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Determining and Estimation of Antibody Production in the Bubble Eye, Goldfish

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Abstract

Development of antibody production technologies is necessary for diagnostic treatments and drug discovery. In general, mammals are used as host animals to produce antigen-specific antibody. However, such host animals have never produced several specific antibodies because host animals may not recognize foreign proteins. To overcome this problem, we used teleost to produce antibodies because teleost are evolutionally localized in the origin of vertebrates and have an acquired immune system in addition to the innate immune system. In particular, we attempt to produce antibody using "Bubble Eye" as a kind of goldfish (*Carassius auratus*), which has sacs filled with lymph liquid, as an immune animal. In this study, a recombinant EGFP-His was expressed in *E. coli* and then injected into Bubble Eye's sac in every two weeks. The antibodies were collected from sac instead of blood. Furthermore, a sandwich dot blotting was developed for detection of antibodies against EGFP-His. The antigen-specific antibodies were detected after 42 days from first immunization.

Keywords: Antibody; Immunization; Fish; Goldfish; Vaccination

Introduction

Development of antigen-specific antibody production is necessary for research tools, diagnostic treatments, drug discovery, and so on. However, host animals have never produced specific antibodies against well-conserved proteins between human and other mammals, because host animals do not often recognize foreign proteins, so-called immune tolerance. To overcome this problem, the procedures on antibody production have recently been researched using various immune animals such as chicken, ostrich, shark, camel, and so on. In general, mammals are used as host animals to produce polyclonal antibodies. These host animals have some advantages to produce the antibodies. For example, camelids and sharks are known to possess functional homodimeric antibodies composed of only heavy chains in addition to classical heterodimeric immunoglobulin (Ig) antibodies [1,2]. Such as small molecular antibodies make it possible to easily produce the recombinant antibody in the heterologous expression system by *Escherichia coli* [3]. On the other hand, chicken and ostrich antibodies can be harvested from their egg yolks instead of blood, and the productivity of antibody in their eggs is much higher than that in a similar sized mammal [4,5].

In this study, we attempted to produce the antigen-specific antibodies using fish. Teleost are evolutionally localized in the primitive and diverse groups of vertebrates, and have an acquired immune system in addition to the innate immune system. Mammals possess five functionally distinct immunoglobulin (Ig) isotypes (IgM, IgD, IgG, IgA and IgE), while fish Ig is identified as IgM in cartilaginous and teleosts [6,7]. In a recent study, several Ig isotypes have been identified in different species of teleosts, such as IgZ in zebrafish (*Danio rerio*) [8], IgT in rainbow trout (*Oncorhynchus mykiss*) [9] and the novel IgH in fugu (*Fugu rubripes*) [10] and chimeric IgM-IgZ in common carp (*Cyprinus carpio*) [11]. Therefore, teleosts have a potentially of unique immune responses. Previously, we reported to produce specific antibody using zebrafish against human leucine-rich repeat-containing G-protein-coupled receptor 3 (LGR3) [12]. However, zebrafish is too small to take blood including antibody. So we focused on goldfish belonging to Cyprinidae, the same species as carp and zebrafish. Goldfish was first domesticated in China more than a thousand years ago, and several distinct breeds have so far been developed. 23 types of goldfish has been produced by selective breeding and certified by Japan Ornamental Fish Association, while more than 100 types were not certified. In particular, we focused on "Bubble Eye" as a new antibody producer which has two large fluid-filled sacs containing lymph under each eye (Figure 1).

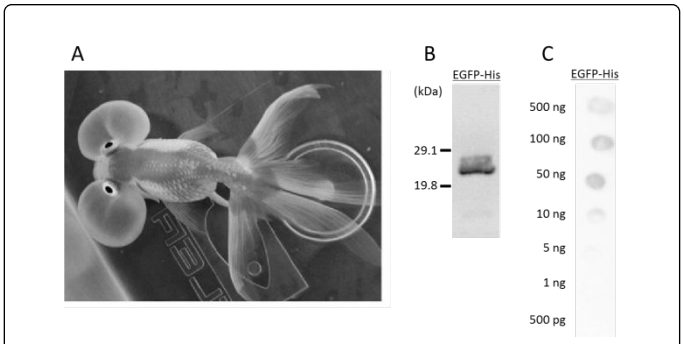

Figure 1: Bubble Eye and its antigen preparation. (A) Immunization to Bubble Eye which has two sacs enlarged cornea and are filled with lymph fluid. (B) Western blotting analysis of the EGFP-His expression in *E. coli* using anti-His antibody. (C) Dot blotting analysis using an anti-GFP antibody. The amounts of solid-phased EGFP-His proteins are shown to the left.

A recent study suggests that sac fluid can be used as a growth-promoting supplement for animal cell cultures [13]. Moreover, Bubble

Eye has several advantages as an immune host, the antigen injection and the antibody collection can be performed through the sacs including lymph, antibody can be continuously collected because the sacs after collecting lymph would recover again within about a week or two, and sampling is easier and less damage to the fish than through blood. Here, we report that Bubble Eyes immunized with EGFP-His as antigen generated antigen-specific antibody.

Material and Methods

Recombinant EGFP-His was used as the antigen in this study. The EGFP-His gene was amplified from pXI-EGFP plasmid [14] with primer set as follows: EGFP_rest9U, 5′-aaaaaacccgggGTGAGCAAGGGCGAGGAG-3′ and EGFP_His6, 5′-ctagtggtggtgatgatgatgCTTGTACAGCTCGTCCATGC-3′ and subsequently amplified with an SfiI restriction site at the downstream of His-tag using these primers; EGFP_rest9U and His6_rest1D, 5′-ttttggccgaggcggccCTAGTGGTGGTGATGATGATG-3′. For expression in *Escherichia coli* (*E. coli*), the EGFP-His gene was cloned into the modified pCold TF DNA (TaKaRa Bio Inc.) vector that the SmaI site was bound to the translation enhancing element (TEE) and the SfiI site was bound to upstream of the transcription terminator using these primers; pCold TF_rest1U, 5′-aaaaaaggccgcctcggccGTAATCTCTGCTTAAAAGCACAGAATC-3′ and 5′-ttttttcccgggCACTTTGTGATTCATGGTGTATTACC-3′. *E. coli* BL21 strain was transformed with pCold TEE EGFP-His plasmid and was cultured at 37°C in 2 × YT medium (1.6% Bacto Trypton, 1% dry yeast extract (Nacalai Tesque, Inc.), and 0.5% NaCl, pH 7.0) containing 50 μg/ml ampicillin. When the OD600 of the culture was reached from 0.4 to 0.5, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at the final concentration of 0.1 μM and was incubated at 15°C for 24 h.

The cells were collected by centrifugation at 3,000 × g for 15 min at 4°C and were resuspended in a Ni-chelating column binding buffer (20 mM sodium phosphate, 0.5 M NaCl, and 20 mM imidazole, pH 7.4). After sonication, the crude extracts were collected by centrifugation at 12,000 × g for 30 min at 4°C. The supernatant was loaded onto the Ni-column charged with Ni-Sepharose 6 Fast Flow (GE healthcare) and was eluted by the Ni-column elution buffer (20 mM sodium phosphate, 0.5 M NaCl, and 500 mM imidazole, pH 7.4). Furthermore, the target fraction was replaced with the DEAE-column buffer (20 mM 2-amino-2-(hydroxymethyl) propane-1,3-diol (Tris)-HCl, pH 8.0) using a ultrafiltration membrane (Millipore). The EGFP-His protein was purified using DEAE-Sepharose Fast Flow (GE healthcare) with the DEAE-column buffer and a linear elution by the buffer (20 mM Tris-HCl, 1 M NaCl, pH 8.0). The eluted fraction was replaced with a divalent ion-free goldfish Ringer's solution (125 mM NaCl, 2.6 mM KCl, 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), pH 7.4).

Purified fractions were separated by an SDS-PAGE (10% gel) and were transferred onto a PVDF membrane. The membrane was blocked in PBS containing 5% skim milk and 0.5% Tween 20, incubated with an anti-His antibody (Amersham Biosciences, 1:3,000) at room temperature for 1 h, and subsequently incubated with an anti-mouse IgG HRP-conjugated antibody (Cell Signaling Technology, 1:25,000) at room temperature for 1 h. The dot blots were developed using an Amersham ECL plus Western Blotting Detection Reagent (GE healthcare) and the chemiluminescent was captured by Light Capture II Cooled CCD Camera System (ATTO).

Adult Bubble Eyes (Figure 1A) were purchased from local breeders at the Maruteru fish farm (Aichi, Japan). Bubble Eyes were maintained in a freshwater at 25°C under light condition of 14-h light period and at 10-h dark period. Bubble Eyes were injected at the 100-μg purified recombinant EGFP-His with an adjuvant directly into Bubble Eye's sacs. Six groups were prepared, i.e., only EGFP-His antigens, antigens with oil-based adjuvant (egg-yolk lecithin, peanut oil, and glycerol) [15], antigens with oil base adjuvant including inactivated *E. coli*, *Aspergillus oryzae*, or *Mycobacterium tuberculosis*, or goldfish Ringer's solution instead of the antigens with oil-based adjuvant as a control. The *E. coli* DH5α strain was cultured in LB-medium at 37°C for 16 h and the *A. oryzae* OSI-1013 strain was cultured in DPY-medium at 28°C for 18 to 20 h. These bacteria were sonicated for inactivating. *M. tuberculosis* H37 Ra (dry, Difco Laboratories) was used as inactivated cells. Each bacterial cells were added to the oil base in an amount of 0.5 mg/ml and mixed with antigen in a volume ratio of 1:1 using 1-ml syringe (Termofisher Scientific) and an 18 G-reagent mixing needle to prepare with water-in-oil emulsions. The bacterial cells were used as only for an initial immunization, and only the oil-based adjuvant was subsequently used. Booster injections were then given for every 14 days. The Bubble Eye's lymph was collected from their sacs before immunization and 14 days after each injection up to 70 days. The collected lymph was centrifuged at 1,500 × g for 10 min at 4°C to remove precipitates and stored at -30°C.

For inferring detection limit of the dot blotting using an anti-GFP antibody, dot blotting solid-phased EGFP-His recombinant protein was performed. The PVDF membrane was soaked in methanol and was transferred to distilled water and soaked in PBS with shaking. A total of 2-μl EGFP-His solutions diluted with PBS were spotted onto the membrane and dry-up overnight. The membrane was blocked with TBST (0.05% [v/v] Tween 20, 20 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 5% skim milk at room temperature for 1 h. After washing with TBST, the membrane was incubated with anti-GFP antibody (MBL) diluted 1:3,000 with Can Get Signal Solution 1 (TOYOBO) at room temperature for 1 h. Next, the membrane was washed and incubated with an anti-rabbit IgG HRP-linked antibody diluted (1:100,000) with Can Get Signal Solution 2 (TOYOBO) at room temperature for 1 h. After through washing, the dot blots were developed using an Amersham ECL plus Western Blotting Detection Reagent and detected by Light Capture II Cooled CCD Camera System.

For detecting specific antibodies against EGFP-His of immunized Bubble Eye's lymph, sandwich dot blotting was performed. 2-μl aliquots of Bubble Eye's lymph diluted to 1:100 with PBS were spotted onto the membrane prepared as same, as the above method and dry-up overnight. The membrane was blocked in TBST including 5% skim milk at room temperature for 1 h. After washing, the membrane was incubated with the EGFP-His protein solution diluted with Can Get Signal Solution 1 at room temperature for 1 h, incubated with an anti-GFP antibody (MBL) diluted 1:3,000 with Can Get Signal Solution 1 at room temperature for 1 h, and subsequently incubated with an anti-rabbit IgG HRP-linked antibody with the dilution (1:100,000) in Can Get Signal Solution 2 at room temperature for 1 h. The dot blots were developed by using an Amersham ECL plus Western Blotting Detection Reagent and detected by Light Capture II Cooled CCD Camera System.

Results

The recombinant EGFP-His protein was successfully expressed in *E. coli* transformed by the pCold TEE-EGFP-His plasmid, with molecular mass of around 28 kDa. The identity of expressed proteins was further confirmed by Western blotting using the anti-His antibody. This result suggested that the purified proteins were detected by the His-tagged EGFP protein (Figure 1B).

For calculating detection limit of the dot blots using the anti-GFP antibody, dot blotting solid-phased by the EGFP-His recombinant protein was performed. The results showed that anti-GFP antibody detected over 5-ng EGFP-His proteins under the condition (Figure 1C). For detecting antigen as specific antibodies, the dot blotting of solid-phased Bubble Eye's lymph fluid was performed. Dot blotting showed a significant response to the EGFP-His protein in experimental group B, while the antigen with oil base adjuvant, and group C, and the antigen with oil-based adjuvant including *E. coli*. Both groups indicated that the titer of antigen specific antibodies were clearly higher after 42 days from first immunization (Figure 2).

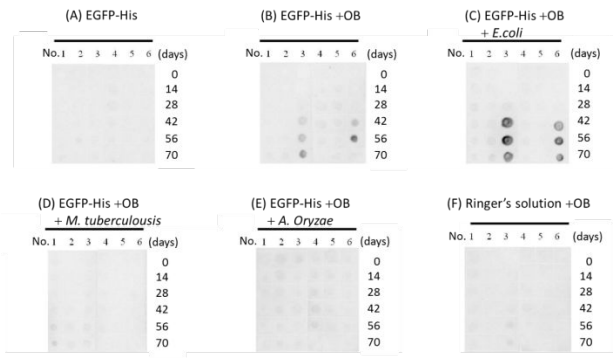

Figure 2: Specific antibody production against the recombinant EGFP-His. (A) Immunization to Bubble Eyes which have their sacs filled with lymph fluid. (B) Specific antibody production against 100-μg EGFP-His proteins on each dose was detected and evaluated by sandwich dot blotting. Group A, only EGFP-His; group B, EGFP-His including oil-based adjuvant (OB); group C, EGFP-His including OB with *E. coli* cells; group D, EGFP-His including OB with *M. tuberculousis* cells; group E, EGFP-His including OB with *A. oryzae* cells; group F as a control of Ringer's solution and OB. The days for collecting lymph fluids from each sac are shown onto the right.

However, experimental groups which were injected only antigen, the antigens with the adjuvant including *A. oryzae* or *M. tuberculosis*, and Ringer's solution with those adjuvant was detected with no signals.

Discussion

To our knowledge, this is the first description of a successful method to generate the antigen-specific antibody by employing Bubble Eye's sacs. Therefore, the recombinant EGFP-His proteins were directly injected into their sacs. The results on dot blotting suggested that the adjuvants at a first antigen injection were needed to produce some specific-antibody in Bubble Eyes. In addition, the antigen-specific antibodies were raised after third antigen injection in the experimental group B, the EGFP-His proteins with an oil-based adjuvant, and group C as EGFP-His with an oil-base adjuvant including *E. coli*. Gram-negative bacterium in outer membrane of cell walls consists of lipopolysaccharides which are involved in some immunostimulation. Mycobacterium is also known to have the adjuvanticity, although the present study suggested that the antibody's titers were higher when the adjuvant including *E. coli* was used of injection into their sacs. Some of high antigenicity could be blamed on the existent of other gram-negative bacteria, through the freshwater such as a Gram-negative bacterium such as Aeromonas spp. which was caused on the opportunistic infection. Bubble Eyes have a potential of immune animals as alternative to mammals, because the antibodies of immunoglobulin M (IgM) could be easily collected through their sacs without any killing. Therefore, these fish would be no more out of animal welfares. Further work is needed to generate other antigens such as GPCRs and developing more highly sensitive assay methods. Cyprinidae contributes over 20 million metric tons to fish production in the worldwide and accounts for approximately 40% of total global aquaculture production and 70% of total freshwater aquaculture production [16]. However, goldfish farming have declined in recent years, so it is hoped that the researching of goldfish utilization as an immune hosts contributes to the revitalization of goldfish farming.

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