High-Efficiency Generation of Monoclonal Antibody for *Vitreoscilla* Hemoglobin Protein

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Bacterial hemoglobin from *Vitreoscilla* (VHb) is recognized as a good fusion protein for the soluble expression of foreign protein. In this study, we generated a monoclonal antibody (MAb) against VHb for its detection. For the rapid screening of MAb, a protein chip technology based on the Alexa-488 (A488) dye labeling method was introduced. In order to fabricate the chip, the VHb protein was chemically coupled to the chip surface and then the culture supernatants of 84 hybridoma cell lines were spotted onto the VHb chip. The bound MAbs were measured by A488-modified anti-mouse IgG. A single spot (MAb A10) exhibited significantly high signal intensity. The immunoblot analysis evidenced that the MAb A10 can detect VHb-fused proteins with high specificity.

Keywords: Alexa-488, fusion, monoclonal antibody, protein chip, VHb

Bacterial hemoglobins (Hbs) are oxygen-binding proteins expressed under oxygen limitation or oxidative stress conditions [1]. The Hb protein from an obligate aerobic bacterium, *Vitreoscilla* (VHb), is one of the best studied Hb proteins. VHb is a small heme-containing protein with the molecular mass of 15.8 kDa. It is expressed under hypoxic condition and functions as an oxygen storage to provide molecular oxygen to the host respiratory system [9]. Because of this, VHb has been employed and coexpressed as an oxygen supplier in the commercial production of bioactive metabolites and pharmaceuticals to support large-scale and high-cell-density culture where oxygen demand is high [1, 8].

Besides its role of oxygen storage, VHb has been employed as a new fusion protein system in the production of foreign proteins. The red color of VHb facilitates visualization of any VHb-fused protein, which enables rapid detection of the target protein and therefore simplifies protein purification steps [6, 7]. Fusion of VHb with D-amino acid oxidase (DAO) showed that VHb not only provides benefits in the purification but also enhances the activity and stability of DAO in the bioconversion of cephalosporin C [4]. Fusion with superoxide dismutase (VHb-SOD) showed that VHb can function as a peroxidase and thus the chimeric protein can facilitate the detoxification of cellular reactive oxygen species [2]. Since VHb-fused proteins have been proven to be functionally active, the fused chimeric proteins can be employed for the fabrication of a biosensor chip. In the fabrication of protein biosensor chips, immobilization of the protein on a solid surface with proper orientation is the most important step. Proteins with fusion tags, including glutathione S-transferase (GST), maltose binding protein (MBP), and 6×His, have been immobilized on the solid chip surface that is modified with glutathione-, cyclodextrin-, Ni²⁺-iminodiacetic acid-linked solid surfaces, respectively [3]. Similarly, VHb-fused proteins can be displayed on the solid surface of biosensor chips through anti-VHb antibodies. Accordingly, this work focused on the generation of an anti-VHb monoclonal antibody (MAb) with the purified VHb proteins. Screening of the specific MAb was facilitated by the protein chip technology as previously reported [5]. The performance of anti-VHb MAb was evaluated by Western blot and enzyme-linked immunosorbent assay (ELISA).

In order to prepare the purified VHb protein, *Escherichia coli* BL21 (DE3) carrying pPosKJ [6] was grown in LB at 37°C. When the culture reached the exponential phase, the expression of VHb protein was induced by addition of
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1 mM isopropyl β-D-thiogalactoside (IPTG). The cells were grown for an additional 12 h and were collected by centrifugation. The collected cells were suspended in 30 mM Tris-HCl buffer (pH 7.5) and disrupted by sonication. The supernatant of cell extract was loaded onto a Q-Sepharose column (Amersham). The VHb protein was eluted at 0.3 M NaCl (Fig. 1A and 1B). The fractions containing the VHb protein were easily identified by their red color. The VHb protein was purified to near-homogeniety after Ni-NTA column chromatography (Fig. 1C).

To generate anti-VHb MAb-producing cell lines, 0.2 mg of the purified VHb protein was injected to a female Balb/c mouse. An equal quantity of the VHb protein was injected again with Freund’s incomplete adjuvant (Sigma) after 3 weeks. Lastly, the VHb protein was immunized after an additional 3 weeks. Generation of hybridoma cell lines was performed by the fusion of the spleen cells from the immunized mouse with myeloma cells, as previously reported [5]. The hybridoma cells (84 cell lines) were distributed in 96-well culture plates and cultured for 10 days. Screening of the VHb-specific MAb-producing cell lines was conducted by a fluorescence protein chip as described in the scheme in Fig. 2B. Employment of the protein chip technology simplified and accelerated the laborious screening procedure. To make a VHb-specific protein chip surface, the VHb protein solution in PBS buffer (1 mg/ml) was layered onto an aldehyde-modified slide glass (NuriCell, Korea) on which the proteins are chemically coupled to the chip surface by the formation of

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**Fig. 1.** Purification of VHb. (A) Elution profile of VHb in the Q-Sepharose column chromatography. The arrow indicates the VHb peak. (B) SDS-PAGE analysis of the eluted fractions. (C) Further clarification of VHb by Ni-NTA column chromatography.

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**Fig. 2.** Protein microarray analysis of the culture supernatants of hybridoma cells. (A) The MAbs in the culture supernatants bound to the VHb protein, which was coated on a solid surface. The bound MAb was detected by Alexa-488 dye-labeled anti-mouse IgG. The white spots on G4 are positive controls with Alexa 488-IgG alone, which were spotted after the washing steps. (B) The scheme for fluorescence detection. (C) Western blot analysis with selected culture supernatants. The VHb protein (200 ng in each lane) was briefly resolved in SDS-PAGE. The protein was transferred to a PVDF membrane. The lanes in the membrane was cut and incubated with the corresponding culture supernatant.
Schiff’s base bond between the aldehyde groups on the slide glass and the amino groups in the protein. For the screening of MAb, the culture supernatants of hybridoma cells were mixed with PBS containing 20% glycerol at a ratio of 1:1 ratio and were spotted onto the VHb-coupled slide using a protein arrayer (Proteogen, Korea). The slide was incubated for 1 h at 25°C at 80% humidity and then washed with PBS containing 0.1% Tween-80. The bound MAb was detected by a fluorescence chip scanner (Genepix, Axon Instruments, USA) using Alexa-488-goat anti-mouse IgG (5 µg/ml; Invitrogen). As shown in Fig. 2A, only the cell line A10 produced high fluorescence intensity as an indication of highly specific MAb against VHb. This was further confirmed by Western blot analysis in the detection of VHb with some selected cell culture supernatants. The cell line A10 produced the strongest signal, whereas the others produced weak or no signals (Fig. 2C).

The sensitivity of the anti-VHb A10 MAb was investigated by ELISA. The VHb protein solutions at various concentrations in PBS were added to the wells of an ELISA plate and then incubated overnight at 4°C. The wells were washed 3 times with PBS. After blocking with 5% skim milk, 100 µl of the MAb A10 (0.1 mg/ml) solution was introduced to each well. The plate was incubated for 2 h at room temperature and then the bound MAb was detected by anti-mouse IgG-HRP. A shown in Fig. 3A, the absorbance at 492 nm increased proportionally with the increase of the VHb concentration until the concentration reached 300 ng/ml. The absorbance became saturated above 600 ng/ml. The detection limit was less than 10 ng/ml of VHb.

Lastly, the MAb A10 was employed for the detection of various VHb-fused proteins. Six two-component response regulator genes of Salmonella enterica ATCC9150 were cloned into pPosKJ vector right after the VHb gene, so that the response regulator proteins produced as chimeric proteins with VHb at the N-terminus. The sonicated extracts of E. coli cells harboring the expression vector were analyzed by SDS-PAGE and Western blot. The Coomassie-stained protein gel image showed that the concentrations of the chimeric proteins in the cell extracts were in the range of 0.01 mg/ml – 0.05 mg/ml (Fig. 3B). The anti-VHb MAb A10 successfully detected the presence of VHb-fused proteins in the Western blot analysis (Fig. 3C) with the detection limit lower than 0.01 mg/ml, proving the usefulness of the MAb in the detection of VHb protein.

In this work, we demonstrated that protein microarray is a potent tool in the high-throughput screening of monoclonal antibodies from a large pool of hybridoma cell lines. The anti-VHb MAb A10 was applicable for both ELISA and Western blot analysis for VHb or VHb-fused proteins. The MAb could detect less than 10 ng/ml of VHb in ELISA. The developed MAb will be employed in the fabrication of protein biosensors to assemble monolayers of VHb-fused proteins onto solid surfaces.

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References


