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Identification of peptides that selectively bind to myoglobin by biopanning of phage displayed-peptide library

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ABSTRACT

Biopanning of phage displayed-peptide library was performed against myoglobin, a marker for the early assessment of acute myocardial infarction (AMI), to identify peptides that selectively bind to myoglobin. Using myoglobin-conjugated magnetic beads, phages that bound to myoglobin were collected and amplified for the next round of screening. A 148-fold enrichment of phage titer was observed after five rounds of screening relative to the first round. After phage binding ELISA, three phage clones were selected (3R1, 3R7 and 3R10) and the inserted peptides were chemically synthesized. The analysis of binding affinity showed that the 3R7 (CPSTLGASC) peptide had higher binding affinity (K_d = 57 nM) than did the 3R1 (CNLSSSWIC) and 3R10 (CVPRLSAPC) peptide (K_d = 125 nM and 293 nM, respectively). Cross binding activity to other proteins, such as bovine serum albumin, troponin I, and creatine kinase-MB, was minimal. In a peptide-antibody sandwich ELISA, the selected peptides efficiently captured myoglobin. Moreover, the concentrations of myoglobin in serum samples measured by a peptide–peptide sandwich assay were comparable to those measured by a commercial antibody-based kit. These results indicate that the identified peptides can be used for the detection of myoglobin and may be a cost effective alternative to antibodies.

1. Introduction

During acute myocardial infarction (AMI), detection and measurement of creatine kinase-MB (CK-MB), cardiac troponin I or T, and myoglobin are commonly used for the diagnosis of AMI. CK-MB and cardiac troponin I are elevated after 3–6 h of onset of symptoms. Myoglobin, a 17.8 kDa heme protein normally present in muscles, is elevated within 1 h to 3 h and peaks within 6–9 h after the onset of AMI. The early increase of serum myoglobin may help the measurement of myoglobin as a major diagnostic utility for the early detection of AMI (de Winter et al., 2000; Moe and Wong, 2010; Sallach et al., 2004). As per National academy of clinical biochemistry (NACB) laboratory medicine practice guidelines, clinicians test for myoglobin in conjunction with cardiac troponin during AMI, and the combined use of myoglobin with cardiac troponin or CK-MB is helpful in early diagnosis of myocardial infarction (Morrow et al., 2007).

Currently antibody-based immunoassays are a widely-used tool for diagnosing various biomolecules (Borrebaeck, 2000). This is due to its high affinity and selectivity against the antigen. However, antibody-based immunoassays seem to have disadvantages. For example, polyclonal antibodies have cross reactivity to antigens if the animal has exposed to various antigens in the past, while monoclonal antibodies are highly specific but sensitive to different environmental conditions (Petrenko and Vodyanoy, 2003; Shone et al., 1985). Other drawbacks of antibodies are high production cost and less chemical stability (Ruigrok et al., 2011). Nevertheless, antibody-based immunoassays are still commonly used for diagnosing cardiac biomarkers during AMI.

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As an alternative approach, synthetic peptides may have advantages over antibody-based diagnostics, since it is easy to produce, cost less, and is readily modified chemically (Ladner et al., 2004; Meloen et al., 2003). Using the diversity of phage library, varieties of peptides have been produced in the past two decades. The selection process using a phage peptide library or biopanning is simple and cost effective. Based on an affinity selection, phages bound to the immobilized target were enriched and amplified. After several rounds of biopanning, the selected phage clones were picked and analyzed individually (Paschke, 2006; Willats, 2002). Using phage displayed-peptide library, we have identified a peptide that binds to the interleukin-4 receptor that is exposed on atherosclerotic plaques and cancer cells and employed it for imaging and drug delivery (Hong et al., 2008; Namgung et al., 2014). Also, we have identified a peptide that binds to histone H1 that is exposed on the surface of apoptotic cells and employed it for *in vivo* imaging of apoptosis and drug delivery (Wang et al., 2011, 2010).

The aim of this study was to identify peptides that selectively bind to myoglobin and develop a peptide-based diagnostic assays for the detection of serum myoglobin. For this, we screened phage-displayed-peptide library to identify peptides that bind to purified myoglobin. Rather coating the target protein onto micro titer plates, we conjugated myoglobin to magnetic beads to perform the screening process three-dimensionally and also to employ the magnetic separation to remove the unbound phages. The binding of selective clones was confirmed by phage binding ELISA and their selectivity or binding affinity of the positive clones were confirmed by ELISA using synthetic peptides. Further, peptide-based sandwich assay was developed using clinical serum samples to validate the measurement of myoglobin by the selected peptides.

2. Materials and methods

2.1. Materials

Ph.D-C7C phage peptide library was purchased from New England Biolabs (#E8110SC; New England Biolabs, Beverly, MA, USA). This contains a structurally constrained 7-mer random peptide library with complexity of 1.2×10^9 and *E. coli* ER 2738 as a host cell. Surface activated Dynabeads M-270 Epoxy was purchased from Life Technologies (Carlsbad, California, USA). Human myoglobin was purchased from Abcam (#ab96036; Cambridge, Massachusetts, USA). Anti-myoglobin monoclonal antibody (#sc-65982) and goat anti-mouse IgG-HRP (#sc-2005) were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Myoglobin ELISA kit was purchased from DRG International Inc. (#EIA 3955, Springfield, New Jersey, USA). ELISA plates were purchased from Corning (#3590; New York, USA), black-colored ELISA plates for fluorescent assay were from SPL life sciences Korea (Gyeonggi-do, Korea), bovine serum albumin (BSA) was from Bovogen (Bovostar #BSA100; East Keilor, Australia), gelatin was from Sigma (St. Louis, Missouri, USA), horse radish peroxidase (HRP)-conjugated anti-M13 antibody was from GE Healthcare (#45-001-419, New Jersey, USA), and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was from Komabiotech (#K0331070; Seoul, Korea). Absorbance was measured using the Sunrise Basic microplate reader (Tecan group Ltd, Männedorf, Switzerland).

2.2. Overall experimental setup

The overall experimental setup is shown in Fig. 1. This consists of five parts: (1) biopanning of phage library, (2) Sequencing and amino acid sequence analysis, (3) evaluation of phage binding, (4) evaluation of peptide binding, and (5) development of peptide-based sandwich assays.

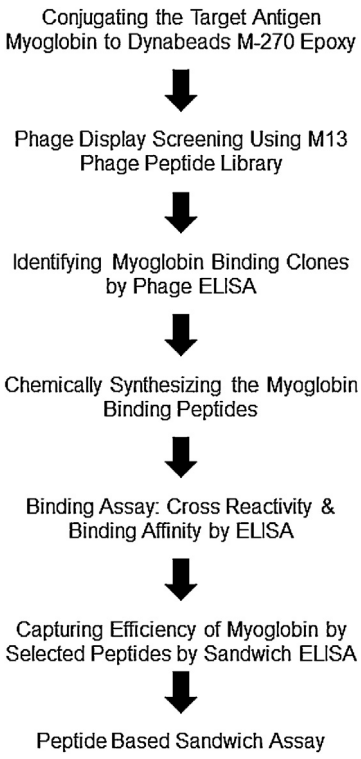

Fig. 1. A flow chart of experiments.

2.3. Biopanning of phage library

Myoglobin was conjugated to surface activated Dynabeads M-270 Epoxy for biopanning. After Dynabeads were resuspended and washed as per the manufacturer's instruction, 60 μg of myoglobin was immobilized to 105 μl of beads (2×10^8), and 60 μl of 3 M ammonium sulfate is added to make a final volume of 180 μl. The mixture was incubated at 4 °C for 16–24 h in Eppendorf tube rotator. After incubation, the myoglobin coated beads were collected using magnetic separator and the supernatant was removed and the coated beads were washed for 4 times with 1 ml of 0.1% PBST. Then the coated beads were blocked for 30 min at room temperature (RT) using 0.5% BSA in phosphate-buffered saline (PBS).

Each biopanning round consists of negative selection or subtraction of phages that nonspecifically binds to the Dynabeads and subsequent positive selection of phages that binds to myoglobin and amplification of the eluted phages. In the first round, 5 μl of 1×10^{11} M13 phage library in PBS was added to the 0.5% BSA-blocked Dynabeads and incubated at 4 °C for 1 h with gentle shaking for subtracting the phages that binds to the beads. After incubation, unbound phages in the supernatant were collected using magnetic separator and were incubated with myoglobin coated on the beads at 4 °C for 1 h with gentle shaking. After incubation, the unbound phages were extensively washed using PBS containing Tween (PBST) and the concentration of Tween was gradually increased from 0.1% up to 0.5% in further rounds, in order to minimize the non-specific binding of phages. The bound phages were eluted by incubating with 0.1 M citrate (pH 3.1) at RT for 2 min and immediately neutralized with 1 M Tris-HCl (pH 9.1). The phage particles in the elute were precipitated using 20% polyethyleneglycol (PEG)/2.5 M NaCl and then suspended in Tris-buffered saline (TBS). The phage titration was performed by serially diluting the elute and plated on LB media containing isopropyl-β-D-thiogalactoside and 5-bromo-4-chloro-3-indolyl-β-D-galactoside. The remained phages were amplified