Generation of functional single-chain fragment variable from hybridoma and development of chemiluminescence enzyme immunoassay for determination of total malachite green in tilapia fish

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ABSTRACT
To determine malachite green (MG) and its major metabolite, leucomalachite green (LMG) residual levels in tilapia fish, chemiluminescent enzyme immunoassay (CLEIA) was developed based on a single-chain variable fragment (scFv)-alkaline phosphatase (AP) fusion protein. At first, VH and VL gene sequences were cloned from hybridoma cell lines secreting monoclonal antibody against LMG, and then thoroughly by database-assisted sequence analysis. Finally, the productive VH and VL were assembled to an intact scFv sequence and engineered to produce scFv-AP fusion protein. The fusion protein was further identified as a bifunctional reagent for immunoassay, then a sensitive one-step CLEIA against LMG was developed with a half-maximal inhibitory concentration (IC50) and limit of detection (LOD) of 1.3 and 0.04 ng/mL, respectively. The validation results of this novel competitive CLEIA was in line with those obtained by classical HPLC method for determination of total MG in spiked and field incurred samples.

1. Introduction
Malachite green (MG), a triphenylmethane dye, has been used as an antimicrobial and antiparasitic agent for treatment of skin and gill flukes, protozoans, and fungi in global aquaculture production since the 1930s. Once the MG has been absorbed by fish, it is reduced to a colorless and lipophilic metabolite, leucomalachite green (LMG) (Plakas, ElSaid, Stehly, Gingerich, & Allen, 1996). Previous studies have suggested that both MG and LMG pose significant risks on animal health, because of suspected carcinogenicity (Fernandes, Lalitha, & Rao, 1991), mutagenicity (Rao, 1995), and teratogenicity (Meyer & Jorgenson, 1983). Hence, MG has now been banned for use in aquaculture in most countries. The minimum required detection limit of 2 μg/kg for the sum of MG and LMG set by the European Union has been used as a standard limit for international food trade since 2002. Unfortunately, MG is still used illegally because it is the most cost-effective option with respect to aquaculture diseases. To limit or eliminate the potential adverse effects of MG and LMG contaminated fishery products on human health, a sensitive analytical tool is necessitated for detecting the sum of MG and LMG in aquatic products.

The reported analytical methods used for determination of MG and LMG, include liquid chromatography (Dowling, Mulder, Duffy, Regan, & Smyth, 2007; Mitrowska, Posyniak, & Zmudzki, 2005; Roybal, Pfenning, Munns, Holland, Hurlbut, & Long, 1995), surface-enhanced resonance Raman scattering (Lee, Choi, Chen, Park, Kyong, Seong, et al., 2007; Zhang, Yu, Pei, Lai, Rasco, & Huang, 2015). Compared with traditional HPLC and other assays, immunoassay based on polyclonal or monoclonal antibody against MG/LMG is less expensive technique, doesn’t need any expensive instruments, and rapidly conduct the high-throughput screening (Shen, Deng, Xu, Wang, Lei, Wang, et al., 2011;...
Singh, Koerner, Gelinias, Abbott, Brady, Huet, et al., 2011; Yang, Fang, Kuo, Wang, Huang, Liu, et al., 2007). For instance, a reported rabbit polyclonal antibodies generated by immunization of leucomalachite green/carrier protein conjugate showed group-specific binding activity to its analogs (Xing, He, Yang, Sun, Li, Yang, et al., 2009). It would, therefore, be appropriate to monitor the total triphenylmethane dyes contamination in aquaculture products. However, broad-specific assay is not suitable for quantitative analysis of neither LMG nor MG. Recently, a monoclonal antibody (Mab) against LMG was successfully produced in our laboratory, with no cross-reactivity toward other triphenylmethane dyes (Wang, Yang, Shen, Sun, Xiao, Lei, et al., 2017). Though homemade ELISA kit based on this Mab showed excellent specificity and sensitivity for detection of LMG, it requires an additional step of secondary antibody incubation. Alternatively, the single-chain variable fragment (scFv) derived from conventional IgG possessing a minimal antigen-binding conformation with affinities comparable to those of conventional antibodies. One significant advantage of scFvs is that they could be genetically fused with enzymes (such as alkaline phosphatase (AP)) yielding a bifunctional immunoreagent, which can be easily expressed in bacteria. This fusion protein can shorten the overall run time of immunoassay via avoiding the use of secondary antibodies enzyme conjugate.

Generally, the generation of functional scFvs from hybridoma was performed using a routine cloning protocol. In our previous study, the unique productive VH and VL from hybridoma secreting anti-Ractopamine Mab have been successfully cloned using only a single pair of primers (Dong, Li, Lei, Sun, Ducancel, Xu, et al., 2012). However, due to the complexity of hybridomas (Bradbury, Trinklein, Thie, Wilkinson, Tandon, Anderson, et al., 2018), failure in developing scFv has been most recently encountered in our laboratory. Attempts, such as phage display biopanning (Pan, Wang, Zhang, Liu, Lei, Huang, et al., 2006; Wang, Yang, Liu, Liang, Lei, Shen, et al., 2009) and mass spectrometry-based DNA sequence analysis (Du, Zhou, Li, Sheng, Ducancel, & Wang, 2016; Zhang, He, Zhao, Wang, & Jin, 2016) were proposed to avoid such pitfalls. Unfortunately, phage display technology is a time-consuming process, and mass spectrometry is expensive. Therefore, a highly efficient strategy to generate scFv from hybridomas is crucial for developing a sensitive immunoassay.

Herein, taking hybridoma secreting Mab against leucomalachite green as a model, a series of primers were applied to clone the VH and VL repertoire from hybridomas and select the functional VH and VL based on the database-assisted analysis. The aberrant VH/VL sequences were quickly excluded, and the database-predicted productive sequences were assembled to the scFv fragment that was identified in the format of one-step scFv-AP based ELISA. The functional scFv-AP fusion protein was further prepared to develop a competitive CLEIA for detecting the sum of MG and LMG. To the author’s knowledge, this is the first report of using scFv-AP based one-step CLEIA for determination of MG and LMG residues in tilapia fish.

2. Material and methods

2.1. Reagents and materials

MG, LMG, crystal violet (CV), leucocristal violet (LCV), brilliant green (BG), parafuchsin (PA), methylene blue (MB), 3′,3′,5′,5′-tetramethylbenzidine (TMB), were procured from Sigma Chemical (Shanghai, China). All chemicals acquired from Sigma-Aldrich are of analytical grade unless otherwise specified. p-nitrophenol phosphate and 3′-(2′-s piroadaman tane)-4-methoxy-4′(3′-phosphoryl ox)phenyl-1,2-dioxetane (AMPPD) were acquired from Aladdin-reagent Co. Ltd. (Shanghai, China). Molecular reagents, such as restriction enzyme and DNA polymerase, were supplied by Thermo Fisher Scientific Inc. (Grand Island, NY, USA). The plasmid named pLIP6/GN was a kind gift of Dr. Frédéric Ducancel (CEA-Saclay, France). The hybridoma cell line secreting Mab against LMG had previously been generated in our laboratory (Wang, et al., 2017). E. Coli strain BL21 was stored in our laboratory. A 96-well polystyrene microplates for luminescence test (White, high binding) were obtained from JET Bio-Scientific Inc. (Guangzhou, China). An S1000™ thermal cycler was secured from Bio-Rad Laboratory, Inc. (Hercules, CA, USA). Victor 3 multi-label counter (for CLEIA and ELISA) was purchased from Perkin Elmer (San Jose, CA, USA).

2.2. PCR amplification and analysis of VH and VL genes from hybridoma cells

Total mRNA was obtained from culture hybridoma secreting an anti-LMG antibody using TRIzol reagent (Takara, Dalian, China). The resulting RNA was reverse-transcribed to cDNA first chain using a reagent kit (Promega, CA, USA). The VH/γ1 forward (HB/HF) or VL/γ1 backward/γ1 forward (LB/LF) primer sets (Krebber, Bornhauser, Burmester, Honegger, Willuda, Bosshard, et al., 1997) were used for PCR amplification of all the VH and VL sequences in hybridoma cells. While The HB and LB primer sets contain 19 and 18 different sequences, the HF and LF primer sets contain 4 and 5 oligonucleotides, respectively. For maximum efficiency, two-stage PCR was performed. In the first round, the HF forward primer set mixture was used and combined with each of the HB backward primers in tubes. The HB primers that amplify the VH/γ1 gene were selected and mixed with individual HF primers for PCR in the next round. The same procedure was performed to obtain the immunoglobulin VL/γ1 gene. All the PCR products were cloned into the pEASY-T3 vector for sequencing. The sequence data were trimmed according to the LB/LF and HB/HF primer sets and analyzed by IMGT/HighV-QUEST online program (Giudicelli, Brochet, & Lefranc, 2011) and IgBLAST in NCBI (www.ncbi.nlm.nih.gov/igblast).

2.3. Construction and expression of anti-LMG scFv-AP fusion protein

The productive VH and VL region sequences were selected and reamplified with a corresponding primer set (Table S1). The PCR product was run on agarose gel for purification, then equal VH and VL DNAs were assembled to intact scFv gene by overlap extension PCR. The purified scFv PCR product was digested with Sfi I and Not I and cloned into the pLIP6/GN vector (Fig. S1). The resulting ligation was transformed by heat shock into E. coli strain BL21. The transformed bacterial cells were grown on a selective LB plate containing 100 μg/mL ampicillin. Positive colonies were selected to extract plasmid and sequenced to confirm identity. The confirmed colony was cultured in 2 × YT medium and then induced by adding 1 mM IPTG once the OD600 reached 0.6. The E. coli cells were then harvested by centrifugation. The cell pellet was lysed by the B-PER reagent according to manufacturer protocol and centrifuged at 15,000xg for 5 min to collect the soluble scFv–AP fusion protein. The SDS-PAGE gel of resulting recombinant expressed protein was stained by Coomassie Brilliant Blue and detected by anti-AP Mab in Western blotting.

2.4. Mab-based competitive ELISA and scFv-AP-based competitive ELISA procedure

ELISA with heterologous coating antigen (Table S2) was performed to validate the binding activity of scFv–AP fusion protein compared with parent Mab (Wang, et al., 2017). The coating antigen (LMG hapten H14-OVA) was diluted to 1.2 μg/mL and 100 μl per well was pipetted into the transparent microplates. After overnight incubation at 37 °C, the plates were washed twice with PBST (0.01 M PBS with 0.05% tween 20) to remove any unbound coating antigen. The plate was blocked with 250 μl per well blocking solution (5% (w/v) skim milk in PBS). After discarding the blocking solution, 50 μl per well LMG or single analogs from 729 to 0 ng/mL and 50 μl per well anti-LMG scFv–AP fusion protein (12.5 μg/mL) or anti-LMG Mab (5 μg/mL) diluted in PBST were added successively into the wells. Subsequently, the plates
were washed 6 times with PBST after incubation at 37 °C for 45 min.

For one-step competitive ELISA based scFv-AP, 100 µL freshly pre-
pared AP-ELISA substrate solution (1 mM p-nitrophenyl phosphate, 10 mM MgCl₂, 50 mM ZnCl₂; 1 M Tris-HCl, pH 8.0) was pipetted into
each well for 20 min incubation. The absorbance at 405 nm was mea-
sured by a multi-label counter followed by termination of the enzymatic
reaction with 50 µL 3 M NaOH. For classic two-step ELISA based on
anti-LMG MAb, rabbit anti-mouse IgG-HRP was used as the secondary
antibody tracer with 45 min incubation, and the corresponding TMB
substrate solution (200 µL of 0.6% TMB and 50 µL of 1% H₂O₂ in
12.5 mL citrate buffer, pH 5.5) was used for colorimetric detection
at 450 nm. The standard curve was fitted by OriginPro 8.5 (OriginLab,
MA, USA).

2.5. AP-based competitive CLEIA procedure

The CLEIA was performed similar to ELISA; however, the con-
centration of coating antigen (LMG hapten H14-OVA), was diluted to
0.21 µg/mL, respectively. LMG or single analogs were tested from 50 to
100 µg/mL. In one-step competitive CLEIA, 100 µL AP-CLEIA substrate
solution (1 mM MgCl₂, 0.67 mM AMPD in 50 mM carbonate buffer)
was pipetted into wells following 6 washes with PBST. The plate
was measured in chemiluminescent mode using multi-label counter
after a 25 min incubation. The standard curve was fitted by OriginPro
8.5.

2.6. Assessment of specificity and validation of one-step competitive CLEIA

The specificity of competitive CLEIA was evaluated by testing LMG
analogus under optimized conditions. The cross-reactivity was calcu-
lated by the formula: 100 × IC₅₀(LMG)/IC₅₀(analogs). A validation study
was performed by assessing matrix effects and evaluating recovery from
spiked tilapia samples (obtained from a local market) with MG, LMG or
a mixture of them at a concentration rate of 1, 5, 10 or 20 ng/g. The
tilapia fish samples (2.0 g) were proved to be free from MG and LMG by
HPLC. To eliminate the matrix effects and enrich the MG and LMG from
tilapia fish muscle, liquid-liquid extraction and further solid-phase
clean-up were used and described as follow. Briefly, the samples (2.5 g)
were homogenized with 10 mL acetonitrile (ACN), then 2.25 mL po-
tassium borohydride (0.2 mol/L) was added as a reducing agent, the
converting MG to LMG was accomplished by 10 min shaking in room
temperature. Following liquid-liquid extraction with 5 mL di-
chloromethane, the lower organic layer was evaporated to partial
dryness and then the residue was reconstituted in 500 µL ACN.
The prostate sulfonic acid strong cationic (PAB) solid-phase extraction
columns (Agilent, CA, USA.) were used for further clean-up procedure.
The final extract was evaporated to dryness and redissolved in 2 mL PBS,
followed by 10 min sonication, prior to analysis with competitive CLEIA
and HPLC, respectively. The above extraction and clean-up processes
were performed at room temperature. To determine the matrix effects,
an LMG-free extract obtained from the aforementioned procedure was
used to dilute the LMG standard. The standard curves diluted with
LMG-free extract were compared to the ones diluted with PBST. HPLC
analysis was performed on an Agilent 1290 HPLC system equipped with
a model G1321B fluorescence detector. The analytical procedure is
outlined in Table S3.

3. Results and discussion

3.1. Cloning of the Vₓᵧ and Vᵧᵦ repertoire from hybridoma cells

In general, recombinant scFvs is consists of two variable regions,
heavy (Vₓᵧ) and light chains (Vᵧᵦ); the Vₓᵧ and Vᵧᵦ fragments are linked
through a peptide spacer. In this work, the hybridoma secreting anti-
LMG Mab served as a source for mRNA isolation. The isolated mRNA
was used as a template for synthesis of the first-strand cDNA. Then, PCR
amplification of Vₓᵧ and Vᵧᵦ genes was performed using cDNA and the
HB/HF or LB/LF primer sets. As shown in Fig. S2, Vₓᵧ amplified pro-
ducts with an expected size of 350 bp were obtained using the following
HF2. Similarly, Vᵧᵦ genes of approximately 300 bp were successfully
amplified by PCR using the primer sets of LB1/LF1, LB2/LF4, LB2/LF5,
LB4/LF1, LB5/LF2, LB8/LF1, LB9/LF4, LB11/LF2, LB13/LF1, and
LB17/LF2 (Fig. S3). All of Vₓᵧ/Vᵧᵦ amplified gene products were sub-
cloned individually into the T overhang pEASY-T3 vector for amplifi-
cation and subsequent sequencing.

3.2. IMGT / V-QUEST sequence data analysis

For hybridoma technology, Sp2/0-Ag14 derived from P3X63Ag8
cells has been commonly used as a myeloma fusion partner fused with
spleen cells from an immunized mouse (Shulman, Wilde, & Kohler,
1978). Therefore, hybridomas would contain endogenous and aberrant
variable region transcripts of Sp2/0-Ag14, which are different from
those of spleen cells. However, to prepare recombinant antibodies, all
variable region genes expressed in hybridomas are cloned by PCR from
cDNA. Thus, the products of PCR are often heterogeneous and
sometimes consist exclusively of the aberrant sequences, which might
result in unsuccessful production of engineered antibody. To distin-
guish between the functional and aberrant sequences, a database-as-
isted strategy based on IMGT/V-QUEST (Brochet, Lefranc, &
Giacchini, 2008) and IqBLAST from National Center for Biotechnology
Information (NCBI) was applied in this study. Notably, users utilizing
these web-based tools can search against comprehensive nucleotide or
genomic databases to analyze the functionality or unproductiveness of
the candidate genes, rearrangements of IG V domain framework regi-
nons, and complementarity determining regions.

In our work, two different Vₓᵧ (Vₓᵧ8-2 and Vₓᵧ2-3) sequences (Fig. 1a,
Tables S4, S5) were obtained. Sequence analysis from IGMT/VQUEST
revealed that the Vₓᵧ8-2 was productive without any stop codon. As
shown in Table S5, the Vₓᵧ2-3 sequence was unproductive and should
not be translated into a functional protein. For Vᵧᵦ, three different Vᵧᵦ
sequences (Vᵧᵦ1-1, Vᵧᵦ4-1, and Vᵧᵦ2-4) were obtained (Fig. 1b). The re-
results of sequence analysis indicate that the Vᵧᵦ1-1 gene appears to be
productive. The junction was an in-frame translation with no stop
codon in the V-J region (Table S6), the finding which suggest that the
Vᵧᵦ1-1 sequence was deemed productive and rearranged correctly.
However, the Vᵧᵦ4-1 was found to be an unproductive sequence for light
chain variable region. The V-gene and J- gene of this sequence were
classified to IGKV3-12 and IGKJ2, respectively (Table S7) that was
further identified by searching the entire nucleotide database in Ig-
BLAST. This sequence was first reported by Carroll, Mendel, & Levy,
1988 and was derived from MOPC-21 kappa transcript (Ding, Chen,
Zhu, & Cao, 2010). Unexpectedly, the Vₓᵧ2-4 sequence contains a gene
identical to V glycine of Mus musculus IGKV3-12 (Table S8) with Vᵧᵦ4-1,
although it’s productive sequence reported in IMGT. We noted that the
protein sequence of Vₓᵧ2-4 aligned with Vᵧᵦ4-1 from FR1 to FR3, while
differed at the CDR3/FR4 junction region (Fig. 1b). Therefore, we
assumed that Vₓᵧ2-4 was indeed the variant derived from the Vᵧᵦ4-1 po-
tentially due to the rare rearrangement of V-J region during the pas-
saging of hybridoma cells (Kromenaker & Srienc, 1994). This particular
gene rearrangement was not observed using general sequence analysis
software. Thus, our proposed strategy was efficient for predicting and
identifying the functional and aberrant variable region sequences prior
to expression.

3.3. Construction of the anti-LMG scFv-AP fusion protein and assessment of
its potency by one-step competitive ELISA

To evaluate the function of Vᵧᵦ1-1 and disprove the Vₓᵧ2-4 by
Experimental procedure, both VH and VL fragments were amplified for later assembly of the scFv genes with unique VH8-2, named scFv1 and scFv2. The VH and VL fragments connected by a flexible linker were assembled to the scFv gene by overlap extension PCR. The two scFv sequences were inserted to the pLIP/6GN vector and transferred to E. coli BL21 for further verification by Western blotting (Fig. 2). In lane 3, Bacteria harboring scFv2-AP plasmid. Lane 1, Blank bacteria; Lane 2, Bacteria harboring scFv1-AP plasmid; Lane 3, Bacteria harboring scFv2-AP plasmid.

Fig. 2. Characterization of the total periplasmic protein by SDS-PAGE (A) and Western blotting (B) analysis. Anti-AP Mab and Goat anti-mouse IgG-HRP were used in Western blotting analysis. Lane M, low molecular weight protein standards; Lane 1, Blank bacteria; Lane 2, Bacteria harboring scFv1-AP plasmid; Lane 3, Bacteria harboring scFv2-AP plasmid.

3.4. One-step competitive CLEIA based on the anti-LMG scFv–AP fusion protein and assessment of its specificity

To improve the sensitivity of the assay, a sensitive competitive CLEIA against LMG was developed. AMPPD was used as a substrate for AP in the development of the competitive CLEIA. The enzymatically cleaved phosphate group was converted to an intermediate anion, AMP, which spontaneously forms a carbonyl compound, releasing its energy as a glow-type emission. The enhancement of signal in the CLEIA allowed the use of lower concentrations of coating antigen and scFv1–AP (0.21 µg/mL and 1.2 µg/mL, respectively), the concentrations of coating antigen and scFv1–AP used in the traditional colorimetric ELISA were 0.85 µg/mL and 12.5 µg/mL, respectively. After the optimization of coating antigen and scFv1–AP fusion protein by checkerboard titration, an AP-based competitive CLEIA standard curve was designed (Fig. 3c). The standard curve exhibited an IC50 of 1.3 ng/mL and a linear range of 0.09–11.50 ng/mL along with a low limit of detection (LOD) of 0.04 ng/mL. At variance, the sensitivity of competitive CLEIA was approximately 10 times higher than that of the competitive ELISA (IC50 = 1.3 ng/mL vs. IgG = 14.0 ng/mL, respectively). Thus, the sensitivity was improved by altering the substrate used in competitive ELISA and competitive CLEIA.

The specificity of competitive CLEIA using anti-LMG scFv–AP was determined by testing the cross-reactivity of other structurally related compounds (Table S9). The anti-LMG scFv–AP fusion protein was highly specific for LMG, which is in line with its parent monoclonal antibody (Wang et al., 2017). Only slight cross-reactivity was observed against LCV and MG (4.65% and 1.36%, respectively), and low cross-reactivity was indicated for other tested analogs. Since the scFv-AP against LMG demonstrate limited cross-activity toward MG, it was not possible to simultaneously determine LMG and MG using competitive ELISA. The residues of MG and its metabolite, LMG, in foods of animal origin were monitored in many countries and the minimum required performance limit was set at...
The developed one-step competitive CLEIA had sufficient sensitivity compared to the required performance limit and can be used to monitor the total sum of MG and its metabolite (LMG) in a single run.

3.5. Matrix effects of the competitive CLEIA and correlation between competitive CLEIA and HPLC

Matrix effects might increase or inhibit the binding affinity of antibody, causing false positive or negative results in immunoassay. As fish muscle contains various proteins and lipids, it is necessary to extract lipophilic LMG using solid-phase extraction (SPE) to eliminate the matrix effect. In our study, the standard curve in 1:5, and 1:10 fish muscle extract dilution were overlapped with that in PBST (Fig. 4). The IC₅₀ values of 1:5 and 1:10 extract dilutions were similar to that in PBS; the finding which indicates that matrix effect was minimized after solid-phase cleanup and dilution. Therefore, LMG was extracted from spiked samples using SPE and was diluted 5 folds with PBST before analysis. Blank samples were spiked with MG, LMG, and their mixtures. The MG was reduced to LMG by potassium borohydride (Fig. S4), so it can be detected by scFv-AP specific to LMG. The mean recovery was found to be 73.00 ~ 86.00% for LMG, 73.30 ~ 85.50% for MG, and 76.70 ~ 84.50% for the mixture (Table 1). Comparison with HPLC results shown in Fig. S5 indicates a good correlation (R² = 0.98) in the range of 1 ~ 20 ng/mL.

3.6. Determination of MG/LMG in field incurred samples compared with classical methods

The one-step CLEIA established based on scFv-AP fusion protein was successfully applied for detection of both LMG and MG in real fish samples using a simple reduction treatment. The total MG/LMG residual levels in tilapia fish samples collected from local markets were determined by the proposed CLEIA. The obtained results were verified with those of traditional HPLC and were compared with those of classic Mab-based ELISA that was developed with the parent Mab from the original hybridoma. Out of the tested samples, 2 samples were tested positive with concentration (total MG/LMG) ranged from 2.2 to 9.3 ng/g, as shown in Table 2. The validation results showed that the developed CLEIA method was in a good agreement with Mab-based ELISA and HPLC method, indicating that the scFv-AP based CLEIA was accurate and reliable for residual determination of total MG/LMG in tilapia.

Table 1

<table>
<thead>
<tr>
<th>Analytes Added (ng/g)</th>
<th>Found (ng/g)</th>
<th>Average Recovery (%)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMG 0</td>
<td>ND</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1</td>
<td>0.7 ± 0.04</td>
<td>73.0</td>
<td>5.5</td>
</tr>
<tr>
<td>5</td>
<td>4.0 ± 0.6</td>
<td>80.7</td>
<td>15.5</td>
</tr>
<tr>
<td>10</td>
<td>8.2 ± 0.8</td>
<td>82.0</td>
<td>9.2</td>
</tr>
<tr>
<td>20</td>
<td>17.2 ± 1.2</td>
<td>86.0</td>
<td>7.0</td>
</tr>
<tr>
<td>MG 0</td>
<td>ND</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1</td>
<td>0.7 ± 0.05</td>
<td>73.3</td>
<td>6.2</td>
</tr>
<tr>
<td>5</td>
<td>4.2 ± 0.3</td>
<td>83.6</td>
<td>6.2</td>
</tr>
<tr>
<td>10</td>
<td>8.2 ± 0.6</td>
<td>81.9</td>
<td>7.4</td>
</tr>
<tr>
<td>20</td>
<td>17.1 ± 0.8</td>
<td>85.5</td>
<td>4.5</td>
</tr>
<tr>
<td>LMG + MG (1:1) 0</td>
<td>ND</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1</td>
<td>0.8 ± 0.09</td>
<td>76.7</td>
<td>11.3</td>
</tr>
<tr>
<td>5</td>
<td>3.9 ± 0.5</td>
<td>79.7</td>
<td>12.4</td>
</tr>
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<td>10</td>
<td>8.2 ± 0.5</td>
<td>82.0</td>
<td>6.0</td>
</tr>
<tr>
<td>20</td>
<td>16.9 ± 0.6</td>
<td>84.5</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Table 2

Detection of the total LMG/MG (ng/g) in tilapia fish obtained from local markets (n = 3).

<table>
<thead>
<tr>
<th>Samples</th>
<th>scFv-AP based CLEIA</th>
<th>Mab-based ELISA</th>
<th>HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO.1</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>Not detectable</td>
</tr>
<tr>
<td>NO.2</td>
<td>2.2 ± 0.03</td>
<td>2.2 ± 0.05</td>
<td>2.3 ± 0.05</td>
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<tr>
<td>NO.3</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>Not detectable</td>
</tr>
<tr>
<td>NO.4</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>Not detectable</td>
</tr>
<tr>
<td>NO.5</td>
<td>9.3 ± 0.04</td>
<td>9.1 ± 0.07</td>
<td>9.4 ± 0.07</td>
</tr>
</tbody>
</table>

Fig. 3. Standard curve of one-step competitive ELISA based on two scFv-AP fusion proteins (a), standard curve of two-step competitive ELISA based on parent Mab from original hybridoma (b) and standard curve of competitive CLEIA based on scFv1-AP(c).

Fig. 4. Standard curves of LMG in PBST and sample extracted with solid-phase extraction (SPE) (n = 5). The matrix effect was evaluated by comparison of standard curves constructed in PBST and extracts purified with SPE then diluted at a ratio of 1:1, 1:5, 1:10.
4. Conclusions

Herein, a database-assisted strategy was proposed to generate a recombinant antibody from an anti-LMG secreting hybridoma, even though it has a complex genomic background mixed with fusion partners. The functional V\textsubscript{H} and V\textsubscript{L} genes were successfully identified and assembled to an intact scFv and then generated an scFv-AP that was characterized by one-step ELISA. It also emphasized that the recombinant antibody fusion protein has similar specificity and sensitivity as the parent Mab from hybridoma. Based on the resulting scFv-AP fusion protein, a one-step competitive CLEIA was developed for detection of LMG, which is 10 times more sensitive than the competitive ELISA based on the same fusion protein. As MG could be easily converted to LMG by reduction during sample preparation, the developed competitive CLEIA can be used as well for detection of total MG. Recovery and validation performance indicate that competitive CLEIA is a powerful tool for determination of MG and LMG in tilapia fish.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References