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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, V_H and V_L gene sequences were cloned from hybridoma cell lines secreting monoclonal antibody against LMG, and then thoroughly by database-assisted sequence analysis. Finally, the productive V_H and V_L were assembled to an intact scFv sequence and engineered to produce

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Declaration of competing interest

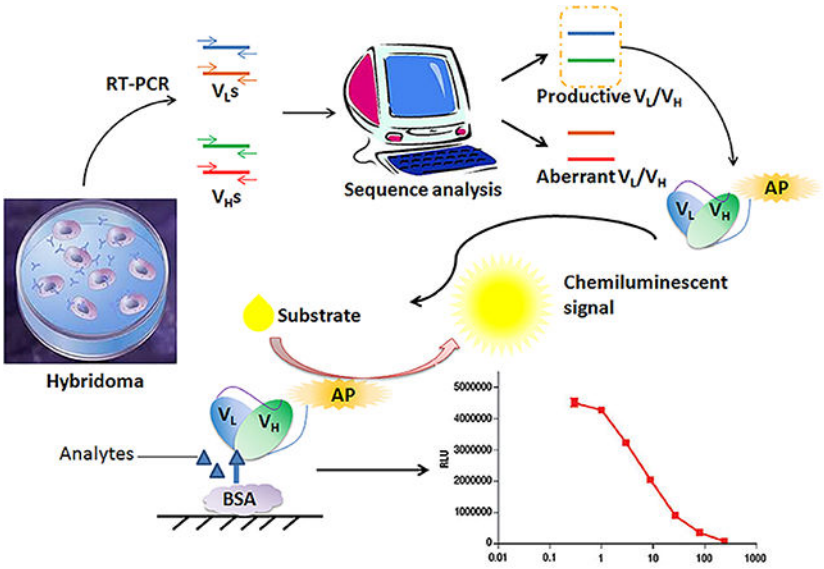
None.

Appendix A. Supplementary data

The supporting information has several aspects of concerns as follows: (1). Schematic of scFv assembly and plasmid construction for scFv-AP expression; Agarose gel electrophoresis of V_H genes amplified by PCR using HB/HF primers and LB/LF primers; Standard curves of LMG in PBST and different dilutions of sample extracted with solid-phase extraction (SPE) and the correlation of recovery of spiked samples determined by competitive CLEIA and HPLC. (2). Primers for scFv overlap extension and plasmid cloning, Structure of LMG, haptens, immunogen, and coating antigen, and the HPLC-FLD conditions for LMG detection. (3). The detail of database-assisted analysis result of V_H8–2, V_H2–3, V_L1–1, V_L4–1, and V_L2–4 sequenced by IMGT/HighV-QUEST online tool.

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 (IC_{50}) 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.

Graphical Abstract



Schematic diagram of Database-assisted workflow to prescreen scFv from hybridomas and the development of one-step CLEIA based on scFv-AP.

Keywords

Database-assisted sequence analysis; ScFv-AP based immunoassay; Malachite green; Residue determination; Tilapia fish

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). Thence, 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, Gelinas, 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 possess 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 V_H and V_L 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; X. Y. 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 V_H and V_L repertoire from hybridomas and select the functional V_H and V_L based on the database-assisted analysis. The aberrant V_H/V_L

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), leucocrystal 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*-nitrophenyl phosphate and 3-(2'-spiroadamantane)-4-methoxy-4-(3'-phosphoryloxy)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 V_H and V_L 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 V_H backward/V_H forward (HB/HF) or V_L backward/V_L forward (LB/LF) primer sets (Krebber, Bornhauser, Burmester, Honegger, Willuda, Bosshard, et al., 1997)were used for PCR amplification of all the V_H and V_L 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 V_H gene were selected and mixed with individual HF primers for PCR in the next round. The same procedure was performed to obtain the immunoglobulin V_L 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 V_H and V_L 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 V_H and V_L 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 OD_{600} 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 15,000*xg* 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 μLper well was pipetted into the transparent microplates. After overnight incubation at 37 °C, the plates were washed twice with PBST (0.01M PBS with 0.05% tween 20) to remove any unbound coating antigen. The plate was blocked with 250 μLper well blocking solution (5% (*w/v*) skim milk in PBS). After discarding the blocking solution, 50 μLper well LMG or single analogs from 729 to 0 ng/mL and 50 μLper 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 prepared AP-ELISA substrate solution (1 mM *p*-nitrophenyl phosphate, 10 mM $MgCl_2$, 50 mM $ZnCl_2$; 1 M Tris-HCl, pH 8.0) was pipetted into each well for 20 min incubation. The absorbance at 405 nm was measured by a multi-label counter followed by termination of the enzymatic reaction with 50 μL 3M 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 % H_2O_2 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 concentration of coating antigen (LMG hapten H14-OVA), was diluted to 0.21 μg/mL, and the working concentration of scFv1-AP was diluted to 1.2 μg/mL, respectively. LMG or single analogs were tested from 50 to 0 ng/mL. In one-step competitive CLEIA, 100 μL AP-CLEIA substrate solution (1 mM $MgCl_2$, and 0.67 mM AMPPD 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 analogs under optimized conditions. The cross-reactivity was calculated by the formula: 100 × IC_{50}(LMG)/IC_{50}(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 potassium borohydride (0.2 mol/L) was added as a reducing agent, the converting MG to LMG was complished by 10 min shaking in room temperature. Following liquid-liquid extraction with 5 mL dichloromethane, the lower organic layer was evaporated to partial dryness and then the residue was reconstituted in 500 μL ACN. The propane sulfonic acid strong cationic (PRS) 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 procedures 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 V_H and V_L repertoire from hybridoma cells

In general, recombinant scFvs are consists of two variable regions, heavy (V_H) and light chains (V_L); the V_H and V_L 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 V_H and V_L genes was performed using cDNA and the HB/HF or LB/LF primer sets. As shown in Fig. S2, V_H amplified products with an expected size of 350 bp were obtained using the following 10 sets of HB/HF primers: HB2/HF3, HB3/HF3, HB4/HF3, HB5/HF3, HB6/HF3, HB8/HF2, HB9/HF2, HB10/HF2, HB11/HF2, and HB13/HF2. Similarly, V_L 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 V_H/V_L amplified gene products were subcloned individually into the T overhang pEASY-T3 vector for amplification 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 the 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 distinguish between the functional and aberrant sequences, a database-assisted strategy based on IMGT/V-QUEST (Brochet, Lefranc, & Giudicelli, 2008) and IgBLAST 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 regions, and complementarity determining regions.

In our work, two different V_H (V_H8–2 and V_H2–3) sequences (Fig. 1a, Table S4, S5) were obtained. Sequence analysis from IGMT/VQUEST revealed that the V_H8–2 was productive without any stop codon. As shown in Table S5, the V_H2–3 sequence was unproductive and should not be translated into a functional protein. For V_L, three different V_L sequences (V_L1–1, V_L4–1, and V_L2–4) were obtained (Fig. 1b). The results of sequence analysis indicate that the V_L1–1 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 V_L1–1 sequence was deemed productive and rearranged correctly. However, the V_L4–1 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 IgBLAST. 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 V_L2–4 sequence contains a gene identical to V gene of Musmus IGKV3–12 (Table S8) with V_L4–1, although it's productive sequence reported in IMGT. We noted that the protein sequence of V_L2–4 aligned with V_L4–1 from FR1 to FR3, while differed at the CDR3/FR4 junction region (Fig. 1b). Therefore, we assumed that V_L2–4 was indeed the variant derived from the V_L4–1 potentially due to the rare rearrangement of V-J region during the passaging 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 V_L1–1 and disprove the V_L2–4 by experimental proced-ure, both V_L sequences were amplified for later assembly of the scFv genes with unique V_H8–2, named scFv1 and scFv2. The V_H and V_L 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 subsequent expression. The scFv–AP fusion protein had produced a distinct band (corresponding to an approximate mass of 75 kDa) on SDS-PAGE and was further verified by Western blotting (Fig. 2). In general, the molecular weight of an scFv is approximately 30 kDa, and the recombinant AP enzyme was approximately 43 kDa (Muller, Lamoure, Le Du, Cattolico, Lajeunesse, Lemaitre, et al., 2001). The results of SDS-PAGE and Western blotting confirmed that the size of the expressed scFv–AP fusion protein was as expected.

To further exploit the potency of the anti-LMG scFv–AP fusion protein, it was used as a dual functional reagent with antigen binding and signal amplification in a one-step heterologous ELISA. The optimized concentration of coating antigen and two scFv–AP fusion proteins were used to obtain the competitive ELISA calibration curve that was compared with the curve of Mab-based ELISA. The IC_{50} for scFv1-AP was 14.0 ng/mL in one-step ELISA (Fig. 3a) and the IC_{50} for parent Mab from original hybridoma was found to be 5.9 ng/mL in a classic two-step ELISA (Fig. 3b). Thus, a recombinant scFv1(V_H8–2/V_L1–1) with comparable binding affinity to its parent monoclonal antibody against LMG was generated. At variance, the scFv2 (V_H8–2/V_L2–4) showed nonspecific binding for LMG, the finding indicated that V_L2–4 was not a functional antigen-binding domain as it might be derived from hybridoma fusion partner as V_L4–1. In our case, this one-step competitive ELISA also served as an efficient approach to confirm the potency of the scFv candidates.

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, $AMP^{-}D$, 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 IC_{50} 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 (IC_{50}=1.3 ng/mL *vs.* IC_{50}=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 selective for LMG, which is in line with its parent monoclonal antibody (Wang, et al., 2017). Only slight cross-reactivity was observed for LCV and MG (4.65% and 1.36%, respectively), and low cross-reactivity (<0.01%) 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, however, MG can easily be reduced to LMG. The residues of MG and its metabolite, LMG, in foods of animal origin are monitored in many countries and the minimum required performance limit was set at 2.0 g/kg. 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 and/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 IC_{50} 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 ranged between 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 (R^2=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 fish.

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_L and V_H 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Database-assisted workflow to prescreen scFv from hybridomas was presented.
- The rearrangement between functional and the aberrant light chain was identified.
- Characterization of scFv candidates was conducted by one-step ELISA.
- A sensitive competitive CLEIA was developed based on scFv-AP fusion protein.
- The developed CLEIA was applied for determination of total MG in tilapia fish.

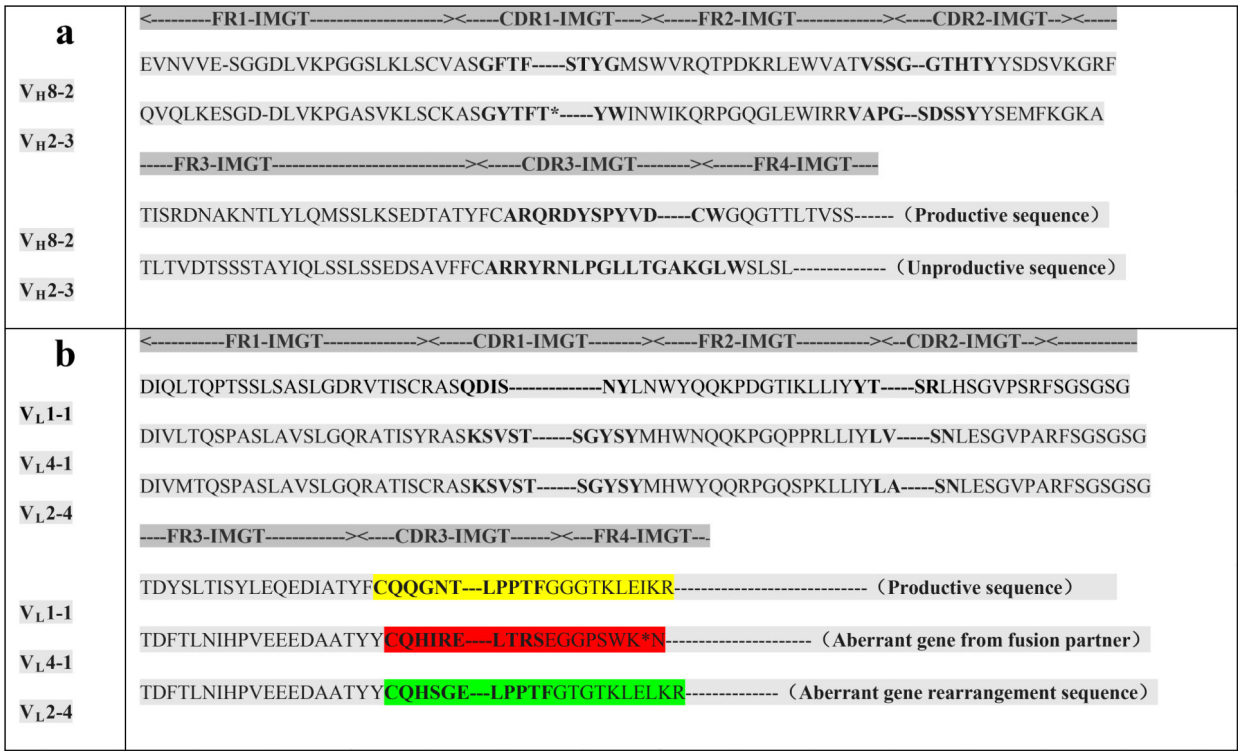

Fig. 1.

Protein sequence alignment of two unique V_H genes (a) amplified by PCR using HB/HF primers and three unique V_L genes (b) amplified by PCR using LB/LF primers. "*"in the protein sequence indicated stop codon occurs during translation. The CDR region was marked by black font. The CDR3/ FR4 junction region in V_L sequences are labeled with different colors.

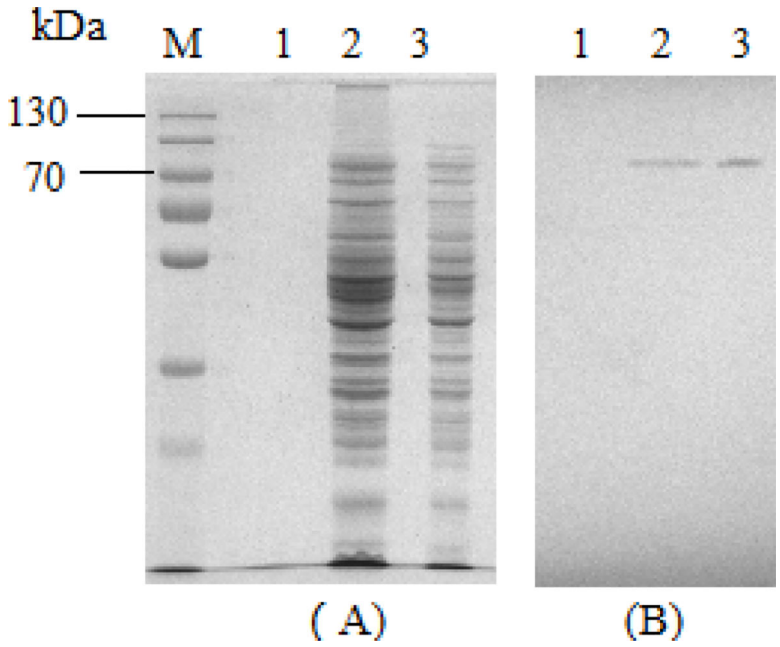

Fig. 2.

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.

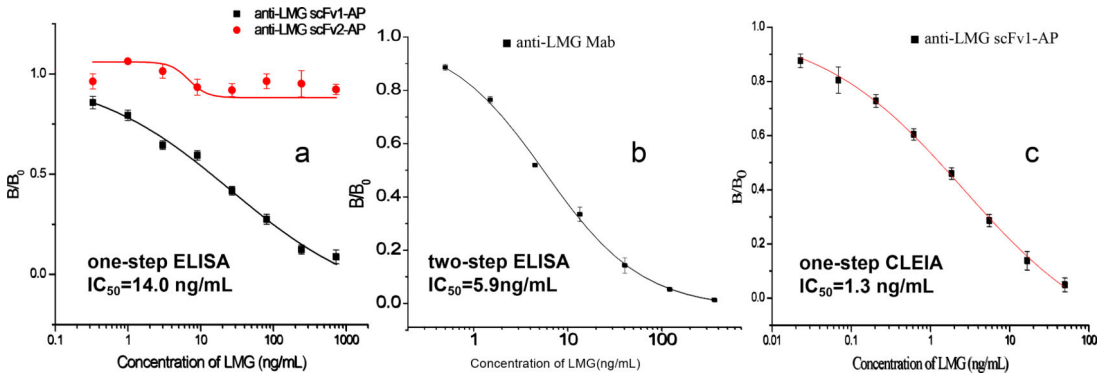

Fig. 3.

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).

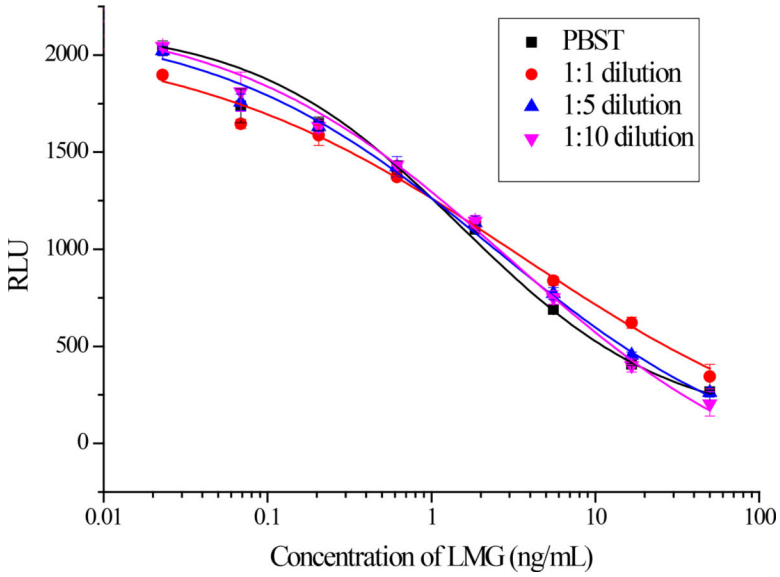

Fig. 4.

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.

Table 1.

Recovery rate of spiked tilapia fish samples determined by competitive-CLEIA (n=5)

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

Table 2.

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

Samples	scFv-AP based CLEIA	Mab-based ELISA	HPLC
NO.1	Not detectable	Not detectable	Not detectable
NO.2	2.2± 0.03	2.2± 0.05	2.3± 0.05
NO.3	Not detectable	Not detectable	Not detectable
NO.4	Not detectable	Not detectable	Not detectable
NO.5	9.3± 0.04	9.1± 0.07	9.4± 0.07