

The matrix interference to the immunoassay of food samples: The effect of some proteins in aquatic products

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ABSTRACT

The elimination of matrix interference is now a task for further development and application of immunoassays. Here the effect of proteins in aquatic samples on immunoassay was investigated by western blotting and competitive indirect enzyme-labeled immunosorbent assay (ci-ELISA). Some proteins in flatfishes interacted with different antibodies, resulting in significant loss of accuracy of the immunoassays. Moreover, these matrix proteins prefer to interact with enzyme-labeled antibodies than label-free ones. Such a matrix protein-immunoglobulin interaction was validated with different fish and shrimp samples and similar results were observed, which demonstrated a universal significance of the protein-induced matrix interference to immunoassays. Besides, the strategies to prevent such interference were preliminarily discussed.

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1. Introduction

There is now an increasing application of immunoassays for the analysis of diverse food-stuffs. Besides the excellent specificity and sensitivity, the relative simplicity and low cost are also considered as the main advantages of such techniques, which make them especially suitable for fast screening of large amounts of samples (Krska et al., 2012; Richman, Karthikeyan, Bennett, Chung, & Lee, 1996; Wang, Zhang, Yang, Wang, & Zhang, 2005; Xu, Sui, Cao, & Lin, 2010). Despite enormous advances in immunoassays, the unwanted interference from the matrix (so-called matrix effect) is still an important obstacle to overcome (Selby, 1999). The complex and variable chemical components in food samples might greatly interfere with the immunological reactions, resulting in significantly reduced accuracy and sensitivity, and even false results (Mitchell & Lowe, 2009; Tiwari et al., 2010; Zhang et al., 2007). In past years such matrix interference has been reported in a wide range of food samples, including vegetable, tea, fruit, chocolate, dairy, nuts, confectionery, shellfish, animal muscles and fishes (Liu, Fang, Zhang, Zheng, & Wang, 2009; Naar et al., 2002; Tate & Ward, 2004). However, due to very limited information about the mechanism of the matrix effect, till now it still seems difficult to develop reasonable and simple techniques to solve the problem. In most cases complex pretreatments of the samples were exploited in trying to diminish the

interference (Chiu et al., 2010; Mitchell & Lowe, 2009) at the expense of significantly reduced simplicity and sensitivity of the immunoassays.

Considering the diversity in sample composition and analytical procedures, multiple factors may be involved in the matrix effect in various immunological systems (Selby, 1999; Tiwari et al., 2010). In comparison to lipid, carbohydrates, nucleic acid, salts and other coexistent components, some matrix proteins were indicated more important sources of the interference, which were assumed to affect the immunological reactions by nonspecific protein–surface or/and protein–protein interactions. Jeyachandran, Mielczarski, Mielczarski, and Rai (2010) once reported that the difference in polystyrene microplates could greatly affect the absorption of proteins (BSA, IgG and SpA) on the plate surface, therefore may result in a significant variation in the result of immunosorbent assays. Tiwari et al. (2010) indicated that one of the reasons for the matrix interference in immunorecognition of amandin might be attributed to the direct interaction between food proteins and rabbit polyclonal antibodies, but such an assumption has not been validated by any experiments. More interesting information was provided in the studies of Dory et al. (1998) in which a significant cross-reaction was observed between some proteins from codfish with an anti-parvalbumin monoclonal antibody in western blotting. However, it is not sure that, such a nonspecific binding has universal significance as a source of interference for other immunoassays, or it is just due to the high conformational similarity between the proteins and the antigen (parvalbumin). In our previous study (Wang, Lin, Sui, & Cao, 2013), some proteins from flatfishes were also demonstrated to interfere strongly with the enzyme-linked immunosorbent assay (ELISA) of an antibiotic (norfloxacin); moreover, they could result in significant positive bands in western blotting in the presence of antibody reagents, indicating that the fish protein–IgG interaction might play an important role in the matrix interference observed.

Here using ELISA and western blotting, the interaction between the fish proteins and different immunoglobulins (including IgG and IgE) was further validated, the reaction path was preliminarily clarified, and the mechanism for the matrix interference was proposed. Moreover, the distribution of the matrix protein and its interference to ELISA in different aquatic samples were investigated, and the universal significance of such a protein–protein interaction to ELISA was discussed.

2. Materials and methods

2.1. Materials

Iced fresh *Scophthalmus maximus*, *Paralichthys olivaceus*, *Cynoglossus robustus*, *Cyprinus carpio*, *Ctenopharynxodon idellus*, *Penacus orientalis*, *Lateolabrax* sp and *Pseudosciaena crocea* were purchased from Liqun supermarket (Qingdao, P.R. China), and stored at -20°C until use. The ELISA test kits were purchased from Wanger Biotechnology Co. Ltd (Beijing, P.R. China) and preserved at 4°C . Horseradish peroxidase (HRP), HRP-labeled goat anti human IgG and alkaline phosphatase (AP)-labeled goat anti-rabbit IgG were purchased from Sigma-Aldrich Co. LLC. (Saint Louis, MO, USA) and preserved at -20°C . HRP-labeled monoclonal mouse IgG of V5-Tag, HRP-labeled goat anti-mouse IgG and HRP-labeled human IgE were purchased from Hangzhou HuaAn Biotechnology Co. Ltd (Hangzhou, P.R. China). Bromo-Chloro-Indolyl Phosphate and Nitro Blue

Tetrazolium (BCIP/NBT) were purchased from Boster Bioengineering limited company (Wuhan, P.R. China). Molecular weight markers were purchased from Fermentas Co. Ltd (Vilnius, Lithuania). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore Corporation (Billerica, MA, USA). Ponceau S was purchased from Beyotime Institute of Biotechnology (Shanghai, P.R. China). All other chemicals were of analytical grade unless otherwise stated.

2.2. Instruments

All centrifugations were performed with BR-4i (Jouan, Saint-Herblain, France). Western blotting results were recorded by Tanon180 Gel imaging system (Tanon Science & Technology Co. Ltd. Shanghai, P.R. China). The ELISA results were recorded with a plate reader (Multiskan MK3, Thermo Labsystems Co., Ltd, Shanghai, China). The Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) results were captured by HP Scanjet G4050 (China Hewlett-Packard Co., Ltd, Beijing, P.R. China). The solutions were prepared with deionized water from a Milli-Q system (Millipore, Billerica, MA, USA).

2.3. Preparation of food matrix

The crude fish extracts were prepared according to the procedure described by [Chen and Hwang et al \(2002\)](#). 5 g of fish samples were mixed with phosphate buffer (with an ionic strength value of 0.02 mol L^{-1} , pH 7.4, PBS) at a ratio of 1:1 (w:v) and then homogenized for 1 min. The mixture was then centrifuged for 15 mins at 4°C ($4132 \times g$), and the precipitate was extracted with the same procedure again. The supernatants were combined. Then the fish crude protein extracts were used for further experiments.

And the proteins in *P. olivaceus* extracts were further purified by sephadex G-100 according to our previous studies ([Wang et al., 2013](#)), two forms of protein fractions referred as f1 and f2, respectively, were obtained and dissolved in PBS.

2.4. SDS-PAGE of fish proteins

The protein content in crude fish protein extracts or isolated protein fractions by sephadex G-100 was adjusted to a final concentration of 1 mg mL^{-1} with water. The discontinuous SDS-PAGE was performed according to the procedure described by Wang, Li, Zheng, Liu, and Lin (2011) under the denatured condition. The stacking and separating gels contained 50 and 150 g L^{-1} of acrylamide (Sigma, St Louis, MO, USA), respectively. The initial voltage was 80 volts, and then was increased to 120 volts when the tracking dye reached the separating gel. Gels were immersed in coomassie brilliant blue R-250 (1 g L^{-1} , in a mixture of methanol, acetic acid and water with a volume ratio of 1:2: 7) for 20 mins, and then immersed in a de-staining solution (methanol:acetic acid:water = 1:1:8, v:v:v) overnight. The picture of treated gels was captured by HP Scanjet G4050 (China Hewlett-Packard Co., Ltd, Beijing, P.R. China).

2.5. ELISA procedures

The stocking solution of norfloxacin standard (0.1 mg mL^{-1} , from the test kit) was serially diluted with PBS or fish extracts. The samples were determined by ci-ELISA with a commercial fluoroquinolones ELISA test kit according to the manufacturer's instructions as follows: (1) all the reagents needed were taken out from the storage environment and kept at 25°C for 30 mins; (2) $50 \mu\text{L}$ of PBS solutions or samples containing norfloxacin was added to the microplate followed by adding the mixture of primary antibody solution and HRP-labeled secondary antibody solution with a ratio of 10:1 (v:v), and the microplate was then incubated at 25°C for 25 mins in the dark; (3) the plate was washed with the washing solution (from the test kit) for five times; (4) $50 \mu\text{L}$ of substrate solution A (from the test kit) was added to each well followed by the addition of solution B ($50 \mu\text{L}$) and incubation at 25°C for 15 mins in the dark; (5) the reaction was stopped by the addition of stopping solution ($50 \mu\text{L}$); (6) the absorbance value of each well at 450 nm was finally determined with a plate reader. Blank controls were prepared in the same way, except that PBS was used to replace norfloxacin solutions. Absorbance values with and without norfloxacin were recorded as A and A_0 , respectively, and the inhibition ratio was calculated as follows:

$$\text{Inhibition ratio (\%)} = \left[1 - \left(\frac{A}{A_0} \right) \right] \times 100\%.$$

To investigate the effect of the *P. olivaceus* proteins (f1 and f2) on different steps of the ci-ELISA, five groups of experiments were performed, as given in Table 1. Fifty microliter PBS or norfloxacin-spiked samples were added to the microplate followed by the addition of anti-norfloxacin antibody solutions, and the microplate was incubated for 30 mins. The microplates were washed five times as mentioned above, and then $50 \mu\text{L}$ of secondary antibody (diluted ten times in PBS) and $50 \mu\text{L}$ of PBS (or fish protein solutions) were added sequentially. All other procedures were the same as those mentioned above.

2.6. Western blotting

Five antibodies and two different enzyme labels were prepared as follows: HRP-labeled monoclonal mouse IgG was diluted 1:3000 with PBS; HRP-labeled goat anti-mouse IgG (secondary antibody of anti-norfloxacin antibody) was diluted 1:10 with PBS; HRP-labeled goat anti human IgG was diluted 1:8000 with PBS; HRP-labeled human IgE was diluted 1:1000 with PBS; AP-labeled goat anti-rabbit IgG was diluted 1:1000 with TBS (pH = 9.5, containing 9 g L^{-1} NaCl, 1.2 g L^{-1} Tris); HRP (1 mg mL^{-1}) was diluted 1:1000 with PBS; AP (1 mg mL^{-1}) was diluted 1:1000 with TBS; and BCIP/NBT was diluted 1:50 with TBS.

Table 1. Design of the ELISA in the presence of proteins from *P. olivaceus*.

Group	Incubation with the primary antibody	Incubation with the secondary antibody
1	Anti-norfloxacin antibody and norfloxacin in PBS	Secondary antibody in PBS
2	Anti-norfloxacin antibody and norfloxacin in f1 isolate	Secondary antibody in PBS
3	Anti-norfloxacin antibody and norfloxacin in f2 isolate	Secondary antibody in PBS
4	Anti-norfloxacin antibody and norfloxacin in PBS	Secondary antibody and f1 isolate
5	Anti-norfloxacin antibody and norfloxacin in PBS	Secondary antibody and f2 isolate

Proteins in the crude fish extracts were at first separated by SDS-PAGE as mentioned above, and then electro-blotted onto a PVDF membrane (with an average pore size of ϕ 0.45 μm). The membrane was immersed in Ponceau S solution (containing 2 g L⁻¹ Ponceau S, 30 g L⁻¹ trichloroacetic acid and 30 g L⁻¹ salicylic aldehyde) for about 1 min, until protein binds could be clearly seen after washing with water. Then the membrane was washed with PBS/TBS containing 1 g L⁻¹ Tween 20 (PBST/TBST) for three times (5 mins for each time), cut into strips and blocked in PBS/TBS (containing 1 g L⁻¹ Tween-20 and 50 g L⁻¹ de-fatted milk) for 2 h at room temperature or overnight at 4°C. The membrane was then washed with PBST/TBST again (three times, 5 mins for each time), followed by incubation in the antibody or/and enzyme label solutions for an hour. There are different operating steps for the next procedures: (a) for HRP-labeled antibody and HRP solution, the membrane was washed with PBST three times (5 mins for each time) and immersed in the Pierce enhanced chemiluminescence Western Blotting Substrate solution (Thermo Scientific, Rockford, IL, USA) at room temperature for 1 min. Then the membrane was exposed in darkroom, and the image was recorded by Tanon180 Gel imaging system; (b) for AP-labeled antibody and AP solution, the membrane was immersed in a solution of BCIP/NBT for 10 mins followed by washing with water, and finally the picture was taken by a digital camera (Canon EOS 550D with lens of EFS 18 mm–55 mm, Tokyo, Japan).

2.7. Statistical analysis

All experiments were performed at least in duplicate, and data were expressed as Mean $\pm$ standard error of mean unless indicated otherwise. Data were subjected to analysis with SPSS 11.0 software (SPSS Inc., Chicago, IL, USA). The Student's *t* test was carried out to determine the significant difference ($P = .05$) between test groups and controls. figures were prepared with Origin Pro 7.5 software (OriginLab, Northampton, MA, USA).

3. Results and discussion

3.1. Interaction between fish proteins and different immunoglobulins

To validate the generality of the flatfish proteins to react with immunoglobulins, their interaction with different commercial antibodies was investigated by western blotting, and the results were summarized in Figure 1. Some proteins of the three flatfishes could effectively bind with all enzyme-labeled antibodies, regardless of monoclonal antibody or polyclonal antibody, IgG or IgE (Figure 1). However, no significant interaction was investigated between fish proteins and the enzyme labels (HRP, AP) (Figure 1). Considering the diversity of the antibodies investigated as well as their responding antigens used, such a fish protein–immunoglobulin interaction could not be attributed to the accidental conformational similarity between the fish protein components and some antigens for the immunoglobulins, or the impurity of the polyclonal antibodies, as in our previous studies (Wang et al., 2013). More reasonably it indicated a significant and universal interaction

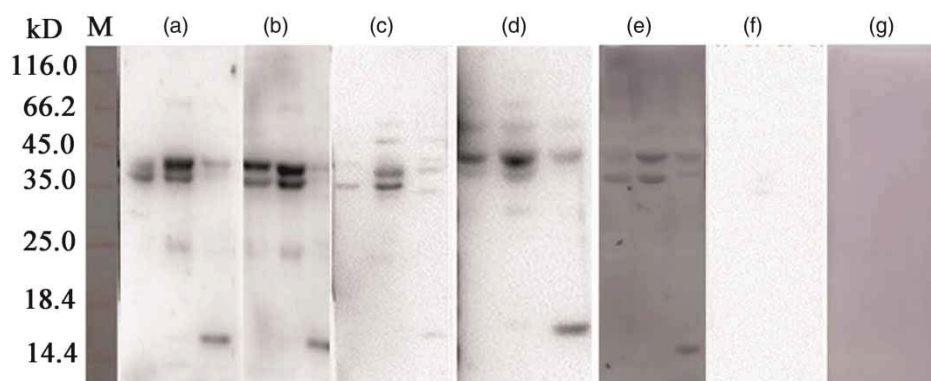


Figure 1. Western blotting results of fish proteins with different antibodies and enzyme labels. (a): HRP-labeled monoclonal mouse IgG; (b): HRP-labeled goat anti-mouse IgG; (c): HRP-labeled goat anti human IgG; (d): HRP-labeled human IgE; (e): AP-labeled goat anti-rabbit IgG; (f): HRP; (g): AP. The three proteins bands were (from the left to right) *C. robustus*, *P. olivaceus* and *S. maximus*, respectively.

between the fish proteins and immunoglobulins, which one can expect to observe in different immunoassays using these labels, other than just in the detection of norfloxacin.

At least two kinds of fish proteins exhibited the ability to effectively bind with the immunoglobulins: for *C. robustus* and *P. olivaceus*, the molecular weight of the proteins was estimated to be about 35–45 kD, and for *S. maximus* a protein of approximately 16 kD was also demonstrated besides the 35–45 kD components. Galland et al. (1998) once also reported a protein component of 41 kD from codfish to bind with an anti-parvalbumin monoclonal antibody and AP-labeled anti-mouse IgG in western blotting. Of course this may be due to the possible conformational similarity between the 41 kD protein and parvalbumin, but much more reasonably it should be another case of fish protein–immunoglobulin (labeled or/and label free) interaction just as we observed here.

3.2. The role of enzyme labels in the protein–immunoglobulin interaction

It seems necessary to provide further information on the interaction among fish proteins, labels and immunoglobulins, especially the role of enzyme labels. Therefore ci-ELISA for norfloxacin were designed and performed with the addition of the fish proteins at different steps (Figure 2). When the fish proteins (f1 and f2 isolated from *P. olivaceus*) were incubated with HRP-labeled secondary antibodies, there was a significant increase in the inhibition ratio in comparison to that of controls ($P < .05$), which confirmed a direct and competitive binding of the fish proteins with the enzyme–antibody conjugate. However, when they were mixed with the label-free primary antibody first, the results demonstrated were more complex: there was a significant increase in the inhibition ratio in the presence of f1 fragment, but not as much as that performed with the HRP-labeled secondary antibody; and no significant difference was observed between the f2 group and controls ($P > .05$).

Based on these results, the scheme of the fish proteins to affect the ci-ELISA was briefly summarized. As shown in Figure 3, the presence of fish proteins could result in a significant increase in the inhibition ratio by their competitive binding with the antibodies. Moreover, such a protein–protein interaction was demonstrated much stronger in the

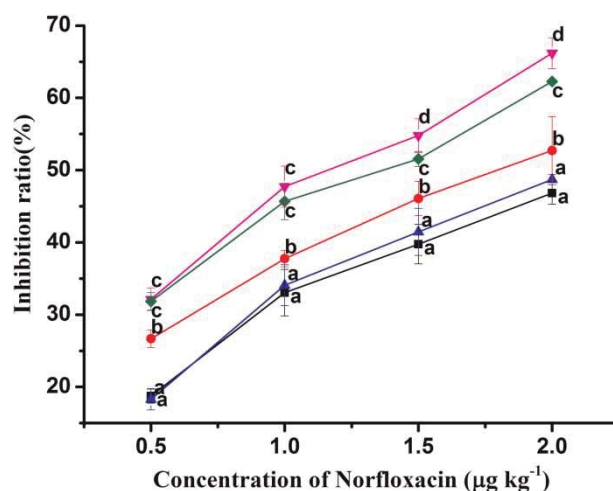


Figure 2. ci-ELISA results for norfloxacin in the presence of flatfish proteins. Data were presented as the average of four measurements. ■ control (PBS); ● *P. olivaceus* fraction f1 with the primary antibody; ▲ *P. olivaceus* fraction f2 with the primary antibody; ▼ *P. olivaceus* fraction f1 with the secondary antibody; and ◆ *P. olivaceus* fraction f2 with the secondary antibody.

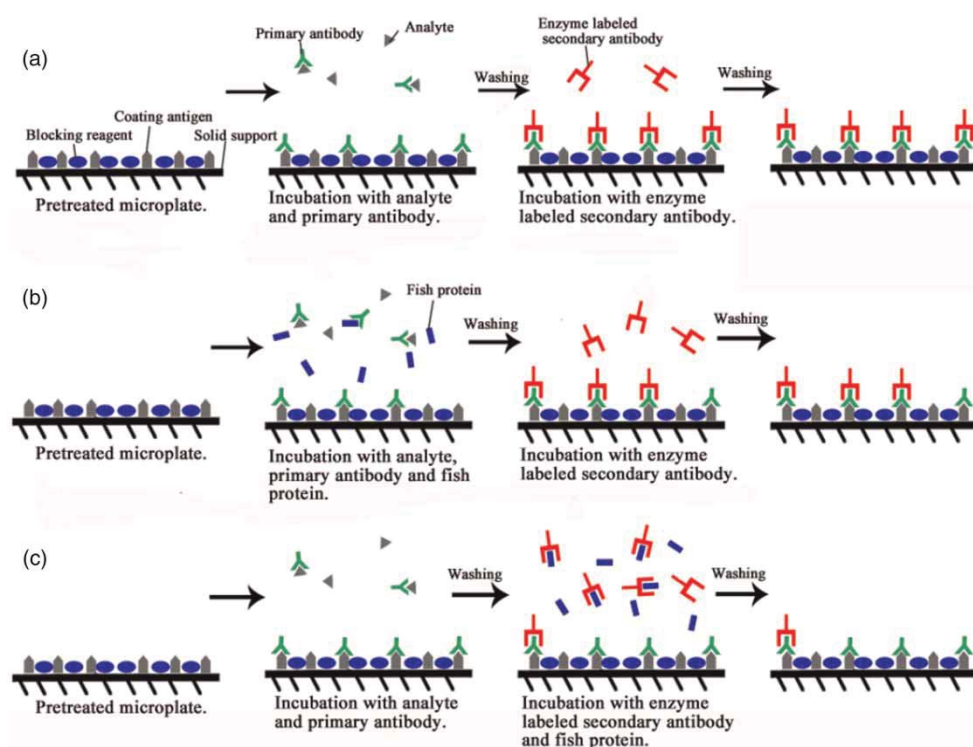


Figure 3. The scheme of the matrix proteins to interfere to the ci-ELISA. (a): control; (b): incubated with primary antibody; and (c): incubated with labeled secondary antibody.

presence of enzyme-labeled antibodies in comparison to label-free antibodies, which indicated the importance of enzyme labeling for the matrix interference observed. The enzyme labels HRP and AP we usually used in immunoassay have large molecular weight of 40 and 140 kD, respectively. Antibodies are usually covalently modified at three modification sites, namely primary amines ($-\text{NH}_2$), sulfhydryl groups ($-\text{SH}$) and carbohydrate residues. These residues are abundant, widely distributed and easily modified due to their reactivity and their location on the surface of the antibody. Thus the nature of the enzyme-immunoglobulin complexes might be different from the unlabeled ones, for example the accessible surface area of newly folded protein is changed for it is simply proportional to two-thirds power of their molecular weight (Chothia, 1976); and the change of surface functional groups might affect the adsorption ability of the protein (Ostuni, Chapman, Holmlin, Takayama, & Whitesides, 2001). To summarize, one can reasonably assume that there may be some conformational changes or charge structure in the immunoglobulins after their conjugation with labeling enzymes, and such a structural modification would greatly enhance their binding efficiency with the fish proteins.

3.3. The universal significance of the protein-induced matrix effect in aquatic samples

The effect of matrix proteins on enzyme-labeled immunoassays was further validated with five aquatic samples from freshwater and marine water. Concerning the ci-ELISA for norfloxacin, a significant shift of the inhibition curve was demonstrated in all samples at the spiking level ranged from $0.2 \mu\text{g kg}^{-1}$ to $5.0 \mu\text{g kg}^{-1}$ ($P < .05$) in comparison to the control, except the result for *P. orientalis* at the concentration of $5.0 \mu\text{g kg}^{-1}$ ($P < .05$) (Figure 4). Such matrix interference was also confirmed by the western blotting (Figure 5), but it seemed to have no significant correlation with the total protein concentration of samples; instead, it was more likely induced by some special protein contents. Just as that for flatfishes, the protein content with molecular weight of 35–45 kD was demonstrated

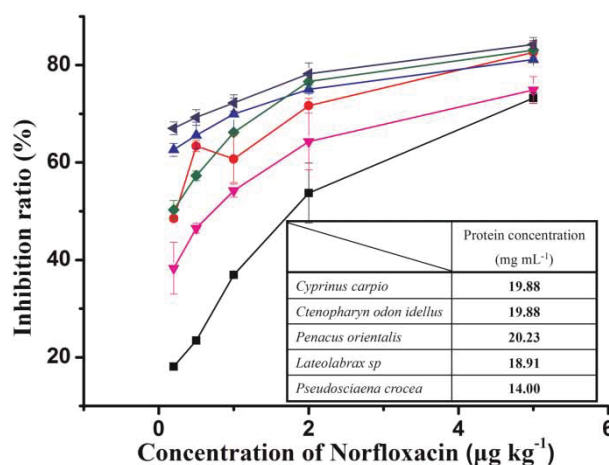


Figure 4. ci-ELISA results for norfloxacin in crude extracts of different aquatic samples. Data were represented as the average of three measurements. ■ control (PBS); ● *C. carpio*; ▲ *Ctenopharynx odon idellus*; ▼ *P. orientalis*; ◆ *L. sp*; and ◀ *P. crocea*.

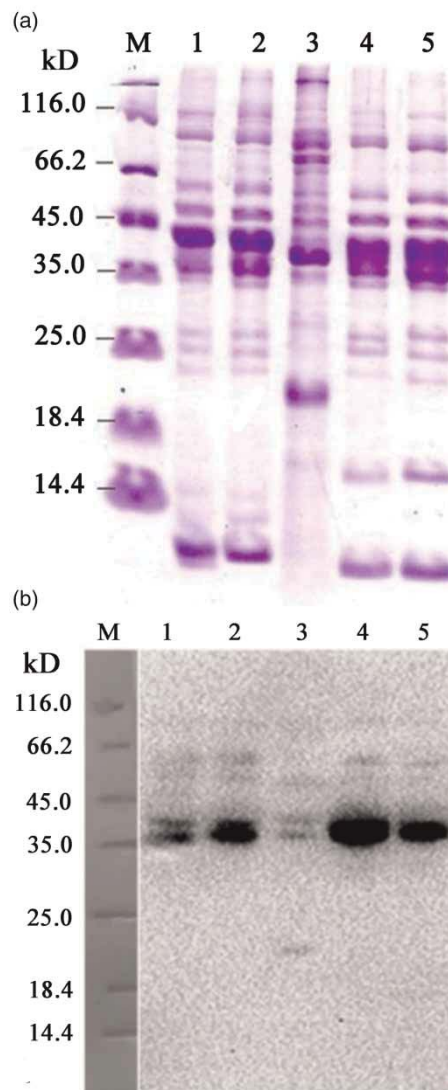


Figure 5. SDS-PAGE (a) and western blotting (b) of crude extracts of aquatic samples. M: marker; 1: *C. carpio*; 2: *Ctenopharynxodon idellus*; 3: *P. orientalis*; 4: *Lateolabrax* sp; 5: *P. crocea*.

the main source of positive results. Moreover, it should be noted that, samples with relatively high concentration of the 35–45 kD component, for example *P. crocea*, *L. sp* and *Ctenopharynxodon idellus*, also exhibited much stronger matrix interference than others. There were some other protein components which may also contribute to the matrix effect investigated, such as the protein of 18.4–25.0 kD in *P. orientalis*, but their influence seemed far less than that of the 35–45 kD component.

Based on the results mentioned above, the universal significance of the matrix protein-induced interference in aquatic samples could be preliminarily concluded, and its influence on the usually exploited immunoassays should be addressed. For those performances in which the aquatic samples directly react with enzyme-labeled antibodies, such as western

blotting, the interference would greatly affect the accuracy of the results, for example the identification of seafood allergens (Sicherer & Sampson, 2010). In fact such an assumption could be well supported by many previous studies: Sicherer and Sampson (2010) once indicated that food-specific IgE (sensitization) does not necessarily indicate clinical allergy, due to the possible interference of nonspecific interactions; Galland et al. (1998) reported an unexpected cross-reaction between the 41 kD protein in codfish and anti-parvalbumin monoclonal antibody in western blotting, as we mentioned above; based on the positive binding with labeled IgE in western blotting, many protein components were indicated of allergenic activity, such as the 50 and 42 kD proteins in uncooked Spanish mackerel (*Scomberomorus commerson* Lacepede) (Misnan, Murad, Arip, Abdullah, & Mohamed, 2005), 15–138 kD proteins in swimming crabs and red crabs and the 16.5 and 40 kD proteins in shrimps (Hajeb & Selamat, 2012), but now these results might be carefully checked to take the possible matrix protein-induced interference into full account.

There is also an increasing application of different ELISA techniques for quantitative or semi-quantitative analysis of aquatic samples or foodstuffs containing aquatic components. For these performances especially the analysis of trace analyte, at least the following considerations should be emphasized to prevent significant loss of accuracy: (i) the interference-inducing proteins should be sufficiently removed from the matrix by reasonable pretreatments and (ii) the ELISA procedures should be well evaluated and optimized. There is an emerging trend to develop one-step ci-ELISA in which the sample label-free primary antibody and enzyme-labeled secondary antibody were mixed and incubated together to simplify the procedures, but considering the preference of the matrix proteins to bind with enzyme-labeled antibodies, it may result in significantly enhanced interference in comparison to traditional two-step procedures where the sample is incubated with the label-free primary antibody first and then followed by addition of labeled secondary antibody after full washing.

4. Conclusion

Some protein components from aquatic products, especially those with an approximate molecular weight of 35–45 kD, were demonstrated to significantly bind with different immunoglobulins, and therefore interfere with the accuracy of immunoassays. Further experiments by ci-ELISA indicated that, these matrix proteins prefer to bind with enzyme-labeled antibodies other than label-free ones. Such a protein-immunoglobulin interaction was also confirmed in many other aquatic products, which allowed us to propose a universal significance of the matrix protein-induced interference to the immunoassay of food samples containing these components.

Disclosure statement

No potential conflict of interest was reported by the authors.

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