activity and structure of a particular protein to be studied further. In general, immunoprecipitation[10] can be used to detect trace amounts of protein. Protein–protein interactions[11] are of central importance for virtually all processes that occur in a living cell, which are often identified and characterized by co-immunoprecipitation.[12] To perform immunoprecipitation, biological antibodies should be prepared by injecting antigen into mammals and protein A-Sepharose should also be applied. To overcome these shortages, PIPs were used as artificial antibodies taking the place of biological antibodies without using protein A-Sepharose as shown in Figure 1.

Haupt[13,14] reported creating molecular memory using a synthetic polymer by assembling recognition monomers and template into a complex, and we introduced assistant recognition polymer chains[15–18] (ARPCs) as extenders of monomers to enhance the recognition specificity. The amount of template protein required reduced markedly from gram range in conventional molecular imprinting to milligram range currently. In our recent studies on MIPs,[15,16] we successfully isolated the low-abundance protein cyclophilin 18 (CyP 18),[17,18] which has a low molecular weight of 18 kDa, from cytosol of pig. In this study, we attempted to isolate a low-abundance protein with a higher

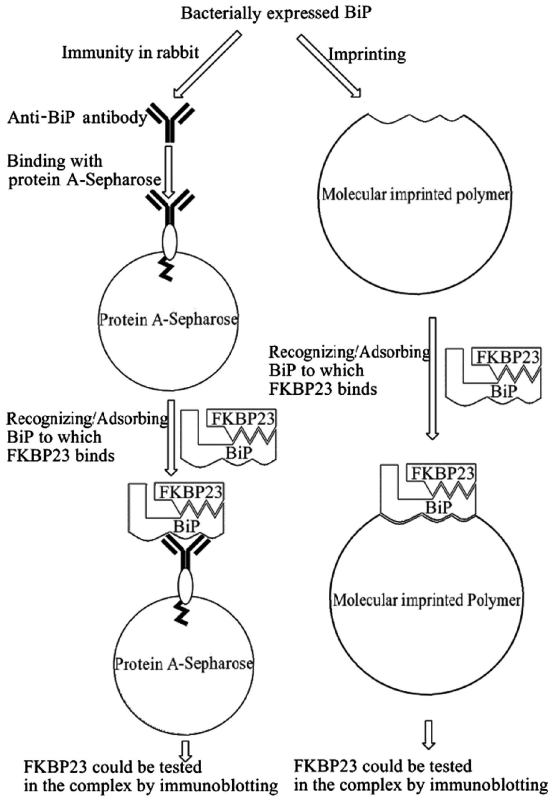
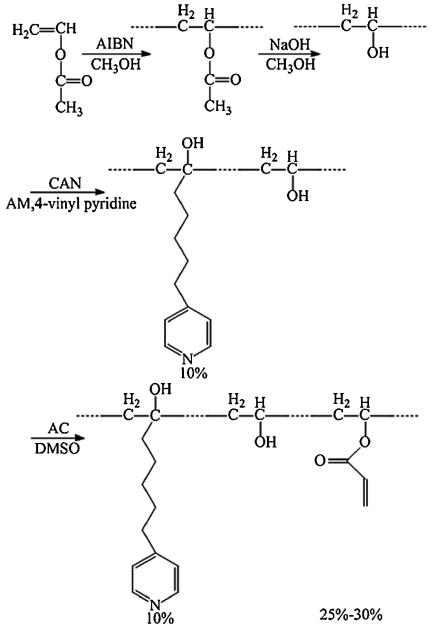


Figure 1. Comparison of the isolation of BiP and investigation of the interactions between BiP and FKBP23 by immunoprecipitation (left) and by using PIP (right).

molecular weight, immunoglobulin binding protein (BiP),[19–21] which has a molecular weight of 78 kDa in endoplasmic reticulum (ER) by using PIP. The BiP was selectively assembled with ARPCs (Scheme 1) with randomly distributed recognition sites and immobilizing sites. The assemblies of BiP 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 BiP in size, shape, and the position of recognition groups were exposed, and their confirmation was preserved by the cross-linked structure (Figure 2). In addition, we were able to use PIP rather than biological antibody to detect an interaction between BiP and FK506 binding protein (FKBP23), which we had previously shown[22] to bind BiP in a [Ca^{2+}]-regulated manner by immunoprecipitation.

Initially, bacterially expressed BiP was prepared with molecular cloning methods. Plasmids to express mBiP were constructed[16,22] using reverse transcription-polymerase chain



Scheme 1. Strategy for the synthesis of the ARPCs.

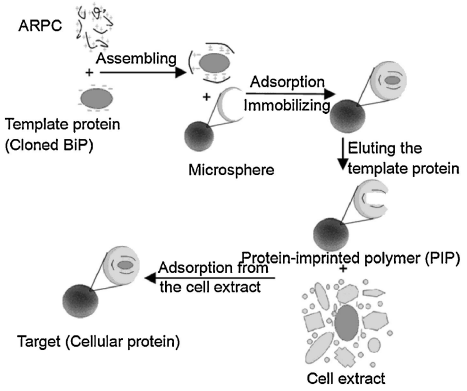


Figure 2. Strategy for the synthesis of the PIP.

reaction (RT-PCR), and the recombinant plasmid DNAs were transformed into competent cells of *Escherichia coli* strain DH5α.[26] Glutathione S-transferase (GST) fusion proteins and cloned proteins with no GST tag were purified[22] and identified with SDS-PAGE.[26] Bacterially expressed FKBP23 was also prepared by the same method above.

Next, the use of immunoprecipitation and that of PIP to isolate BiP and to study the interactions between BiP and FKBP23 were compared.

To perform the immunoprecipitation assay, polyclonal anti-FKBP23 and anti-BiP antisera were prepared primarily by immunizing rabbits with purified recombinant FKBP23 and BiP, respectively.[26] ER extracts were obtained from mouse liver as described by Borgeson and Bowman.[23,26] Then FKBP23 that was bound to BiP was co-immunoprecipitated by incubating 500 µL of ER extract with 40 µL of anti-BiP antibody followed by protein A-Sepharose overnight. The beads were washed five times with 500 µL of washing buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40 and 10% glycerol) to remove non-

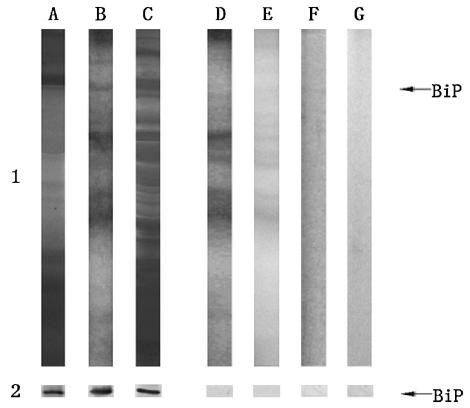


Figure 3. Gel-electrophoretic analysis of proteins adsorbed onto the PIP. 1) Silver staining of the gel slide and 2) Immunoblotting of the gel slide using anti-BiP antiserum. A) Protein A-Sepharose 4B binding with anti-BiP antibodies which adsorbed BiP from ER extract. B) 200 µL of PBP_{500}-2 elution from the ER extract incubated with PIP synthesized in the presence of both ARPCs and the BiP template. C) 15 µL of ER extract. D) 200 µL of PBP_{500}-2 elution from the ER extract incubated with PIP synthesized in the presence of ARPCs but no template protein as a control. E) 200 µL of PBP_{500}-2 elution from the ER extract incubated with PIP synthesized in the presence of ARPCs and other template protein (CyP 18). F) 200 µL of PBP_{500}-2 elution from the ER extract incubated with PIP synthesized in the presence of BiP template but no ARPCs. G) 200 µL of PBP_{500}-2 elution from only PIP synthesized in the presence of both ARPCs and the BiP template without incubation of ER extract.

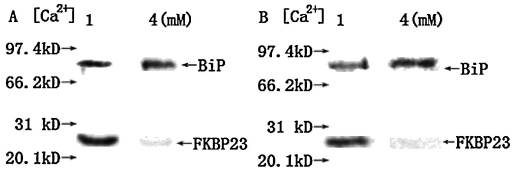


Figure 4. Immunoblotting of binding of FKBP23 to BiP. A: Immunoprecipitation. BiP and mFKBP23 were coprecipitated by adding anti-BiP antibody and protein A-Sepharose to the ER extract. B: Immunoblotting using PIP. BiP and mFKBP23 were coisolated by using PIP. The $[Ca^{2+}]$ was 1 or 4 mM.

specifically bound proteins. BiP in the sample was detected by silver staining and immunoblotting as shown in Figure 3A. The interactions between BiP and FKBP23 were detected by immunoblotting. To test the effects of $[Ca^{2+}]$ on the interactions between BiP and FKBP23, $CaCl_2$ was added to the ER extract to a final concentration of 1 or 4 mM, and then the extract was subjected to immunoprecipitation as before. The results showed that the binding of BiP to FKBP23 was regulated by $[Ca^{2+}]$ and that the interactions could not be detected in the presence of high $[Ca^{2+}]$ (4 mM) as show in Figure 4A.

To produce the PIP, ARPCs were synthesized by grafting acrylamide (AM) and 4-vinylpyridine (4-VP) onto a poly(vinyl alcohol) (PVA) backbone as shown in Scheme 1.[24,26] Modified porous PVA microspheres (Figure S1 and Figure S2),[26] which were hydrophilic and biocompatible, were cross-linked to the ARPCs and cloned BiP by adding AM and *N*,*N*,*N*′,*N*′-tetra-

methylethylenediamine. PIPs were obtained by eluting the cloned BiP as shown in Figure 2. Then 0.9 mL of ER extract containing 2.25 μg of BiP (Figure 3C) was incubated with 2 g of wet PIP at 4 °C for 12 h with end-over-end rotation. The spheres were washed with a series of 1.5 mL of phosphate buffers (PB) that contained increasing amounts of potassium chloride (phosphate buffer: 12 mM Na_2HPO_4, 8 mM NaH_2PO_4, pH 6.95) once, PBP_{100} (phosphate buffer with potassium chloride: 14 mM Na_2HPO_4, 6 mM NaH_2PO_4, 100 mM KCl, pH 7.15: the subscript number represents the millimolar concentration of KCl in this buffer and in the following buffers) once, PBP_{150} once, PBP_{200} once, PBP_{300} once, and PBP_{500} twice. Low-salt buffer (PB, PBP_{100}, PBP_{150}, and PBP_{200}) could remove largely proteins while most of BiP were still retained. Then BiP could be isolated by high-salt buffer (PBP_{300} and PBP_{500}) (Figure S3)[26] and the proportion of BiP in the sample was increased, as is the case for immunoprecipitation as shown in Figure 3B. 1.5 mL of PBP_{500}-2 elution contained 180 ng of BiP. In the controls, BiP was not detected when no template protein (Figure 3D) or unrelated template protein (Figure 3E) was included in the original PIP synthesis and adsorption of ER extract. BiP was not detected when ARPCs were omitted (Figure 3F) in the original PIP synthesis and adsorption of ER extract. These indicated that the adsorption of BiP by the PIP was not due to a nonspecific ionic effect but rather to imprinting of the PIP with the cloned BiP template. Furthermore, BiP was also not detected when both template protein and ARPCs were included in the original PIP synthesis but without adsorption of ER extract as shown in Figure 3G, which indicated that BiP in the elutions (Figure 3B) came from the ER extracts instead of template cloned proteins. The BiP protein contains a polar N-terminal end followed by a strongly hydrophobic 18-amino-acid region.[25] The hydrophobic surface of BiP may be a factor that favores the imprinting by using ARPCs with pyridinyl recognition sites to keep BiP's geometric structure.

To test the effects of $[Ca^{2+}]$ on the interactions between BiP and FKBP23, $CaCl_2$ was added to the ER extract to a final concentration of 1 or 4 mM, and then the extract was subjected to immunoprecipitation. As shown in Figure 4, BiP could be isolated using the PIP under low or high $[Ca^{2+}]$, but no FKBP23 was detected when the $[Ca^{2+}]$ was 4 mM (Figure 4B) which was in accordance with the results of immunoprecipitation (Figure 4A).[22] The results obtained with two methods in accordance with each other; both confirmed that the binding between BiP and FKBP23 was regulated by $[Ca^{2+}]$ and that the binding could not be detected at a $[Ca^{2+}]$ of 4 mM. It also indicated that the adsorption of BiP by the PIP was not due to a nonspecific ionic effect.

In summary, we have used PIP to isolate the relatively large protein BiP and study BiP–FKBP23 interactions. The results by using PIP and by immunoprecipitation both showed that the binding between BiP and FKBP23 was regulated by $[Ca^{2+}]$. Compared with biological antibodies, these artificial antibodies have excellent chemical stability and compatibility with organic solvents. The design of ARPCs and production of PIPs are easy. More importantly, the use of PIPs removes the need to prepare antibodies by injecting antigen into mammals or to use expensive protein A-Sepharose. Therefore, PIPs provide a feasible alternative to biological antibodies in biochemical research. Further studies on optimizing the design of the ARPCs and the size of the macrospore adsorbent spheres are currently underway.

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