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Detection of tilapia metallothionein using antibody-immobilized quartz crystal microbalance sensor

Namsoo Kim [a,*], So-Hee Son [a], Woo-Yeon Kim [b]

[a] Functional Materials Research Group, Korea Food Research Institute, Seongnam 463-746, Republic of Korea
[b] Department of Biotechnology, Chung-Ang University, Anseong 456-756, Republic of Korea

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ABSTRACT

Metallothionein (MT) was induced in tilapia by three subcutaneous injections of $CdCl_2$ and the recovered MT protein was purified to homogeneity through DEAE-cellulose ion exchange chromatography and Sephacryl S-100 gel filtration. For use as biological component, a polyclonal anti-tilapia MT antibody was prepared using the purified tilapia MT. During the preparation of the tilapia MT sensor, topological changes pertinent to immobilization of the thiolated antibody, subsequent blocking of the surface with BSA and final immune reaction were observed by AFM imaging. Specificity of the sensor was confirmed through comparison of the responses of defective and intact sensor chips, and the responses of the sensor chips that were immobilized with the antibody and BSA. Concentration-dependent responses of the sensor showed a linear relationship of Y ($\log_{10}$ sensor response) = 0.3438X ($\log_{10}$ tilapia MT) + 0.7368 (r = 0.9606) in double-logarithmic plot with a limit of detection of 20 ng/mL. When the serum from a tilapia induced with $CdCl_2$ was compared with the control serum, induction of MT was detectable even at 20,000-fold dilution of tilapia serum.

1. Introduction

Tilapia that inhabit diverse freshwater habitats, including shallow streams, ponds, rivers and lakes, is the common name for nearly a hundred species of cichlid fish from the tilapiine cichlid tribe and is, nowadays, the fifth most important fish in fish farming, with production reaching 1.5 million metric tons in 2000 [1]. Various species of *Oreochromis*, *Sarotherodon* and *Tilapia* belong to tilapiine cichlid tribe. Like other large fish, they are good source of protein and are low in saturated fat, calories, carbohydrates and sodium [1–3]. At the same time, tilapia is a useful fish model for eco-toxicological studies and its metallothionein (MT) level may be a potent biomarker of exposure to metal ions in the waters [4,5]. In consequence, safety issue for tilapia with respect to heavy metals has a critical importance not only for food intake but also for management of the inland ecosystems. One way to evaluate the contamination of tilapia with heavy metals is to measure MT that is specifically induced by extraneously added heavy metal ions and is a sum parameter of the presence of these chemicals [6], which will greatly reduce the necessity to carry out fastidious high-precision instrumental analyses because the positive samples from preliminary MT screening are only required to undergo those analyses.

MT that has a monomeric molecular mass of 6000–8000 Da and holds a strong affinity to heavy metals due to thiol groups of cysteine residues is over-expressed in fish when exposed to heavy metal ions in rivers and estuarine waters [7,8]. It has been reported that the induction of MT gene with accumulated xenobiotics in fish, including crab, shrimp, catfish and eel, provides an over-expression of MT up to several hundred μg/g body weight [7,9,10]. Until now, MT has been determined by spectrophotometric assays that comprise Cd-hem assay, Sephadex G-75/atomic absorption spectrometry, immunoassays such as ELISA, capillary electrophoresis and HPLC [11–13]. However, these methods are suitable for laboratory analyses because they are complex in measuring procedure, labile to interference caused by coloring substances and dependent on use of radioisotopes or expensive reagents [12,13]. In light of these, it is necessary to develop a new method such as biosensing to measure level of MT in tilapia for preliminary screening purpose.

Recently, some studies that include immobilization of MT [14], electrochemical analyses of MT based on different principles [2,15–23], preparation of samples [24] and real-time detection using scanning electrochemical microscopy combined with surface plasmon resonance [25] have been reported. In the present study, MT was induced by three subcutaneous injections of $CdCl_2$ into tilapia and the recovered MT protein was purified by

* Corresponding author. Tel.: +82 31 780 9131; fax: +82 31 709 9876.
E-mail address: k9130sen@hanmail.net (N. Kim).

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DEAE-cellulose ion exchange chromatography and Sephacryl S-100 gel filtration. An anti-tilapia MT antibody that was prepared using the purified MT as immunogen was coated to the surface of 9-MHz quartz crystal microbalance (QCM) to produce a sensor chip. A direct-binding immunosensing for tilapia MT, including sample measurements, was conducted with the QCM sensor prepared and the experimental results are first reported.

2. Experimental

2.1. Reagents and transducer

A heterobifunctional thiolation cross-linker, sulfosuccinimidyl 6-[3-(2-pyridyldithio)propionamido]hexanoate (sulfo-LC-SPDP), and dithiothreitol for immobilization of antibody were obtained from Pierce (Rockford, IL, USA). Protein G was purchased from Sigma–Aldrich (St. Louis, MO, USA), and all other chemicals to prepare buffers and to operate the sensor system were guaranteed reagents from various suppliers. Double distilled water was used throughout the study. An AT-cut piezoelectric quartz wafer (QA 9RP-50, Seiko EG & G, Matsudo, Japan), attached with two gold electrodes of 5 mm diameter, was used as transducer. It has a fundamental resonant frequency of 9 MHz with reproducibility of ±0.1 Hz.

2.2. Preparation of tilapia MT

Healthy tilapia (1,000–1,500 g) that were purchased from a local market and stabilized in a fish culture basin were treated by three subcutaneous injections of $CdCl_2$ (2 mg/kg in 1st day, 1.3 mg/kg in 3rd day and 12.5 mg/kg in 5th day of fish culture) to induce production of MT. The blood of tilapia induced with MT was retrieved using a syringe needle that was pricked into the main artery along a lateral line of the fish. A protease inhibitor cocktail was added to the retrieved blood to prevent proteolysis and the treated blood was centrifuged at 3,000 rpm for 30 min to acquire the serum of tilapia. The induction of MT was confirmed by 15% SDS-PAGE against the sera of the induced and control tilapia [26]. Tilapia MT in the induced serum was purified by DEAE-cellulose ion exchange chromatography that used DE-52 resins (Whatman, Kent, UK) at 0.25 M NaCl gradient and then by Sephacryl S-100 gel filtration. The protein concentrations in the purified MT fractions were determined by the microprotein assay of Bradford [27].

2.3. Preparation of anti-tilapia MT antibody

An anti-tilapia MT antibody for use as biological component was prepared using the tilapia MT purified as immunogen in rabbit, with referring to a previous report [26]. For the final anti-serum recovered, reactivity of antibody was measured by an indirect ELISA in which tetramethyl benzidine was used as substrate [26]. In this case, 2 μg/mL of the purified tilapia MT and the anti-serum diluted 1000–100,000 fold were used during the procedure to form an immune complex. The immune reaction owing to the antibody in the anti-serum was further confirmed by Western blotting in which an electrophoretic separation in 18% SDS-polyacrylamide gel and then an electro-transfer from the gel to a polyvinylidene fluoride membrane was carried out [26].

For use as biological component, a portion of the retrieved anti-serum was purified by ammonium sulfate precipitation as follows. Twenty milliliters of the anti-serum and 5.4 g of $(NH_4)_2SO_4$ were combined and the mixture was stirred slowly at 4 °C overnight. The resultant suspension was centrifuged at 14,500 rpm for 30 min and the pellet obtained was washed with 10 mL of 2 M $(NH_4)_2SO_4$, pH 7.0. After suspending, stirring at room temperature for 30 min and the centrifugation as above were further conducted. The pellet was re-suspended in about 4 mL of a dialysis buffer (15 mM KH_2PO_4, pH 7.0, comprising 50 mM NaCl) and the resultant suspension was dialyzed against 1 L of the same buffer at 4 °C for 15 h, with changing the buffer solution four times. Finally, the dialyzate was centrifuged as above to remove the precipitate. The partially purified IgG fraction by the above procedure was further purified by an affinity chromatography that used protein G-bound agarose beads [28].

2.4. Immobilization of antibody

The anti-tilapia MT antibody was immobilized to the QCM according to the method of Park and Kim [29] with slight modification. In brief, the surface of the QCM was treated with 1.2 M each of NaOH and HCl for 5 min successively with rinsing after the treatments with distilled water. Then, it was treated with aliquot of 20 μL of conc. HCl for 1 min with special care to keep the acid from touching the electrode leads, rinsed again with distilled water and dried finally inside a convection oven for 20 min. In the present work, sulfo-LC-SPDP was used to thiolate the antibody because it provided a good sensitivity and reusability to QCM sensors [29]. Aliquots of 30 μL of antibody solutions, dissolved in 0.1 M PBS (pH 7.0, comprising 0.0027 M KCl and 0.138 M NaCl), were separately reacted with the same volume of 20 mM sulfo-LC-SPDP, dissolved in distilled water, at room temperature for 1 h after gentle mixing. The resultant mixture was incubated with aliquot of 20 μL of 15 mg/mL dithiothreitol, dissolved in 0.1 M sodium acetate buffer (pH 4.5), at room temperature for 30 min to reduce disulfide bonds of the thiolated antibody. Aliquot of 7 μL of the final mixture was spread to one gold electrode of the cleaned QCM, and the chemisorption between the thiolated antibody and surface of gold continued at room temperature for 1 h. The surface of the sensor was blocked with 1% BSA for 1 h, and then was rinsed consecutively with distilled water and the reaction buffer of the immunosensor system, 0.1 M sodium phosphate buffer (pH 7.0). The sensor chip coated with the antibody was dried at room temperature and stored at 4 °C until use.

2.5. AFM imaging

The surface of the QCM sensor was imaged during the procedure of immobilization of the antibody and following immune reaction using an Agilent 5500 atomic force microscope (Agilent Technologies, Santa Clara, CA, USA) in Acoustic AC Mode in 3D scales (the total dimensions of *X*, *Y* and *Z* axis were 1 μm, 1 μm and 50 nm, respectively). Silicone probes that were 4-μm thick, 30-μm wide (mean) and 125-μm long, with a force constant of 42 N/m and a resonance frequency of 330 kHz, were used for AFM imaging that was conducted at a scan rate of 1 line/s with 512 data points/line. The amplitude change of the probe was sufficiently small, so that the imaging process was essentially not destructive to the samples. At least three regions of the surface of the sensor were scanned to acquire images optimally and first order 'flattening' was applied to correct the images obtained.

2.6. System setup and operation

The QCM sensor immobilized with the anti-tilapia MT antibody was installed inside a dip cell (QA-CL3, Seiko EG & G) and a batch-type immunosensor system was constructed through connection of the dip cell, an oscillator circuit (QCA 917-11, Seiko EG & G) and a quartz crystal analyzer (QCA 917, Seiko EG & G). An analog frequency signal from the quartz crystal analyzer was converted to a digital one through a GPIB interface of a system PC [30].