



Novel monoclonal antibody-sandwich immunochromatographic assay based on Fe₃O₄/Au nanoparticles for rapid detection of fish allergen parvalbumin

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ABSTRACT

In this study, a rapid sandwich immunochromatographic assay (ICA) was developed to detect parvalbumin (PV). Firstly, two optimum primary monoclonal antibody (mAb) against PV had been screened out: mAb1 was used as the capture antibody, and mAb2 conjugated to Fe₃O₄/Au nanoparticles (Fe₃O₄/AuNPs) that served as a detection reagent. Using this pair of mAbs, a sandwich ICA strip based on Fe₃O₄/AuNPs was developed. The results showed that the color intensity of test line positively correlated with the PV concentration in the standard or spiked sample. The limit of detection for qualitative (LOD) and quantitative detection (LOQ) were 2 ng/mL and 0.691 ng/mL, respectively. Besides, the detection time of this ICA strip was within 15 min. The recovery rates ranged from 104.0% to 117.4%, within an acceptable level (80–120%). Moreover, the developed assay also showed high cross reaction in different fish species. These results demonstrated that the established test strip has the potential to be used as a rapid screening tool for large scale determination of PV in foodstuffs.

1. Introduction

Fish has been recognized as one of eight food groups causing human allergies by Food and Agriculture Organization of the United Nations (FAO) (Gallo, Ferrara, Calogero, Montesano, & Naviglio, 2020). It was estimated that the prevalence rates of fish allergy ranged from 0.17 to 2.29% in the general population (Sharp, & Lopata, 2014). Even milligram dose of fish seems to be sufficient to trigger allergic reactions for supersensitive sufferers (Untersmayr, et al, 2007). As the consumption of fish has been growing worldwide due to its considerable nutritional value and health benefits (Dale, Madsen, & Lied, 2019), it is highly necessary to detect the fish allergens in foodstuffs, thus improving risk assessment and management in food products.

Parvalbumin (PV), the major fish allergen, belongs to the family of calcium-binding sarcoplasmic protein with low molecular weight (10-kDa to 12-kDa) (Kobayashi, et al, 2006). It was reported that 94% of patient with fish allergy are related to PV, which show a wide range of cross-reactivity among various fish species (Griesmeier, et al, 2010). Due to its high thermal stability, water solubility and resistance to enzymatic

digestion, PV is hard to be eliminated by cooking or industrial processing (Kobayashi, et al, 2016; Bugajska-Schretter, et al, 2000). To date, the methods for PV detection have greatly advanced, including real-time PCR (Herrero, Vieites, & Espiñeira, 2014), HPLC-MS/MS (Sun, et al, 2019; Carrera, Cañas, & Gallardo, 2012), enzyme-linked immunosorbent assay (ELISA) (Shibahara, Uesaka, Wang, Yamada, & Shiomi, 2013; Fæste, & Plassen, 2008; Cai, et al, 2013), immunoblotting (Sap-tarshi, Sharp, Kamath, & Lopata, 2014), and biosensor (Lu, Oshima, & Ushio, 2004; Jiang, et al, 2014). However, most of these methods are not suitable for rapid screening of samples in large scales due to the requirement of professional operating techniques, complex sample pretreatment and long analysis times. Therefore, there is an increasing demand for rapid, economical and visible methods to accurately detect fish PVs.

The immunochromatographic assay (ICA) based on the existing ELISA has been developed into a popular platform for qualitative, quantitative detection, and rapid sample screening (Zhao, Zhang, Liu, & Dong, 2018; Nguyen, Song, Park, & Joo, 2020). As compared with ELISA and other analysis methods, ICA method has unique advantages: the

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operation (after sample pretreatment) can be accomplished in less than 20 min, and the final results can be qualitatively detected by the naked eye without equipment and skilled technicians (Kong, Xie, Liu, Song, & Kuang, 2017; Li, et al, 2014), making it a highly promising candidate for on-site detection and home self-test. The development of a reliable ICA system involves several parameters, namely the type of ICA format (competitive vs non-competitive sandwich assay), the selection of antibodies (monoclonal antibody vs. polyclonal antibody) and the type of labels to be used (Diaz-Amigo, 2010; Huang, et al, 2019). So far, there are some reports focusing on the application of ICA in the detection of PV (Zheng, Wang, Lu, & Liu, 2012; Du, Lin, Sui, Wang, & Cao, 2014), most of them were based on the competitive format using polyclonal antibody. However, these methods were limited by the fact that the polyclonal antibody had poor reproducibility and stability. In addition, it was reported that the sandwich format was commonly used to detect macromolecular analytes, which was more sensitive and specific compared with the competitive format (Segura-Gil, et al, 2019). Besides, our previous study showed that the $\text{Fe}_3\text{O}_4/\text{AuNPs}$ could conjugate with antibodies and exhibited good immunocompetence as immunoprobe (Xu, Wang, Pan, Wei, & Wang, 2017). Compared with the colloidal gold nanoparticles (AuNPs) typically used in the ICA system, the $\text{Fe}_3\text{O}_4/\text{AuNPs}$ have superparamagnetism which makes it easy to be separated from the excess antibody by magnetic separation (Zhang, et al, 2020). These works motivated us to explore a novel sandwich ICA for the detection of fish allergen based on the sandwich format using the combination of $\text{Fe}_3\text{O}_4/\text{Au}$ nanoparticles and mAb.

In this study, we employed anti-PV mAb to develop a novel sandwich ICA based on $\text{Fe}_3\text{O}_4/\text{AuNPs}$ for rapid detection of PV. There are four major steps in this study: (a) two mAbs against different epitopes was prepared by hybridoma cell lines via cell fusion; (b) the magnetic nanoparticle functionalized with polyetherimide was synthesized by one-pot method and coated with two layers of gold nanoparticles on the surface to synthesize $\text{Fe}_3\text{O}_4/\text{Au}$ nanoparticles; (c) the two prepared anti-PV mAbs, one was conjugated with the $\text{Fe}_3\text{O}_4/\text{Au}$ nanoparticle to develop a detection immunoprobe, another one was as capture mAb; (d) a ICA strip for rapid detection of PV was constructed, and its performance for the detection of PVs was evaluated, including the qualitative detection with naked eyes and the quantitative detection with a strip scan reader, respectively.

2. Experiment

2.1. Materials

Freund's complete adjuvant, Freund's incomplete adjuvant and HAT (Hypoxanthine, Aminopterin and Thymidine) were purchased from Sigma-Aldrich (USA). The fetal bovine serum (FBS) was supplied from Sijiqing (Hangzhou, China). Bovine serum albumin (BSA), IMDM (Iscove's Modified Dubecco's Medium) culture medium, horseradish peroxidase (HRP), and goat anti-mouse immunoglobulin horseradish peroxidase (IgG-HRP) were purchased from Solarbio (Shanghai, China). Polyethyleneimine (PEI) and ethylene glycol were obtained from Aladdin Chemistry Co., Ltd. (Shanghai, China). Other reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Eight-week-old female BALB/c mice were purchased from SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Live silver carps were purchased from the local market.

2.2. Solutions

PV extraction buffer (pH 8.0) was composed of 20 mM Tris-HCl and 0.02 mM phenylmethylsulfonyl fluoride (PMSF). Buffers and solutions used in ELISA were prepared as follows: (1) carbonate buffer (CBS, 0.05 M, pH 9.6) for coating; (2) phosphate buffered saline (PBS, 0.01 M, pH 7.4) containing 1%(w/v) gelatin for blocking; (3) PBS containing 0.05% (v/v) Tween 20 (PBST) for washing; (4) PBS containing 0.1% (w/v, g/L)

gelatin for antibody dilution; (5) substrate buffer [0.1 M citrate-phosphate buffer containing 0.03% of 30% H_2O_2]; (6) 3,3',5,5'-Tetramethylbenzidine (TMB) solution (60 mg of TMB dissolved in 100 mL glycol); (7) TMB- substrate solution [5:1 (v/v) mixture of substrate buffer and TMB solution] for color development; and (8) 2 M H_2SO_4 solution for stopping the color development reaction.

2.3. Preparation of fish PV

Fish PV was prepared as follows: Live silver carps were skinned, gutted, boned and rinsed with ultrapure water, and the white muscle tissues were collected and homogenized with PV extraction buffer (w/v, 1:5) for 2 h at room temperature. After centrifugation, the supernatant was boiled for 10 min. After centrifugation at 4 °C, the boiled supernatant was transferred into a beaker and precipitated with 60% ammonium sulfate. The mixture was incubated at 4 °C overnight. After centrifugation, the supernatant was precipitated at 4 °C overnight for a second time with 100% ammonium sulfate. After centrifugation, the precipitate was resuspended and dialyzed with ultrapure water for 2 days. The dialyzed protein solution was lyophilized and stored at -20 °C for further use.

2.4. Preparation of mAb against fish PV

The extracted fish PV was used as the immunogen in mice to generate antibodies against fish PV. Briefly, 100 µg of PV with Freund's complete adjuvant (v/v, 1:1) was subcutaneously injected into BALB/c mice for the primary immunization, and 50 µg of PV with Freund's incomplete adjuvant (v/v, 1:1) was used for the next five booster immunizations. The blood was collected after each booster immunization and serum antibody titer was determined by an indirect ELISA. Then the mice with the highest serum antibody titer were intraperitoneally injected with 25 µg of PV (dissolved in 0.9% sodium chloride saline). Three days later, their spleen lymphocytes were isolated and fused with SP 2/0 cells using PEG1450 to produce hybridoma cells. The target hybridoma cells were screened with HAT medium by indirect ELISA and intraperitoneally injected into BALB/c mice for mass production of mAbs. The antibodies from ascites were purified with the caprylic acid-ammonium sulfate precipitation method (Xiang, Zhai, Tang, Wang, Liu, & Guo, 2010). The prepared mAbs were characterized by SDS-PAGE and Western-blotting, respectively. The subtypes of mAbs were determined by a commercial kit called "mouse monoclonal antibody isotyping identified reagents" (Shanghai Shifeng Biological Technology Co., Ltd).

2.5. Labelling of mAb against PV with HRP

The mAbs were labelled with HRP using a modified sodium periodate oxidation method (Kuang, et al, 2013). Briefly, the hydroxyl groups on HRP were oxidized to aldehyde groups by 60 mM NaIO_4 at 4 °C. The reaction was suspended by adding 160 mM ethylene glycol at room temperature for 30 min. The purified antibodies were added into the system, then CBS buffer (50 mM, pH 9.6) was added to adjust the pH to 9.0, and incubated at 4 °C overnight. After that, NaBH_4 (5.0 mg/mL) was added and incubated at 4 °C for 2 h. Then, an equal volume of 100% ammonium sulfate solution was added and incubated at 4 °C for 4 h, the mixture was centrifuged and the precipitate was resuspended and dialyzed with PBS (0.01 M, pH 7.4) for 3 days. The dialysate was centrifuged at 4 °C and the supernatant were stored with equal volume glycerin at -20 °C for further use. All the reactions and dialysis were performed in the dark.

2.6. Indirect ELISA

An indirect ELISA was used to determine serum antibody titers after booster immunization and select hybridoma cells. Briefly, the 96-well microplates were coated with 100 µL/well of PV (10 µg/mL) diluted

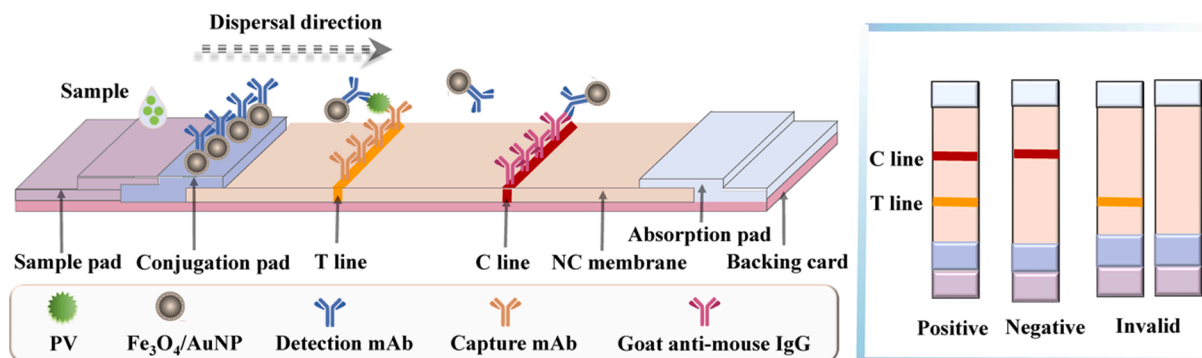


Fig. 1. Schematic diagram of the sandwich ICA test strip based on Au/Fe₃O₄NPs for the rapid detection of parvalbumin.

with coating buffer and incubated at 37 °C for 2 h. Then, the microplate was washed three times with washing buffer. Next, blocking buffer was added (200 µL/well) and incubated at 37 °C for 2 h. Then, 100 µL/well of mice serum or cell supernatant diluted with antibody dilution buffer was added and incubated at 37 °C for 30 min. After washing, 100 µL of HRP-labelled goat anti-mouse IgG (diluted 2000 times with antibody dilution buffer) was added to each well, and incubated at 37 °C for 30 min. After the plate being washed three times, 100 µL of substrate solution was added to each well and incubated at 37 °C for 15 min in the dark. The reaction was stopped by stop buffer (50 µL/well) and the absorbance was measured at 450 nm.

2.7. Pairwise interaction analysis

The prepared mAbs and HRP-labelled mAbs were analyzed as capture antibodies and detection antibodies, respectively. The optimal mAb pairing was evaluated by a sandwich ELISA described as following: A 96-well microplate was coated with capture antibody diluted with coating buffer (100 µL/well) overnight at 4 °C. Next, the microplate was washed three times with washing buffer, blocking buffer was added (200 µL/well) and incubated at 37 °C for 2 h. 100 µL/well of PV (10 ng/mL) was added and incubated at 37 °C for 1 h. After washing, HRP-labelled detection antibody was added (100 µL/well) and incubated at 37 °C for 1 h. After the plate being washed three times, substrate solution was added (100 µL/well) and incubated at 37 °C for 15 min. Then, the reaction was stopped by stop buffer (50 µL/well) and the absorbance was measured at 450 nm. PV (10 ng/mL) and PBS (10 mM) were as positive and negative controls, respectively. The highest OD₄₅₀ ratio of the positive and negative controls (P/N value) was regarded as the optimal pair for further study.

2.8. Development of specific anti-PV probe labelled with Fe₃O₄/Au nanoparticles

Firstly, the bilayer AuNPs-assembled Fe₃O₄/Au nanoparticles (Fe₃O₄/AuNPs) were prepared according to our previous work (Wang, Xu, Zhang, & Wei, 2013). Secondly, the Fe₃O₄/AuNPs-labelled anti-PV immunoprobes were obtained by the conjugation of anti-PV detection antibody and Fe₃O₄/AuNPs using the modified sodium periodate method. Briefly, after being washed three times with Tris-HCl buffer (0.05 M), Fe₃O₄/AuNPs (4 mg) were added to the prepared anti-PV antibody solution. The mixture was incubated at room temperature for 1 h. The Fe₃O₄/AuNPs conjugated with anti-PV mAbs were separated from the solution using a magnet, and then rinsed with PBSB (5% BSA in PBS) for 2 h to block the uncombined sites. Finally, the prepared immunoprobes (Fe₃O₄/AuNPs conjugated mAb) were rinsed three times with PBST (0.05% Tween-20 in PBS) and re-suspended in preserving solution at 4 °C for further use.

2.9. Optimization of Fe₃O₄/AuNPs-labeled anti-PV probe

To obtain the optimal immunoprobes for the detection of PV, the reaction conditions for the conjugation of anti-PV detection antibody and Fe₃O₄/AuNPs were optimized, including pH value (6.0, 6.5, 7.0, 7.4, 8.0 and 8.4), antibody dose (6, 12, 24, 36, 48, 60 and 72 µg/mL), reaction time (20, 30, 40, 50, 60 and 70 min). The content of mAb contained in the supernatant was detected before and after conjugation and the coupling rate was set according the formula as follows:

$$\text{The coupling rate} = (A_{\text{total-mAb}} - A_{\text{unbound-mAb}}) / A_{\text{total-mAb}} \quad (1)$$

where $A_{\text{total-mAb}}$ was the the absorbance of total mAb used to react with Fe₃O₄/AuNPs, and $A_{\text{unbound-mAb}}$ was the the absorbance of free mAb after conjugation and washing procedures at 280 nm, respectively.

2.10. Fabrication of immunochromatographic assay strip

The immunochromatographic assay strip based on a sandwich format was fabricated in our laboratory, utilizing two primary antibodies against PV and one secondary antibody (goat anti-mouse IgG). The schematic view of the ICA strip was shown in Fig. 1(left). It can be seen that the test strip was composed of five sections: sample pad, conjugation pad, nitrocellulose (NC) membrane, absorption pad and plastic backing plate. Typically, one of the primary anti-PV mAbs (capture reagent) and the goat anti-mouse IgG antibody (2 mg/mL) were sprayed onto the NC membrane to form the test line (T Line) and control line (C Line), respectively. Besides, the conjugation pad was immersed in the Fe₃O₄/AuNPs-labeled anti-PV mAb solution (detection reagent). After drying at 37 °C, the NC membrane was pasted onto the center of the plastic backing plate. The sample and absorption pad were glued to the bottom and upper section of the plastic backing plate with a 5-mm overlap of the NC membrane. Finally, the backing plate was cut into 3-mm wide individual test strips and stored in a desiccator for further use.

2.11. Working principle of the sandwich ICA strip for the detection of PV

The working principle of the sandwich ICA strip was primarily based on a one-step lateral flow format as follows: sample solution (100 µL) was dropped onto the sample pad, and allowed to migrate along the NC membrane due to capillary action. The analyte would form a mobile sandwich complex with Fe₃O₄/AuNPs-labeled mAb (detection reagent) in the conjugation pad zone. The immobilized capture antibody in the T line would capture the sandwich complex out of the sample flow. If sufficient PV was present, a test line was getting visible within the test line zone. As free Fe₃O₄/AuNPs-labeled mAb can be captured by goat anti-mouse IgG antibody within the C line zone, the control line would always exist with each analysis. Therefore, as illustrated in Fig. 1(right), a positive result could be judged if both T line and C line appear; a negative result could be judged only if the C line appears; the strip is

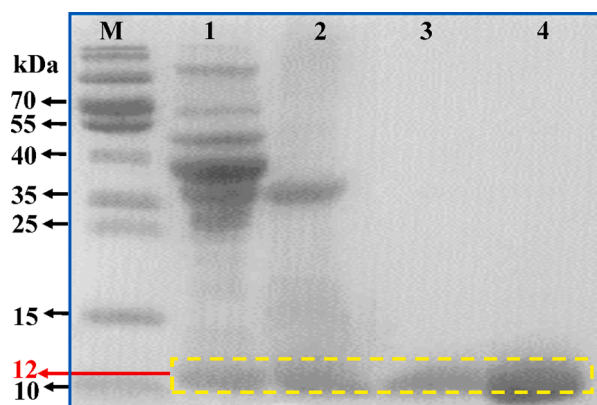


Fig. 2. SDS-PAGE analysis of parvalbumin isolated from the silver carp muscle. M: Protein marker; 1: Crude protein extract; 2: Supernatant after boiling; 3: Supernatant after ammonium sulfate fractionation; 4: Purified parvalbumin after lyophilization.

invalid if the C line does not appear.

2.12. Performance evaluation of ICA strip

The sensitivity of the established ICA strip was determined using serial dilutions of PV (100 ng/mL, 80 ng/mL, 60 ng/mL, 40 ng/mL, 20 ng/mL, 10 ng/mL, 5 ng/mL, 4 ng/mL, 3 ng/mL, 2 ng/mL, and 0 ng/mL as blank control). For qualitative analysis, the visual limit of detection (LOD) value was defined as the lowest concentration of PV that T line could be seen with naked eyes. Quantitative analysis was performed using a strip scan reader. The color intensity of T line and C line was measured, and a calibration curve was established taking the concentration of the PV as the abscissa and the optical intensity ratio of T line to C line (A_T/A_C) as the ordinate.

2.13. Sample treatment

The food samples (fish) were homogenized with 20 mM Tris-HCl extraction buffer (within 0.02 mM PMSF, pH 8.0), and then the mixtures were boiled for 10 min and centrifuged at 10,000 rpm for 20 min. The supernatants collected were used as the food extract samples.

3. Results and discussion

3.1. Isolation of PV

The purity identification of PV was performed by SDS-PAGE (Fig. 2). Lane 1 was the crude protein extract using weakly alkaline extraction buffer with low ionic strength. It suggested that the target protein and some heteroproteins can be separated effectively by changing the composition of buffer. Lane 2 was the supernatant after boiling. Compared with the crude protein extract of Lane 1, the sample showed that the residual thermosensitive proteins were removed by boiling. However, there still existed undesired proteins in the samples after boiling, especially the one with a molecular weight of 37 kDa. As shown in Lane 3, it was obvious that the 60–100% ammonium sulfate fractionation effectively removed the impure protein of 37 kDa completely and played a positive role in the accumulation of the target proteins of about 12 kDa during the purification process. Lane 4 indicated that the highly purified PV was obtained after lyophilization.

3.2. Screening and characterization of mAbs against PV

In this study, purified PV from silver carp was used as antigen to prepare corresponding anti-PV mAb. After a series of screening, three well-grown hybridoma cell lines secreting mAb against PV were named 2C3, 3C3, and 2A1, respectively. The corresponding antibody titers of three ascites were more than 1/128,000 by indirect ELISA, which indicated that isolated hybridoma cells could produce sufficient target antibodies. After purification of ascites, three mAbs were obtained and identified by SDS-PAGE. The mAbs against PV secreted from 2C3, 3C3, and 2A1 were named mAb1, mAb2, and mAb3, respectively. As shown in Fig. 3A, the analysis of SDS-PAGE indicated the obtained three mAbs were highly purified and composed of a heavy chain (52 kDa) and a light chain (28 kDa). Besides, the subtypes of all the three mAbs were identified as IgG1.

The specificity of mAb was analyzed by Western blot. As shown in Fig. 3B, the mAbs only were recognized by target proteins with molecular weight around 12 kDa, indicating the high specificity of both mAb1 and mAb2 against PV.

3.3. Pairwise interaction analysis

In our study, a sandwich format based on two mAbs against different epitopes was established, one antibody for capture and another for detection. The complex “mAb1-PV-[mAb2-HRP]” with the strongest conjugation was requested. In order to obtain the best pairwise mAbs, a

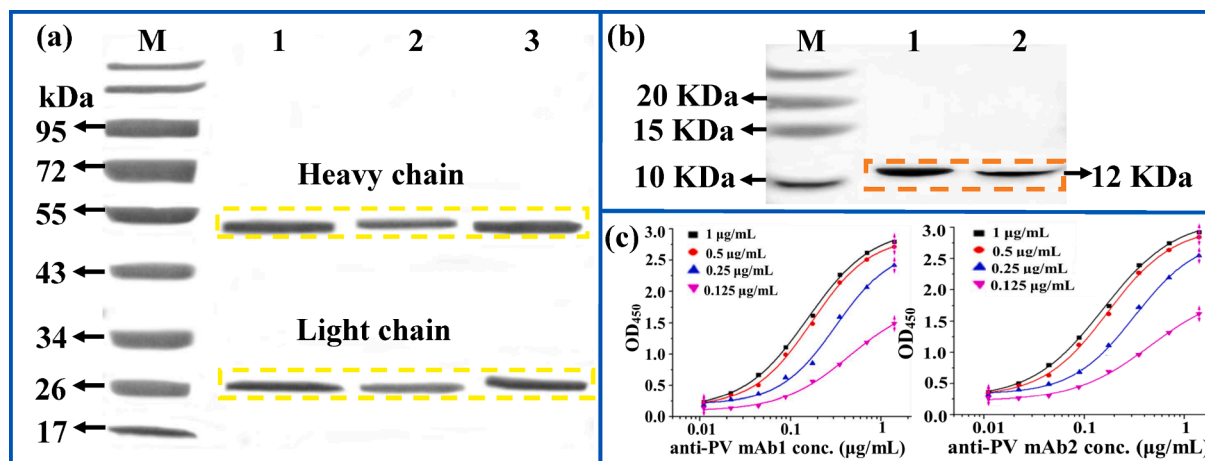


Fig. 3. (a) SDS-PAGE analysis for the purity of mAb against parvalbumin. (b) Western-blot analysis for the specificity of mAb against parvalbumin. (c) The affinity analysis of mAb1 (right) and mAb2 (left) against parvalbumin, respectively. M: protein marker, 1: mAb1; 2: mAb2; 3: mAb3.

Table 1

P/N value in sandwich ELISA for pairwise analysis.

Detection mAb	Capture mAb		
	mAb1	mAb2	mAb3
mAb1-HRP	–	15.3	2.14
mAb2-HRP	14.05	–	2.84
mAb3-HRP	2.05	3.03	–

sandwich ELISA was developed based on mAbs (capture antibody) and HRP-labelled mAbs (detection antibody). As shown in Table 1, the highest P/N value was obtained in the format of “mAb2-PV-[mAb1-HRP]” among nine combinations. Therefore, the combination of mAb1 (detection antibody) and mAb2 (capture antibody) was selected as the

optimal pair for further experiments.

Besides, the affinity constant (K_{aff}), which reflects the binding strength of antibody for monovalent epitope, was determined by indirect ELISA (Loomans, Roelen, Damme, Bloemers, Gribnau, & Schielen, 1995). Briefly, the microplate was coated with different concentration of PV (1, 0.5, 0.25, 0.125 $\mu\text{g/mL}$) and added with mAbs in a serial of diluted concentration as the primary antibody. According to the curves in Fig. 3C, D, the K_{aff} of mAb1 and mAb2 were calculated to be 3.0×10^8 L/mol and 2.1×10^9 L/mol, respectively. These results exhibited that both mAb1 and mAb2 had high affinity, which were highly consistent with the with the high P/N value shown in Table.1, thus could bind large amount of PV antigen with a great stability (Lipman, Jackson, Trudel, & Weis-Garcia, 2005).

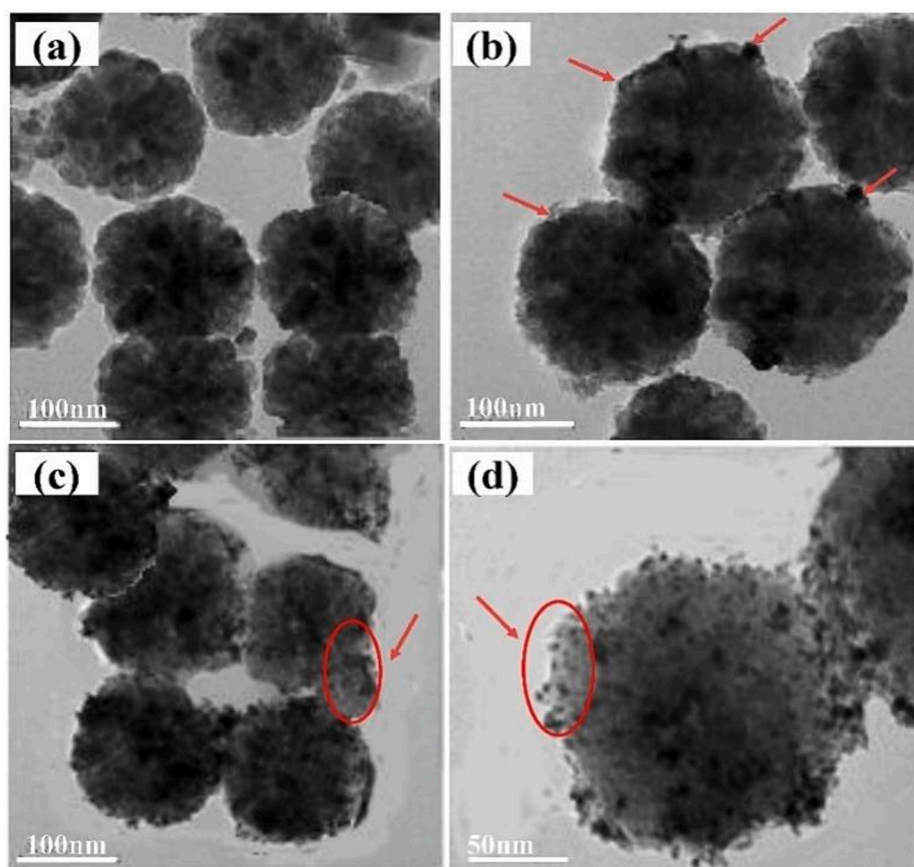


Fig. 4. TEM image of $\text{Fe}_3\text{O}_4/\text{Au}$ nanoparticle synthetic process in different steps: (a) The microstructure of $\text{Fe}_3\text{O}_4\text{-PEI}$. (b) The microstructure of $\text{Fe}_3\text{O}_4\text{-PEI-Au}_1$. (c, d) The structure of $\text{Fe}_3\text{O}_4\text{-PEI-Au}_1\text{-PEI-Au}_2$.

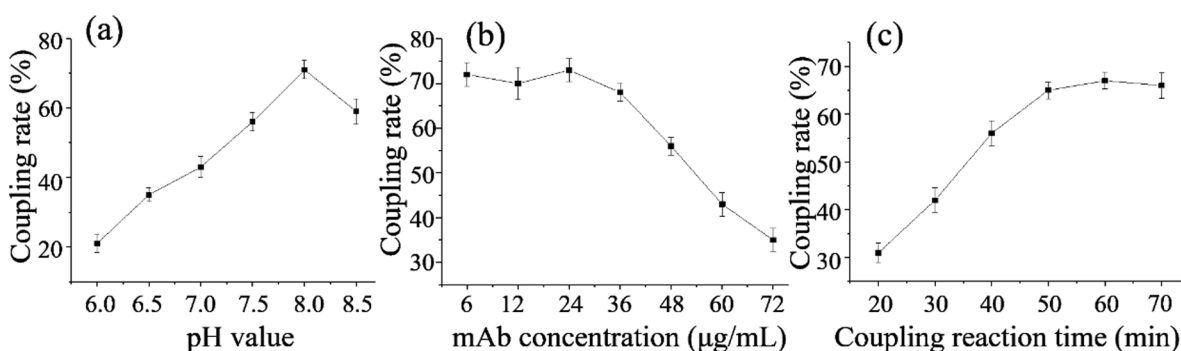


Fig. 5. Optimization for the preparation of $\text{Au}/\text{Fe}_3\text{O}_4\text{NPs}$ -labelled immunoprobes. Effects of (a) pH value, (b) mAb concentration and (c) coupling reaction time on the coupling rate of $\text{Fe}_3\text{O}_4/\text{AuNPs}$ and mAb against parvalbumin.

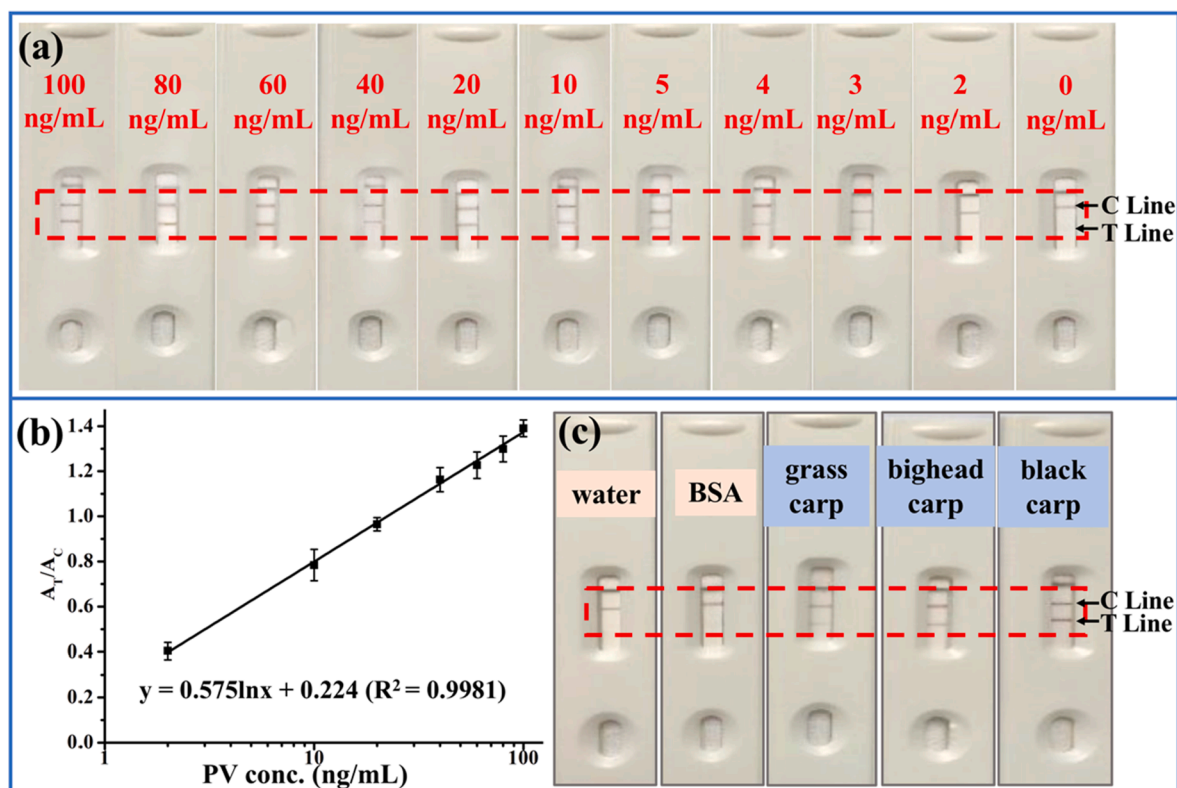


Fig. 6. (a) The sensitivity analysis with a serial concentration of parvalbumin. (b) Calibration curve for parvalbumin. (c) The cross-reactivity analysis of PVs extracted from different species of fish.

3.4. Preparation and characterization of $\text{Fe}_3\text{O}_4/\text{AuNPs}$

According to our previous work, the $\text{Fe}_3\text{O}_4/\text{AuNPs}$, constructed by coating two layers of small-sized gold nanoparticles (AuNPs) onto the surface of PEI-modified magnetic cores (Fe_3O_4), conjugated with antibodies exhibited a great potential for immunoassay applications in vitro (Xu, Wang, Pan, Wei, & Wang, 2017). Here, the $\text{Fe}_3\text{O}_4/\text{AuNPs}$ was constructed and characterized by TEM (Fig. 4). Firstly, the structure of $\text{Fe}_3\text{O}_4\text{-PEI}$ was shown in Fig. 4A, it was obvious that a light-colored organic cationic polyelectrolyte layer of PEI was coated on the surface of Fe_3O_4 core. Furthermore, Fig. 4B was the structure of $\text{Fe}_3\text{O}_4\text{-PEI-Au}_1$, it can be seen that only a small of Au particles were absorbed on the surface of the magnetic core. The $\text{Fe}_3\text{O}_4\text{-PEI-Au}_1\text{-PEI-Au}_2$ composite were shown in Fig. 4C, D, it was observed that abundant small-sized AuNPs were uniformly distributed on the surface of the magnetic cores. Due to the second layer of PEI, more amino groups were introduced to the system, thus helping to combine more AuNPs through electrostatic interaction (Hu, Li, Shen, & Shi, 2014). These results indicated the successful synthesis of $\text{Fe}_3\text{O}_4/\text{AuNPs}$ as well as the uniformity and good dispersion of the particles, and the average diameter was approximately calculated as 150 nm.

3.5. Optimization of $\text{Fe}_3\text{O}_4/\text{AuNPs}$ -labelled immunoprobes

To obtain optimum immunoprobes, the major factors affecting the coupling of $\text{Fe}_3\text{O}_4/\text{AuNPs}$ to mAb, including pH value, mAb concentration, and coupling reaction time, were investigated (Fig. 5). For the pH effect, as shown in Fig. 5A, the coupling ratio increased with the pH until it reached a peak at approximately pH 8.0, and then began to decrease. It was found that $\text{Fe}_3\text{O}_4/\text{AuNPs}$ -labelled immunoprobes exhibited the most stable state at pH = 8.0, mainly due to the electrostatic interaction between the negative charges on the surface of the AuNPs in a weakly alkaline solution. Accordingly, pH = 8.0 was selected

as the optimum pH value for the subsequent preparation of immunoprobes.

Under the optimal pH value, mAb concentration was further optimized. As shown in Fig. 5B, the coupling rates had relatively slight increase with the increase of mAb concentrations. However, the coupling rates declined significantly when the mAb concentration exceeded 24 $\mu\text{g/mL}$, largely because that the surface of the $\text{Fe}_3\text{O}_4/\text{AuNPs}$ was saturated with mAb. Given the efficient binding and cost saving, the mAb concentration was optimized at 24 $\mu\text{g/mL}$ for the conjugation on the $\text{Fe}_3\text{O}_4/\text{AuNPs}$.

For the coupling reaction time, as shown in Fig. 5C, it was observed that the coupling rate gradually increased with reaction time. However, the coupling rate was maintained after 50 min, indicating that the coupling reaction reached saturation. Thus, the optimal reaction time was 50 min.

3.6. Sensitivity analysis of ICA strip

The sensitivity of the established ICA strip was evaluated with a series of PV concentrations. As shown in Fig. 6A, a grey color in the test line was observed with an intensity proportional to PV concentration. The color intensity of the T line completely disappeared at 2 ng/mL, which was estimated as the LOD of the established ICA for PV. This qualitative detection based on the visual evaluation with naked eyes did not require any equipment, and the result was obtained by the naked eyes within 15 min.

PV quantitative detection data was illustrated in Fig. 6B. The calibration curve of ICA for quantitation of PV was plotted by the A_T/A_C ratio against PV concentration. It showed a linear range from 0 ng/mL to 100 ng/mL, and the fitting equation was: $y = 0.575\ln x + 0.224$ ($R^2 = 0.9981$). The LOD of quantitative detection was calculated by substituting y_L into standard curve formula. The value of y_L was estimated as the arithmetical mean of the blank sample (y_b) plus three times

Table 2

Comparison with the existing PV immunoassays.

Methods	Linear range (µg/L)	Detection limit (µg/L)		Detection time	Reference
		LOD	LOQ		
Sandwich ELISA	0.49–500	0.11	0.22	1–2 h	Fæste, & Plassen, 2008
Lateral-flow immunoassay	10–105	500	46	20 min	Zheng, Wang, Lu, & Liu, 2012
Competitive ELISA	0.625–10,240	0.8	5.59	1–2 h	Cai, et al, 2013
Sandwich ELISA	0.78–50	0.58	1.76	1–2 h	Shibahara, Uesaka, Wang, Yamada, & Shiomi, 2013
Capillary ICA	50–1000	70	40	30 min	Du, et al, 2014
ICA strip	2–100	2	0.691	15 min	This work

Table 3

The recovery rates of PV using developed ICA strips.

Spiked level (ng/mL)	Intra-assay (n = 5)		Inter-assay (n = 5)	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)
4	107.5 ± 12.7	12	104.0 ± 12.8	12.4
8	106.4 ± 7.1	6.7	108.3 ± 8.9	8.3
10	117.4 ± 10.2	8.7	108.2 ± 10.4	9.6

the standard deviation obtained on the blank sample (s_b), as the following equation (Zheng, Wang, Lu, & Liu, 2012; Klewitz, Gessler, Beer, Pflanz, & Scheper, 2006):

$$y_L = y_b + ks_b \quad (2)$$

where k is a numerical factor chosen according to the confidence level desired ($k = 3$). In this study, $y_b = 0.00729$ (3 replicates), and $s_b = 0.00126$. According to the calculation result, the LOD of the calibration curve was 0.691 ng/mL.

Besides, the analytical performance of the present ICA strip had been compared to other determination methods reported (Table.2). Compared with the reported immunoassays against PV (Fæste, & Plassen, 2008; Zheng, Wang, Lu, & Liu, 2012; Shibahara, Uesaka, Wang, Yamada, & Shiomi, 2013; Cai, et al, 2013; Du, Lin, Sui, Wang, & Cao, 2014), the established ICA strip displayed a better performance in term of test time and sensitivity. These results demonstrated that our developed sandwich ICA strip seemed sufficient as a rapid and effective tool for routine monitoring of trace PV in food stuffs as well as screening large numbers of samples, thus minimizing the risk for fish allergic consumers.

3.7. Recovery test with spiked samples

Based on the standard curve of quantitative detection for PV, the food samples were spiked of different concentrations (4, 8 and 10 ng/mL) and recovered to evaluate the accuracy and precision of strips. The spiked levels and recovery results for PV were shown in Table.3. The average recoveries for PV ranged from 105.8% to 117.4% for intra and 104.0% to 108.3% for inter, respectively, which were within the criteria of acceptance (80–120%) for the repeatability of the method (Costa, Ansari, Mafra, Oliveira, & Baumgartner, 2015; Du, Lin, Sui, Wang, & Cao, 2014), indicating good accuracy. The precision of the method was validated by performing five replicates. The RSD for intra- and inter-assay were less than 12.0% and 12.4%, respectively. These results indicated that the established ICA strip exhibited good stability and high accuracy.

3.8. Detection of PVs from different species of fish

PVs extracted from grass carp, bighead carp and black carp were determined using the ICA strip. Water and BSA were utilized as negative controls. As shown in Fig. 6C, the results of grass carp, bighead carp and black carp were positive, and the results of water and BSA were negative, respectively. However, at the same concentration of PV (20 ng/

mL), it was observed that there existed obvious differences in relative color intensities of T line for the three species of fish. This may be due to the low homology of the identification sites of mAbs with the PVs from different species of fish (Griesmeier, et al, 2010). These results suggested that the developed ICA strip in this study was suitable in detecting the major allergen in various species of fish.

4. Conclusion

In this study, a novel mAb-sandwich immunochromatographic assay based on $Fe_3O_4/AuNPs$ was established for rapid detection of PV in fish. Our data have demonstrated that this assay was highly efficient to determine the PV in simple matrixes, with the advantages of superior sensitivity, time saving and great accuracy and precision. The test could be accomplished within 15 min without complicated handling procedures. This rapid and effective tool is suitable for sensitive and on-site mass sample screening and would be a potential candidate in future analytical applications of food safety. However, it remains to be further studied on its application to complex food matrixes.

CRedit authorship contribution statement

Mengke Zhang: Writing - original draft, Software, Data curation, Visualization. **Mengyin Li:** Investigation, Methodology. **Yan Zhao:** Formal analysis. **Naifeng Xu:** Resources. **Lanlan Peng:** Visualization. **Yuanfeng Wang:** Supervision. **Xinlin Wei:** Funding acquisition, Supervision.

Declaration of Competing Interest

There are no conflicts of interest to declare.

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