Monoclonal antibody based immunodot for specific detection of proteins of the shrimp *Penaeus* species

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Abstract Frozen shrimp continued to be the single largest item of export from India in terms of value accounting for about 44% of the total marine export earnings. Headless, peeled frozen shrimp is a common and dominant item in the market and there is need for differentiating peeled *Penaeus* sp from *Metapenaeus*, *Parapenopsis* and *Macrobrachium* sp as consumer preference and price vary. Furthermore, there is need to find out original species used in value addition of shrimp products. Hence, it is essential for development of simple and consumer friendly technique for the identification of shrimp and their products in the market. Two monoclonal antibodies (MAbs) C-15 (IgG3) and C-52 (IgG2a) reacting with 65 and 47 kD proteins of *Penaeus monodon* respectively in the Western blot were selected. In epitope analysis by immunodot, the two MAbs reacted and recognized specific proteins of *P. monodon*, *Fenneropenaeus indicus* and *Litopenaeus vannamei* and not that of *Metapenaeus*, *Parapenopsis*, *Macrobrachium rosenbergii*, crabs and fishes. The immunodot required 120 min for completion. The sensitivity of the immunodot to detect proteins of *P. monodon* was 0.225 mg with MAb C-15 and 0.028 mg with MAb C-52. The MAb based immunodot developed, could be used for identifying and differentiating meat of *P. monodon*, *F.*

indicus, and *L. vannamei* from that of *Metapenaeus*, *Parapenopsis*, *M. rosenbergii*, crabs and fishes.

Keywords Monoclonal antibody · *Penaeus* · Shrimp · Proteins

Introduction

Global shrimp and prawn production reached 1,329,372 t with a value of about 8,608,652 \$ US and India contributed 93,513 t of frozen shrimp and prawn with a total value of 734,481 \$ US (FAO 2004). Frozen shrimp continued to be the single largest item of export from India in terms of value accounting for about 44% of the total export earnings (MPEDA 2007). Furthermore, a good number of value added shrimp products having high consumer preference such as breaded and battered shrimp, shrimp balls, belly cut butterfly shrimp, skewed shrimp, shrimp cake and easy peel headless shrimp are also available in the market. In the course of value addition many analogue products have also appeared in the market as substitute for shrimp. Alaska Pollack is now used in Japan, USA and other countries for the manufacture of shellfish substitutes of which crab sticks is the most widely known product (Piggot 1986). Among all the products headless, peeled frozen shrimp is a common and dominant item in the market.

Authenticity of food and fish products is gaining importance in view of value addition and expanding market worldwide. Authenticity issues with meat and fish products generally falls into 4 categories- i) Origin (ii) substitute (iii) treatment (iv) miscellaneous issues (Hargin, 1996). Authenticity of meat and meat products by implementation of chemometrics for quality control has been highlighted (Arvanitoyannis and Koukaliaroglou 2003). Despite the

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precision and accuracy of robust analytical, protein and DNA based techniques, Arvanitoyannis et al. (2005a) stressed importance of multivariate analysis for fish authenticity. Implementation of quality control methods in conjunction with chemometrics has been highlighted for dairy products (Arvanitoyannis and Tzouros 2005) and honey (Arvanitoyannis et al. 2005b). There is need for differentiating peeled *Penaeus* sp from *Metapenaeus*, *Parapenopsis* and *Macrobrachium* sp; as consumer preference and price vary. Furthermore, there is need to find out original species used in value addition of shrimp products. Hence, it is essential for development of simple and consumer friendly technique for the identification of shrimp item and the products in the market.

Methods for identifying shrimp from a piece of muscle tissue include nucleic acid based techniques such as DNA fingerprinting (Foote et al. 1989, Thomas and Backenbush 1989) and protein based techniques such as electrophoresis and chromatography (Mackie 1990, Yman 1992, Stoleo et al. 1993, Mackie et al. 2000). Antibody based immunoassays are also used as an alternative means of differentiating muscle proteins of different fish species and detection of a major fish allergens (Patterson 1985, Hitchcock 1988, Gajewski and Hsiesh 2009). Immunoassay such as Enzyme Linked Immunosorbent Assay (ELISA) has been widely used for identification of animal meat (Asensio et al. 2007), analysis of milk and detection of tissue bound metabolites of antibiotics in prawn tissue (Cooper et al. 2004), because it is less expensive and suitable for routine analysis of large number of samples. Shrimp is a major commodity in the world fish market. However, there are very few immunoassays for detection of shrimp meat at the present. A monoclonal antibody (MAb) based ELISA for rock shrimp (*Sicyonia brevirostris*) identification has been developed (Haejung et al. 1990). Here, we report development of a MAb based immunodot which specifically detects and differentiates proteins of *Penaeus* from that of *Metapenaeus*, *Parapenopsis*, *Macrobrachium rosenbergii*, crabs and fishes.

Materials and methods

Source of hybridomas

Hybridomas secreting antibodies against shrimp proteins used in this study were obtained in our laboratory while developing MAbs to white spot syndrome virus (WSSV) of shrimp *P. monodon* (Anil et al. 2002). Panel of frozen parental hybridomas secreting antibodies to proteins of *P. monodon* were selected, revived, screened and mini cloned thrice following standard procedure to get stable hybridomas specifically reacting to WSSV free shrimp tissue proteins on immunodot assay (Anil et al. 2002). Finally monoclonal

antibodies secreted by C-15 and C-52 hybridomas were taken up for immunoglobulin isotyping and characterization by Western blot analysis.

Screening of hybridomas by immunodot

Hybridoma secreting MAbs were cultured 25 cm² tissue culture flasks with RPMI medium supplemented with 20% fetal bovine serum (FBS). Cell culture supernatant was harvested from each flask after 4 d. MAbs reacting to *P. monodon* tissue homogenate proteins and not reacting to WSSV proteins were screened using nitrocellulose membrane based immunodot procedure with following modifications. Briefly, *P. monodon* (2 step PCR negative for WSSV) procured from fish landing centre, Mangalore was thoroughly washed and stored at −20 °C. About 0.3 mg of muscle from abdomen, head and tails were dissected and homogenized in pestle and mortar with 3 ml of TNE buffer (20 mM Tris sodium, 400 mM Sodium chloride, 20 mM of disodium EDTA, pH 7.4) followed by centrifugation at 1800×g for 10 min. Supernatant of homogenate (3 µl) was dotted on to a nitrocellulose paper (0.2 µm Biorad, USA). Purified WSSV¹ and TNE buffer were used as positive and negative control respectively along with normal shrimp samples. Dotted samples were air dried for 5 min and incubated with blocking buffer (3% Bovine serum albumin in 50 mM Phosphate buffer saline pH 7.4) for 1 h at room temperature (RT) and 3 times using wash buffer (10 mM PBS incorporated with 0.05% of tween 20). MAb from each hybridoma were reacted with membrane by incubating at RT for 2 h. Unbound MAb was washed by wash buffer. Enzyme conjugate rabbit anti-mouse IgG (1:1000 prepared in blocking buffer) was then added and incubated for 30 min, excess secondary antibody was again washed with wash buffer. Following washing step, chromogen-substrate complex 4-chloro- 1 naphthol/H₂O₂ (Sigma, USA) was added which upon reacting with enzyme HRP yields distinct precipitating purple dot at the site of antigen spotted. The reaction was stopped by washing the nitrocellulose with PBS. Membranes showing purple dot at the site of shrimp protein and showing no colour at the site of WSSV and PBS were selected for further characterization by immunoglobulin isotyping and Western blot analysis.

Characterization of MAbs

Immunoglobulin isotype of the MAbs was determined by ELISA according to manufacturer's protocol (Sigma USA).

Determining protein specificity of MAbs by Western blot

Shrimp *P. monodon* tissue sample prepared for immunodot test was mixed 1:1 volume of sample buffer (0.5 M Tris–HCl

pH 6.8, 0.8 ml glycerol, 1.6 ml 10% SDS, 0.4 ml 2- β mercaptoethanol, 0.02 ml 0.05% bromophenol blue) and boiled for 5 min at 90 °C. Protein samples along with molecular weight marker (Sigma, USA) were loaded on to 4% stacking gel and electrophoresed in 12.5% separating gel at 100 V till the dye front reached the bottom of the gel (Haejung et al. 1990). Two reference lanes of the gel (molecular weight marker and shrimp protein) were separated and stained with coomassie blue (10% acetic acid, 40% methanol, 0.1% coomassie blue) for 5 h and destained with destaining solution (10% acetic acid, 40% methanol). Protein from the remaining lanes of the gel, were transferred to nitrocellulose membrane at 250 mA for 1 h using a mini-transblot apparatus (Biorad, USA). Protein lanes on the nitrocellulose were separated and reacted with blocking buffer (3% BSA in PBS). The lanes were then reacted separately with MAbs overnight at 4 °C. After washing three times with wash buffer (PBS Tween-20) and once with PBS, membrane lanes were reacted with rabbit anti-mouse IgG HRP (1:1000, Sigma USA) prepared in blocking buffer for 30 min. After washing three times with wash buffer, chromogen-substrate complex 4-chloro- 1 naphthol/H₂O₂ (Sigma, USA) was added which upon reacting with enzyme HRP yields distinct precipitating purple band at the site of MAb reaction (Fig. 1). The reaction was stopped by washing the Western blot strips with PBS and protein bands identified by comparing with molecular weight markers.

Epitope analysis of muscle proteins of different species of shrimp, prawn, crab and fish by MAb based immunodot

Samples of shrimps (*Penaeus monodon*, *Litopenaeus vannamei*, *Fenneropenaeus indicus*, *Metapenaeus dobsoni*, *Parapenopsis stylifera*, *Aristeus alcocki*), prawn (*Macrobrachium rosenbergii*), crabs (*Charybdis lucifera*,

Fig. 1 Determination of protein-specificity of MAbs by Western blot Lane 1: Molecular weight markers, Lane 2: *Penaeus monodon* proteins from muscle extract, Lane 3, 4: Reaction of C-15 and C-52 respectively with shrimp protein

Charybdis cruciata, *Scylla serrata*, *Portunus pelagicus*, *Portunus sangienolentus*) and fishes (*Sardinella longiceps*, *Rastrelliger kanagurta*, *Scomberomorus guttatus*, *Priacanthus harmur*, *Priacanthus boops*, *Sillago sihama*, *Pampus argenteus*, *Cyanoglossus sp*, *Etroplus suratensis*, *Oreochromis mossambicus*,) were collected from the fish landing center at Mangalore. *Catla catla* a freshwater carp was collected from the farm of Fisheries College Mangalore. The samples were brought to laboratory on ice, washed with distilled water. Immediately, about 0.3 g of muscle tissues were dissected from abdomen and tail from each shrimp collected and homogenized using 3 ml of TNE buffer as explained earlier. Rest of the shrimp samples were stored frozen until further use. After brief centrifugation, clear supernatant (3 μ l) of each sample was dotted on nitrocellulose membrane and air dried (Fig. 2). Similarly two set of nitrocellulose strips were also 'prepared' one each for panel of crabs samples (Fig. 3) and fishes (Fig. 4). Empty sites on antigen spotted membrane strips were blocked using blocking buffer for 1 h, excess wash buffer was washed thrice using wash buffer. Each membrane strip reacted separately with the MAbs C-15 and C-52 for 2 h and immunodot assay carried out as mentioned above and results were recorded based on colour reaction observed at the site of antigen dots.

Detection of protein in cooked or steamed *P. monodon* by immunodot

Peeled *P. monodon* samples were added to boiling water and sample collected at 3, 10, 20 min. *P. monodon* samples were also steamed in a pressure cooker for 30 min. The boiled and steamed samples (0.3 g) were homogenized separately with 3 ml of TNE buffer and the supernatant (3 μ l) was dotted on

Fig. 2 Reaction of the MAbs with muscle proteins of *Penaeus*, *Metapenaeus* *Parapenopsis* and *Macrobrachium rosenbergii* in the immunodot

Fig. 3 Reaction of MAbs with proteins of crabs in the immunodot

to nitrocellulose membrane. Immunodot was carried out as mentioned above (Fig. 5).

Evaluation of sensitivity of MAbs for detection of *P. monodon* protein by immunodot

Shrimp *P. monodon* muscle (0.3 g) was homogenized using 3 ml of distilled water and protein concentration of the supernatant was determined Lowry et al (1951) using protein estimation kit (Bangalore Genei, India Pvt Ltd). Later, serial dilutions of the supernatants were made and from each dilution 3 μ l dotted on to nitrocellulose strip. A negative control with PBS was also dotted. The nitrocellulose strip was then reacted with MAbs C-15 and C-52 and immunodot assay performed as mentioned above. Positive with visible purple blue dots developed with lowest concentration of protein preparation was recorded.

Fig. 4 Reaction of MAbs with proteins of various fishes in the immunodot

Fig. 5 Reaction of MAbs with proteins of *P. monodon* subject to boiling or steaming in Immunodot. Muscle tissue boiled for 3 min (a), 10 min (b), 20 min(c) and steamed (d)

Results and discussion

Characterization of MAbs reacting with muscle proteins of *P. monodon*

Two MAbs C-15 and C-52 were finally selected after screening by immunodot. In Western blot, the MAbs C-15 and C-52 reacted specifically with 65 and 47 kDa proteins of *P. monodon* respectively (Fig. 1). The MAb C-15 was found to be IgG3 isotype and C-52 IgG2a isotype according to immunoglobulin isotyping kit (Sigma USA). Isotyping results determined that both the hybridoma are distinct.

Epitope analysis of proteins of shrimp, prawn, crab and fish by the MAb based immunodot

The two MAbs reacted strongly with proteins of *P. monodon* as indicated by bright purple colour in the immunodot (Fig. 2). The MAbs also reacted with proteins of *L. vanamei* and *F. indicus* but very lightly confirmed by pale purple dots. However, both the MAbs did not react with proteins of other species of shrimp such as *M. dobsoni*, *A. alcocki*, *P. stylifera* and proteins of freshwater prawn *M. rosenbergii*. The reaction pattern indicate that epitopes of 65 and 47 kDa proteins are different in these species and could be used for specific detection and differentiation of species of *Penaeus* from *Metapenaeus*, *Parapenopsis* and the Paleamonid (*Macrobrachium rosenbergii*). This could be an ideal antibody probe particularly for specific detection or differentiation of peeled frozen *Penaeus* or *Palaemonids* which look alike in the market. Furthermore, the MAbs also did not react with proteins of crabs showing that the epitope may be absent although the crabs belong to crustacea. MAbs as expected did not react with the proteins of fish from fresh, brackish and marine waters.

Detection of proteins of *Penaeus sp* by immunodot

An immunodot was developed for specific detection of *Penaeus sp* proteins. It could be clearly seen that the two MAbs reacted with *P. monodon* proteins in the immunodot were also lightly reacting to the proteins of *L. vanamei* and *F. indicus*, which confirms that the two MAbs also recognize proteins of other *Penaeid* species. This indicates that the 65 and 47 kDa proteins of the penaeids share common epitopes. Monoclonal antibody has been developed for rock shrimp muscle protein 17.7 kDa which recognized epitopes present both on the native and heat denatured protein. This MAb was successfully used in ELISA to detect the presence of rock shrimp in 26 sea foods and meat mixture samples (Haejung et al. 1990). Our MAbs could also detect *P. monodon* protein 10 min after boiling. The MAb C-52 could react with *P. monodon* protein even after the sample was boiled for 20 min and steamed for 30 min as compared with other MAb C-15 (Fig. 5). Similarly, MAbs developed against proteins of Red snapper (*Lutjanus campechanus*) meat was used in ELISA for specific identification of the species in the mixed products with other snappers (Huang et al. 1995). The differential reaction of MAbs with proteins of *Metapanaeus* and *Parapenopsis* indicate that the MAb based immunodot can also be used for specific detection of *Penaeus sp*.

Sensitivity of immunodot

In immunodot MAb C-52 detected 0.028 mg and C-15 0.225 mg of proteins of *P. monodon* (Figs. 6 and 7). C-52 was sensitive than C-15. The sensitivity achieved through immunodot was lower compared to that of ELISA for detection of protein of rock shrimp (*Sicyonia brevirostris*) which ranged from 0.19 to 13.6 ng (Haejung et al. 1990).

Fig. 6 Determination of sensitivity of MAb C-52 for detection of proteins of *P. monodon* by immunodot

Fig. 7 Determination of sensitivity of MAb C-15 for detection of proteins of *P. monodon* by immunodot

Generally ELISA is more sensitive than immunodot. However, ELISA is more cumbersome, costly and often confusing with a false positive. On the other hand, immunodot is very simple, with consistent results and ideal for field detection. In recent years there has been increase in adulteration of fish and shellfish products (Asensio et al. 2003a, b). The present study will be helpful to differentiate *Penaeus sp* within various shrimp and not to differentiate within *Penaeus sp*. There is further scope for developing immunodot or lateral flow test kit using these MAbs for detection of *Penaeus* shrimp meat in the market.

Conclusion

Two monoclonal antibodies C-15(IgG3) and C-52(IgG2a) reacting with 65 and 47 kDa proteins of *P. monodon* respectively were produced. The MAbs could also detect *P. monodon* protein after boiling or steaming the meat. The MAbs did not react with proteins of *Parapenopsis*, *Metapanaeus*, *Macrobrachium rosenbergii*, crabs and fishes used in the assay. An immunodot was developed which required 120 min for completion. The sensitivity of MAbs C-15 and C-52 to detect proteins of *P. monodon* was 0.225 and 0.028 respectively. The MAb based immunodot can identify *Penaeus sp* in frozen and value added shrimp products. The study differentiates the shrimp species, particularly useful for identifying headless, peeled and deveined shrimp in the market. Furthermore, the monoclonal antibodies could detect the proteins in both frozen and

cooked shrimp. The present study has commercial implications in identifying *Penaeus* sp using MAbs both in frozen and cooked form.

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