Appl Biochem Biotechnol (2010) 162:1206–1213
DOI 10.1007/s12010-009-8888-1

Magnetic Screening of the Potential Targeted Protein of Salvianolic Acid B Using T7 Phage Display Library

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Received: 30 August 2009 / Accepted: 8 December 2009 /
Published online: 5 January 2010

Abstract Salvianolic acid B is one of the effective components from the Chinese traditional drug *Salvia miltiorrhiza* (Danshen), which is widely used as a usual clinic drug for atherosclerosis-related disorder patients in China. But the targeting protein of salvianolic acid B is still not known. The possible targeting proteins of salvianolic acid B were explored by high throughput screening in this paper. Attached to the magnetic nanoparticles, salvianolic acid B was used for screening the high-affinity protein from the displaying cDNA peptide library phage. After biopanning, the selected protein or peptide sequences were used to explore the whole proteins containing the selected sequences in the National Center for Biotechnology Information website using blast. One of the selected phages was carried out by affinity analysis with salvianolic acid B using capillary electrophoresis (CE). The CE results indicated that the protein or peptide on the surface of the selected phages could bind the drug salvianolic acid B. The results are helpful to preliminarily explain the pharmacology of salvianolic acid B.

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Keywords Phage display library · Magnetic nanoparticles · Targeted protein · Salvianolic acid B

Introduction

Chinese traditional drugs have clinically been used for curing patients for thousands of years in China. They were proved to be strongly medically effective to patients and were very popular in China and even in Japan or in Korea. In order to explore the mechanism of Chinese traditional drug reaction and improve the clinical effects of the Chinese traditional drug, thousands of bioactive compounds have been isolated, purified, and characterized from Chinese traditional drugs. Many bioactive components were proved to be very effective to diseases, and some which have been clinically used included such drugs as salvianolic acid B and artemisinin.

Salvianolic acid B is a water-soluble polyphenolic antioxidant isolated from the root of red-rooted salvia (*Salvia miltiorrhiza* Bunge) and is the main effective component of *S. miltiorrhiza* for clinical applications. *S. miltiorrhiza*, a Chinese traditional drug, is widely used for the prevention and treatment of atherosclerosis-related disorders in clinical therapy in China.

Some investigators reported that salvianolic acid B had such probable effects on other disorders as protecting brain against injuries [1], the antioxidative effects on cardiac cells [2], and the treatment of liver fibrosis [3]. Although some mechanisms of salvianolic acid B have been explored, the targeting protein of salvianolic acid B is not yet known.

Many effective components of Chinese traditional drugs such as taxol, artemisinin, and ephedrine had been further studied, and they have been used as new drugs, as compound drugs, and even as lead compounds now. For example, livmptothecine, containing the topo-isomerase with its targeting protein being the effective component of Camptothecaacuminata, can be involved in the synthesis procedure of DNA duplication and DNA transcription. Regarded as an anticancer drug, the targeting protein of livmptothecine is helpful for discovering the new anticancer drug. The new anticancer drugs such as topotecan and irinotcan were quickly discovered after their targeting proteins were characterized.

Almost all targeting proteins were found by chance. High throughput screening of the targeting protein of small molecules was only recently reported. Paul P Sche et al. reported that they applied cDNA phage display to screen the binding protein of biotinylated FK506, and the protein FKBP12 was characterized as the binding protein of FK506 [4]. Youngnam Jin reported that the protein hNopp140 was a binding protein of anticancer drug doxorubicin using cDNA phage display [5]. Diane J. Rodi et al. found that the Bcl-2 was the binding protein of anticancer drug paclitaxel using cDNA phage display [6]. Kengo Morohashi et al. reported that the peptide LGDPNSSRIP was the drug motif of an anti-tumor drug NK109 using cDNA phage display [7].

In this paper, the characterization of the magnetic nanoparticles was initially performed in targeting protein screen using cDNA phage display. Subsequently, the magnetic nanoparticles were selected to conjugate with salvianolic acid B, and the specific proteins or peptides binding with salvianolic acid B were screened. The phage clones specially binding salvianolic acid B were selected by biopanning in T7select415-1b placenta cDNA phage display library system. The sequences of the selective

targeting proteins were blasted in the National Center for Biotechnology Information (NCBI) website. The affinity analysis between the selected phage clone and salvianolic acid B bound to the targeting protein was further tested by capillary electrophoresis, and the binding constant between the selected phage clone and salvianolic acid B was calculated based on Hummel–Dreyer method.

Materials and Methods

Preparation of Magnetic Nanoparticles

The magnetic nanoparticles were synthesized using the coprecipitation method in the presence of the polymer matrix [8]. After complete synthesis, the magnetic nanoparticles were lyophilized. Ten milligrams magnetic nanoparticle powder was added in 5 ml 0.1 M 2-[*N*-morpholino]ethane sulfonic acid (MES) buffer (pH4.7), and 10 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was added at room temperature. After 15 min, 2 ml ethylene diamine was added. The reaction buffer was stirred for 3 h at room temperature. The magnetic nanoparticles were separated by magnetic bold. And the magnetic nanoparticles were put in dialysis bag and dialyzed until the dialysate was free of amine by the fluorescamine assay. If needed, the magnetic nanoparticles were lyophilized.

Linkage of Salvianolic Acid B with Magnetic Nanoparticles

The protocol of EDC is based on the instructions of Pierce Biotechnology (www.piercenet.com). The following is the main protocol: (1) EDC was equilibrated to room temperature. (2) Magnetic nanoparticles (20 mg) were added in 1 ml conjugation buffer: 0.1 M MES, pH 4.7. (3) Salvianolic acid B (2 mg) was dissolved in 1 ml conjugation buffer. (4) EDC (5 mg) was dissolved in 500 µl conjugation buffer and immediately added to the magnetic particles solution. (5) The mixture was placed at room temperature for 2.5 h. (6) Then, dialysis was carried out using 0.85% NaCl solution. If needed, the magnetic particles could be lyophilized.

Biopanning

The biopanning protocol depicted simply as follows was based on the book constructions of the library [9, 10]. Magnetic particles (200 µl at 6 mg/ml) were blocked with bovine serum albumin overnight at 4°C, then washed for six times using 750 µl Tris-buffered saline Tween-20 (TBST) (TBS + 0.1% (*v*/*v*) Tween-20). One milliliter TBST was mixed with the magnetic particles and 10 µl phage display library, and the mixture was incubated at room temperature for 60 min. Then, the magnetic particles were separated using magnetic bold, and the supernatant was discarded. The magnetic particles were washed ten times using TBST with five different grads (TBS + 0.1%, 0.2%, 0.3%, 0.4%, 0.5% (*v*/*v*) Tween-20) and the elution buffer salvianolic acid B. The amplification of the eluted phages and polymerase chain reaction (PCR) amplification of plaques were carried out according to the protocol instructions of the suppliers. The PCR products were purified with the PCR Clean-up Kit (V-gene Biotechnology Limited, China) and were placed for sequencing in Shanghai DNA Biotechnology Co. Ltd. Under the same

conditions, the control experiments were also carried out using the magnetic particles without salvianolic acid B. The biopanning processing of salvianolic acid B using magnetic particles was shown in Fig. 1.

Bioinformatics Analysis

In the NCBI website http://www.ncbi.nlm.nih.gov/blast/, the displayed sequences of the selected phages were input, and the Nucleotide–nucleotide BLAST (blastn) was chosen for blast. The highest scores were determined for the potential targeted protein of the Chinese traditional medicine.

Binding Assay Using Capillary Electrophoresis

The phages of the candidate sequences for the targeted protein were also checked by capillary electrophoresis (CE) assay with the Chinese traditional medicine.

Purification of the phages was carried out according to the instructions of the suppliers using Ultracentrifugation (Beckmam LP-80XP, SW41 Ti). Then, the phages in the CsCl solution were dialyzed in Dialysis Tubing (PIERCE, 10000MWCO) in phosphate-buffered saline (PBS) (pH=7.2).

The conditions for CE were concisely as follows: the capillary for CE was filled with 0.30 M PBS (pH=7.2) buffer containing different concentrations of salvianolic acid B mixed with phages. The electrophoresis voltage was 10.00 kV, and the UV detector wavelength was 214 nm.

In this study, the internal calibration of the Hummel–Dreyer method [11] was used to investigate salvianolic acid B and the selected phage binding. The initial method of Hummel–Dreyer used was from conventional gel filtration chromatography. Subsequently, it was extended to high-performance liquid chromatography by Sebille et al. [12, 13] for the study of the binding of different drugs on albumin.

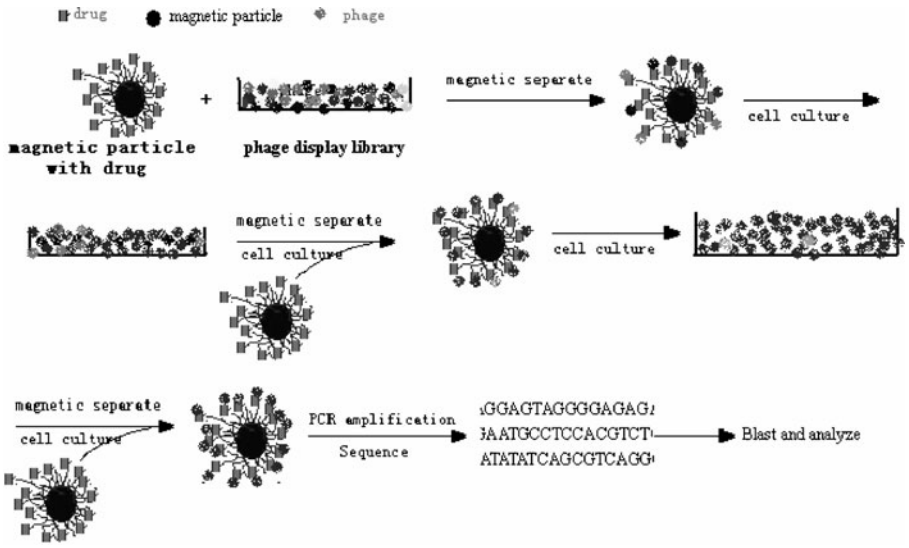

Fig. 1 The biopanning processing of the salvianolic acid B using magnetic particles

The internal calibration of the Hummel–Dreyer method [11] was used for the CE data processing; a simple interpolation allows the determination of the drug concentration whose negative peak vanished, from which the binding drug can be calculated.

To determine the Bmax and Kd, the data were fit to the Scatchard equation using nonlinear regression. The nonlinear regression was performed using GraphPad Prism version 4.03 for Windows, GraphPad Software, San Diego CA, USA, www.graphpad.com.

Table 1 Some selected sequences and the blast results.

Selected sequences	BLAST results
CGTTCGGCACGAGGTAGATAGTCAAGTTCGACCGTC TTCTCAGCGCTCCGCCAGGGCCGTGGGCCGACCCCG GCGGGGCCGATCCGAGGGCCTCACTAAACCATCCAA TCGGTAGTAGCGACGGGCGGTGTGTACAAAGGGCAG GGACTTAATCAACGCA	gi|5714635|gb|AF159295.1|AF159295 Homo sapiens serine/threonine protein kinase Kp78 splice variant CTAK75a mRNA
CAAGCGCTTGAATTCGCACGAGGCGGAGGAGGAGG GAAGGAAGATGGCGGCCGTGGAACTAGAGTGGATC CCAGAGACTCTCTATAACACCGCCATCTCCGCTGTC GTGGACAACTACATCCGCTCCCGCCGAGACATCCGC TCCTTGCCCGAGAACATCCAGTTTGATGTTTACTACA	gi|17390268|gb|BC018121.1| *Homo sapiens* amyloid beta precursor protein (cytoplasmic tail) binding protein 2, mRNA
CGGCACGAGGCCCGAACGGAGCCGTCAAATGACTCCT TTAATGTCTCTTCAGTGGTATCCTCAGACAGGCCTTTGA CAAACAGAGTTTTGGATGGCTGGCTTCTGGCATTAGGT GATCCCCTGGGTCCTTGCAACTCCAGCCTGATTGCTC TGCCCTCAATTTCCCTTTTATTACAGGAATTTAA	gi|4885510|ref|NM_005381.1| *Homo sapiens* nucleolin (NCL), mRNA
CGGCACGAGGAAGCAATTGATATATAACCGTGATTGTC TCTGCTTTTCTTCTGAAATGTAGATAACTGCTTTTTGAC AAAGAGAGCCTTCCCTCTCCCCCACCCCTGTGTTCTTG GGTAGGAATGGGAAAAGGGGCAACCTACAAAGATTGT TGGGGCAAGGGAAGTCACA	gi|42519913|ref|NM_012448.3| *Homo sapiens* signal transducer and activator of transcription 5B (STAT5B)
CGGCACGAGGGTCCATCAACCTCAGATCCCAGGGCTG ACGCTGTCAGTTCACTGACAATGCTGGCCAGTAATCCA CAGGTGTTAGAGACGAGGTGGGAGGAGAAGAGTTCAT TTTCTCTCCTCAGGCTGTTCCTGACTGGTAGCACAACA ATGACCAGCATTTTCTCCAGAACTTCACGGA	gi|7662379|ref|NM_015057.1| *Homo sapiens* MYC binding protein 2 (MYCBP2), mRNA
CGGCACGAGGCCTCGTGCCGAATTCGGCACGAGGG TAAAGGAAGAGTCTGGAGAAACAGGAGCCACCACT GCCTGGGAGTGAGGAGATGGTGGGAAGAGCTTGCA AGAGGAATCAGGAGTGGGGCTCCAGTCAGGCTGCA TGTGGAATGGCTGCTAGACATTCATGCCAAGATGCT GAAGACTCTGATTTTGGA	gi|18543110|gb|AC021678.13| *Homo sapiens* chromosome 8, clone RP11-380I10
CGGCACGAGGGCAGGAGTAGGGGAGAGAAGAGG AGTGGGGAGGGAGGGAGAAAAAGAGACACACAG GGAATGCCTCCACGTCTGGGAAAGACGACGTGGG GGTCCCTTTCCTTTGTTTTCTTAACTATATATCAGCG TCAGGCAGGATTTGAAACCTAAAGCCTTTAAATAG CTGTTTGACAA	gi|28812044|dbj|AP006256.1| *Homo sapiens* genomic DNA, chromosome 9, clone:RP11-433N8,
CGGCACGAGGGCAGGAGTAGGGGAGAGAAGAGG AGTGGGGAGGGAGGGAGAAAAAGAGACACACAG GGAATGCCTCCACGTCTGGGAAAGACGACGTGGG GGTCCCTTTCCTTTGTTTTCTTAACTATATATCAGCG TCAGGCAGGATTTGAAACCTAAAGCCTTTAAATAG CTGTTTGACAA	gi|4982556|gb|AC005921.3|AC005921 *Homo sapiens* chromosome 17, clone hRPK.294_J_22

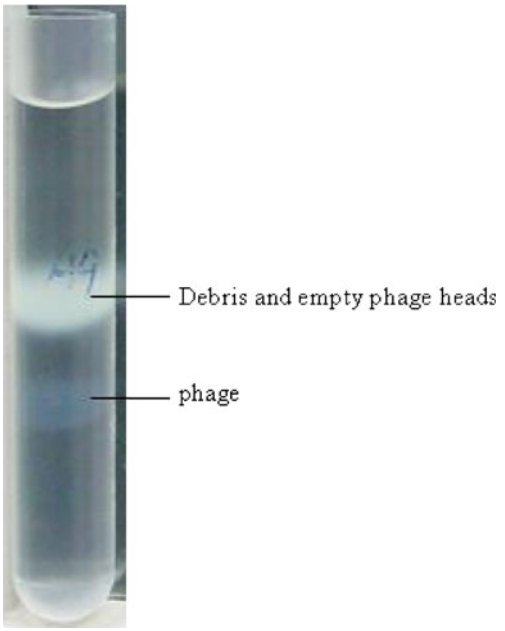

Fig. 2 Purification of T7 phage by CsCl isopycnic gradient ultracentrifuge. The *broad bright band* in the tube contains debris and empty phage heads, and the *narrow dim band* contains the phage particles

Results

The Displayed Sequences of the Selected Phages and the Blast Results

Six repeated experiments with more than 20 plaques were performed for selection and sequence. The phage clones were selected for at least two times and compared to the sequences in the NCBI website using blast. Some results of the sequences and the blast were shown in Table 1.

No phage was selected at the fourth time of biopanning in the control experiment. The magnetic particles without the drug were washed by TBST (TBS + 0.4% Tween-20); no phage clones could be retained on the magnetic particles so that the control sequence was blank.

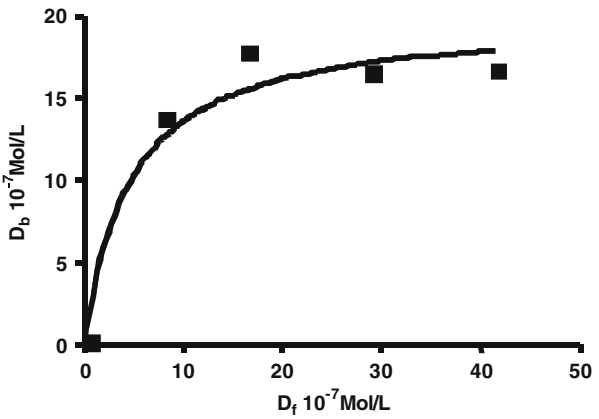

Fig. 3 The binding curve of salvianolic acid B to the selected phage

Affinity by CE Assay

The selected phage was purified by ultracentrifugation (see Fig. 2) and dialyzed. Then, the selected phage was performed for the binding assay with the salvianolic acid B by CE. The injected samples were a series of mixtures with a constant concentration of phage and gradually increasing concentration of salvianolic acid B. The detailed methods and results can refer to the publication [14]. The total drug (D_{total}) was determined using zero interpolation of internal calibration method, and the binding drug (D_b) can be calculated by D_{total}. The curve between the free drug (D_f) and D_b was plotted (see Fig. 3).

The different concentrations of the free drug were applied in the experiment, and the bound drug concentration could be calculated. The drug binding plot was plotted by nonlinear regression using the software (see Fig. 3). The binding constant between the selected phage clone and salvianolic acid B is $Kd = 4.73 \times 10^{-7}$M, and the $Bmax = 2.00 \times 10^{-6}$M.

Discussion

The technology of phage display peptide library systems has been mostly used in epitope mapping of monoclonal and polyclonal antibody [15], generating of immunogens [16], identifying peptides ligands or drugs [17], or evolution of the protein molecule [18]. Phage display cDNA cloning can combine the advantages of gene-linked in vitro protein display with the action of affinity to provide direct access to the genes of natural product receptors of humans.

In our study, cDNA phage display library was used for screening the binding protein or targeted protein of the small drug molecule salvianolic acid B. From CE experiment and data analysis of the binding between the selected phage and the drug salvianolic acid B, we obtained 4.73×10^{-7}M binding constant between the selected phage and the drug salvianolic acid B. The results above suggested that the drug salvianolic acid B was likely to have some specific binding sites to the peptides or protein on the surface of the selected phage, and salvianolic acid B could perform its pharmacological role by binding to the targeting protein containing the sequence above. However, the phage-displayed peptides or proteins could not adequately imitate the relatively exact structure of the binding site of the real targeting protein so that the binding constant is not small enough. Therefore, further work is required to screen the many more binding proteins so as to prove that the selected sequences and the proteins are the targeting sites of salvianolic acid B.

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