Development of Monoclonal Antibodies for Red Snapper (*Lutjanus campechanus*) Identification Using Enzyme-Linked Immunosorbent Assay†

Tung-shi Huang, Maurice R. Marshall, Kuo-jang Kao, W. Steven Otwell, and Cheng-i Wei*

Food Science and Human Nutrition Department, University of Florida, Gainesville, Florida 32611-0370

Two murine IgG1 monoclonal antibodies (McAbs), C1C1 and C2A2, against red snapper protein *a* were produced using hybridoma technique. McAb C1C1 showed strong reactivity with red snapper, vermilion snapper (VS), lane snapper (LS), mutton snapper (MS), and yellowtail snapper (YS), and it could be used in ELISA and immunodot blotting to screen these fish species from 36 commonly consumed seafood and meat samples. Since C2A2 did not react as strongly with VS, LS, MS, and YS as with red snapper, it could be used to separate red snapper from the remaining four species. The combined use of these two McAbs in a blind study using ELISA correctly identified red snapper from 24 various seafood and meat extracts. Furthermore, the identities of 24 commercial fish samples claimed to be red snapper, MS, Caribbean red snapper, and other various red snappers by seafood retailers were verified using ELISA with these two McAbs and thin-layer and slab IEF gel electrophoresis.

Keywords: *Red snapper; monoclonal antibody; ELISA; isoelectric focusing electrophoresis*

INTRODUCTION

Red snapper (*Lutjanus campechanus*) has a rose-red skin and a delicate taste and muscle texture. It is a popular eating fish frequently sold at about $9 per pound in U.S. retail markets. Due to its high cost, popularity, and demand, red snapper fillets are highly susceptible to substitution using less expensive fish species. Hsieh et al. (1993) of the Florida Department of Agriculture and Consumer Services used thin-layer isoelectric focusing (IEF) to check 81 fillets labeled as red snapper from retail markets and fish dealers across Florida between 1988 and 1992. They found that only 24 samples (30%) were labeled correctly as red snapper. Scarlet snapper (*Lutjanus sanguineus*), gray snapper (*Lutjanus griseus*), lane snapper (*Lutjanus synagris*), malabar snapper (*Lutjanus malabaricus*), mutton snapper (*Lutjanus analis*), vermilion snapper (*Rhomboplites aurorubens*), and yellowtail snapper (*Ocyurus chrysurus*) were labeled as red snapper and accounted for 58% of the mislabeling. In Florida, substitution also occurs for other valuable snappers and groupers and, to a lesser extent, for mackerels and swordfish. Analytical methods that identify fish species are therefore important in preventing willful or unintentional substitution with lower priced fish species for red snapper and other more valuable fish species in the marketplace.

Electrophoretic methods such as starch gel-zone electrophoresis, acrylamide disc electrophoresis, thin-layer polyacrylamide gel IEF, and cellulose acetate strip have been accepted as official methods by the Association of Official Analytical Chemists (AOAC, 1990) to differentiate seafood species or seafood products. IEF provides reliable, reproducible, and resolved protein patterns; it is extensively used for identification of closely related seafood species (An et al., 1989; Hamilton, 1982; Lundstrom, 1980, 1983; Toom et al., 1982).

Electrophoretic methods are laborious and time-consuming and require a substantial amount of equipment; therefore, they are limited for field testing. The development of a fast, reliable, and accurate method to identify and quantify species of intact as well as processed seafood for regulatory labeling is necessary to protect consumers' value of purchasing.

Immunological techniques, such as immunodiffusion, hemagglutination inhibition, and enzyme-linked immunosorbent assay (ELISA), have been widely used to detect species origin of fish, beef, pork, and chicken or to detect food components in seafood or meat products (Hayden, 1981; Hitchcock et al., 1981; Whittaker et al., 1983). Lundstrom (1984, 1985) used crude protein extracts from heat-denatured fish muscle to prepare monoclonal antibodies (McAbs) for fish species identification and differentiation of fishery stock. The McAbs failed to demonstrate species specificity. Cross-reactivity occurred with unrelated fish species, possibly because the McAbs reacted with the most prevalent proteins in crude extracts, such as albumin, rather than with species-specific proteins (Scopes, 1970). An et al. (1990) successfully developed a McAb to identify rock shrimp by using species-specific proteins prepared from SDS–PAGE to immunize mice. The McAb correctly detected rock shrimp in a blind study using ELISA. Rock shrimp at 4.3 ng in mixed samples was detected using this technique. McAbs have also been used to identify chicken meat in mixtures of raw beef and pork (Martin et al., 1991) and to quantitate food components in various products for determination of food origin (Brandon et al., 1988; Skeritt and Smith, 1985).

The present study was to develop red snapper-specific McAbs using a red snapper-specific protein isolated from IEF gels as antigen. An ELISA was developed using these McAbs to identify red snapper among various seafood and meat samples. Efforts were also made to investigate the reliability of employing ELISA together with IEF in verifying the identity of red snapper labeled by seafood retailers.

* Author to whom correspondence should be addressed [telephone (904) 392-1991; fax (904) 392-9467].

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MATERIALS AND METHODS

Fish and Meat Samples. Authentic samples of red snapper, vermilion snapper (VS), gray snapper (*L. griseus*; GS), hogfish (*Lachnolaimus maximus*; HF), lane snapper (LS), mutton snapper (MS), and yellowtail snapper (YS) were obtained from the Food Laboratory, Florida Department of Agriculture and Consumer Services, Tallahassee. Red snapper obtained from the Texas and Florida coasts off the Gulf of Mexico were provided by Dr. W. S. Otwell, University of Florida. Onespot snapper (*Lutjanus monostigma*; OS), blackspot snapper (*Lutjanus fulviflamma*; BS), and Madras snapper (*Lutjanus lutjanus*; LL) were obtained from Taiwan and shipped in dry ice to the laboratory at the Food Science and Human Nutrition Department, University of Florida.

Red snapper, VS, GS, HF, LS, MS, YS, pink porgy (*Pagrus pagrus*; PG), white grunt (*Haemulon plumieri*; WG), Atlantic salmon (*Salmo salar*), greater amberjack (*Seriola dumerili*), Gag grouper (*Mycteroperca microlepis*), flounder, bigeye tuna (*Thunnus obesus*), swordfish (*Xiphias gladius*), mahi-mahi (*Coryphaena hippurus*), spot (*Leiostomus xanthusrus*), shark, gray tilefish (*Caulolatilus microps*), spotted seatrout (*Cynoscion nebulosus*), sheepshead (*Archosargus probatocephalus*), gray triggerfish (*Balistes carolinensis*), channel catfish (*Ictalurus punctatus*), Atlantic sturgeon (*Acipenser oxyrhynchus desotoi*), black snapper (*Apsilus dentatus*), white shrimp (*Penaeus setiferus*), oyster (*Crassostrea virginica*), bay scallop (*Argopecten irradians*), and blue crab (*Callinectes sapidus*) were purchased from a local seafood store. Each fish species contained at least two fishes. Fish fillets were prepared after they were scaled, gutted, and headed. The fillets were then cut into small pieces (20 g), put in Whirlpak bags, and stored at −33 °C until needed. Pork, beef, chicken, and turkey were purchased from a local supermarket. They were also cut into small pieces, put in Whirlpak bags, and stored at −33 °C until needed.

Protein Extraction and Sample Preparation. Water-soluble sarcoplasmic proteins were extracted from the center parts (about 6 g) of the defrosted seafood and meat samples as previously described (An et al., 1988; Huang et al., 1995). Samples were homogenized with 3 volumes (w/v) of an aqueous solution (pH 5.14) containing 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 10 mM EDTA, and 0.01% sodium azide at room temperature for 1 min using a Polytron (setting 6.2, Brinkmann Instruments, Westbury, NY). After the homogenized samples were centrifuged at 26900*g* for 20 min at 5 °C, the supernatants were collected and protein concentrations determined (Lowry et al., 1951). Following protein adjustment to 5 mg/mL with water, the supernatants were added with 6% sucrose, placed into small vials in 100 μL aliquots, and stored at −70 °C.

Isoelectric Focusing Electrophoresis and Determination of Protein p*I* Values. A gel mixture containing 4% (w/v) acrylamide [containing 5.3% cross-linker *N*′,*N*′-bis(methyleneacrylamide)], 2% (w/v) Triton X-100, 9.2 M urea, and 2% (v/v) of an ampholyte mixture containing 20% pH 3−10 and 80% pH 4−6.5 (Pharmacia, Piscataway, NJ) was used to compare the protein profiles of red snapper, VS, GS, HF, LS, MS, and YS (Wei et al., 1990). The methods of Huang et al. (1995) for IEF electrophoresis and determining protein p*I* values were followed. Protein standards (broad p*I* kit, pH 3−10, Pharmacia) containing trypsinogen, p*I* 9.30; lentil lectin basic band, p*I* 8.65, middle band, p*I* 8.45, and acidic band, p*I* 8.15; horse myoglobin basic band, p*I* 7.35, and acidic band, p*I* 6.85; human carbonic anhydrase B, p*I* 6.55; bovine carbonic anhydrase, p*I* 5.85; β-lactoglobulin A, p*I* 5.20; soybean trypsin inhibitor, p*I* 4.55; and amyloglucosidase, p*I* 3.50 were included. IEF gels were stained with 0.04% Coomassie blue R-250, destained in a solution containing 0.5% copper sulfate, 12% 2-propanol, and 7% acetic acid (Lee et al., 1987), and then stored in a 7% acetic acid−5% methanol solution. Electrophoretic patterns were recorded using Kodak electrophoresis duplicating paper (Eastman Kodak Co., Rochester, NY).

Purification of Red Snapper-Specific Proteins. Red snapper-specific protein *a* used for immunization in mice was obtained from IEF gels containing 2% pH 5−6 ampholyte. The running conditions for IEF were the same as previously described. After the slab gels were stained with Coomassie blue R-250, the protein profiles were compared to that of authentic red snapper. The band corresponding to red snapper-specific protein *a* was cut off. After elution from the gels, the protein was further purified by rerunning the protein in IEF gels containing pH 5−6 ampholyte. After the gel containing red snapper-specific protein *a* was cut off, it was mixed with a small quantity of water and then homogenized at room temperature in a 2 mL micro tissue grinder. After centrifugation at 2000*g* for 5 min, the clear supernatant was discarded and the blue pellet brought to 0.4 mL with phosphate-buffered saline (PBS, pH 7.2) for animal immunization (Tracy et al., 1983).

Immunization Procedures. Five 6-week-old Balb/c BYJ female mice (Jackson Laboratory, Bar Harbor, ME) were each immunized with 100 μg of red snapper-specific protein *a* freshly emulsified in Freund's adjuvant (Sigma, St. Louis, MO). Complete Freund's adjuvant was used for primary immunization, while incomplete Freund's adjuvant was used for boosting. The mixture was injected in 50 μL aliquots into each of four separate subcutaneous sites on the ventral side near the axillary and inguinal lymphatics and in 100 μL volume into one subcutaneous site between the scapulae (Kao and Klein, 1986). The injection process was repeated three times at 2-week intervals. One week after the third booster, test sera from immunized mice were collected to determine the titers against protein *a* by ELISA. Three weeks after the fourth booster injection, 25 μg of antigen was injected intraperitoneally. Four days later, the animals were sacrificed and the spleens removed for fusion with SP2/0 myeloma cells.

Development of Monoclonal Antibodies. The previously established protocols of Kao and Klein (1986) and An et al. (1990) were followed. Spleen cells harvested from immunized mice were fused with SP2/0 myeloma cells at a ratio of 7.5:1 (spleen cells/myeloma cells) using 50% poly(ethylene glycol) (PEG). The fused cells were suspended in hypoxanthine−aminopterin−thymidine (HAT) selective medium (DME-PC containing 200 μM hypoxanthine, 0.8 μM aminopterin, and 32 μM thymidine) and seeded into 96-well tissue culture plates (Corning Glass Works, Corning, NY). Ten to 14 days later, the supernatants of growth-positive wells were screened for production of anti-protein *a* antibody by testing with red snapper extracts using ELISA. Those hybridomas producing desirable antibody were expanded and rescreened. Those showing continuous production of anti-protein *a* antibody were cloned using the limited dilution method (Harlow and Lane, 1988). McAb isotype was determined using the Immuno Type-Mouse Monoclonal Antibody Isotyping Kit (Sigma) containing isotype-specific antibodies anti-mouse IgG1, anti-mouse IgG2a, anti-mouse IgG2b, anti-mouse IgG3, anti-mouse IgM, and anti-mouse IgA.

Four cloned hybridomas (designated C1C1, A1B2, A2C3, and C2A2) producing antibodies with high reactivities for red snapper extract were propagated intraperitoneally in four female Balb/c BYJ mice already primed by injection with incomplete Freund's adjuvant, to produce McAb-containing ascites. McAbs from mouse ascites were purified by affinity chromatography using MAbTrap G (Pharmacia), a protein G−Sepharose that binds only IgG and its subclasses. The bound antibodies were eluted with 100 mM glycine−HCl buffer (pH 2.7).

Enzyme-Linked Immunosorbent Assay for Red Snapper-Specific Proteins. Each well of a 96-well assay plate (Falcon Micro Test III plate, Becton Dickinson Labware, Oxnard, CA) was coated overnight at 4 °C with 0.1 mL (50 μg/mL) of red snapper or other test proteins. The plate was washed three times with PBS containing 0.1% Tween 20 and 0.02% sodium azide (PBS-T). The unbound sites were blocked for 1 h at room temperature with 200 μL/well PBS containing 1% bovine serum albumin (BSA) and 0.02% sodium azide (BSA-blocking solution). Following addition of 0.1 mL of hybridoma culture supernatant or purified McAb (50 μg/mL) to each well, the plate was incubated at room temperature for 2 h and then washed three times with PBS-T. The plate was subsequently incubated with rabbit anti-mouse IgG conjugated

with alkaline phosphatase (Sigma, 3000-fold dilution in PBS) for 1 h. After the plate was washed, 0.1 mL of *p*-nitrophenyl phosphate (Sigma) was added to each well and it was then left in the dark for color development. Absorbance at 405 nm was determined using an ELISA reader.

Western Blotting. Water-soluble sarcoplasmic extracts of seven fish species were focused with a 20% pH 3−10 and 80% pH 4−6.5 ampholyte IEF gel and then blotted onto a nitrocellulose membrane (Bio-Rad) in a 0.7% acetic acid transfer buffer using an electrophoretic transfer cell (Bio-Rad) at 40 V for 45 min. The membrane was blocked with 5% nonfat milk in PBS containing 0.02% sodium azide for 8 h, followed by 8 and 1.5 h incubations in the respective solution containing McAb and alkaline phosphatase conjugate. Following additional washing with PBS-T, the membrane was incubated in a nitroblue tetrazolium (0.2 mg/mL) and 5-bromo-4-chloroindolyl phosphate (0.2 mg/mL) solution in 0.1 M Tris buffer containing 5 mM $MgCl_2$ (pH 9.5) until color developed.

Immunodot Blotting. Water-soluble sarcoplasmic extracts (10 μg/100 μL) from 22 fish species were blotted onto nitrocellulose membrane assembled in a Bio-Dot (Bio-Rad) microfiltration apparatus and incubated for 1.5 h. Unbound sites were blocked by incubating the membrane in BSA-blocking buffer for 2 h. The membrane was incubated in McAb solution for 1.5 h, rinsed twice in Tris-buffered saline (TBS) containing 0.2% Tween 20 (TTBS), and then incubated with alkaline phosphatase conjugate for 1.5 h. After the membrane was washed in TTBS, it was processed as previously described for Western blotting.

Determination of the Reactivity of McAbs. The reactivity of these McAbs with water-soluble sarcoplasmic proteins of 36 raw seafood and meat samples was performed using ELISA and immunodot blotting. Furthermore, these products (10 g) were broiled at 350 °F for 10 min in a household oven and then retested with ELISA. Water-soluble sarcoplasmic proteins of these diverse samples were extracted following the previously described procedures. The protein content of each extract was determined and adjusted to 50 μg/mL.

Optimization of McAb and Antigen Concentrations for ELISA. *Antibody Concentration.* McAb at 0.5, 1, 5, 10, 25, 50, 100, and 200 μg of protein/mL was tested by ELISA to determine the optimal concentration needed to achieve highly specific reactivity with red snapper proteins. Water extracts of raw red snapper at 5 and 10 μg of protein/mL were used to coat the plate wells.

Antigen Concentration. Water extracts of raw red snapper at 0.1, 0.5, 1, 5, 10, 25, 50, 100, and 200 μg of protein/mL were used to coat ELISA plate wells (0.1 mL/well). McAbs from 25 to 100 μg/mL were tested on these plates to determine the optimal concentration of antigen needed for maximum reactivity.

Blind Study To Identify Red Snapper. A blind study was carried out using ELISA to determine the specificity of McAbs for red snapper. The reactivity of the water-soluble sarcoplasmic proteins of red snappers was tested along with those of 23 other species including VS, GS, HF, LS, MS, YS, OS, BS, LL, PG, Atlantic salmon, Gag grouper, bigeye tuna, swordfish, mahi-mahi, shark, black snapper, sheepshead, gray triggerfish, channel catfish, and chicken. Three red snapper samples including those from Texas and Florida coasts off the Gulf of Mexico and from the Food Laboratory of the Florida Department of Agriculture and Consumer Services, were used. The water-soluble protein extracts of these test samples were prepared as previously described, and the protein concentration of each sample was adjusted to 50 μg/mL. After all of the samples were marked in Arabic numerals by the author and given to an operator, each test sample was divided into two equal portions by the operator. After the sample orders were rearranged, one set of the samples was given back to the author. Both the operator and author conducted the reactivity test with McAbs for these samples at the same time using ELISA. The experiment was repeated once.

RESULTS AND DISCUSSION

IEF Protein Patterns of Various Fish Samples. IEF gels containing a 20% pH 3−10 and 80% pH 4−6.5 ampholyte mixture have been shown to provide better fish protein profiles for species identification (Wei et al., 1990; Huang et al., 1995). This system was employed to compare the protein profiles of seven authentic fish species, including red snapper, VS, GS, HF, LS, MS, and YS. Proteins with p*I* values between 5.0 and 7.16 were well separated (Figure 1), while those with p*I* values greater than 7.16 clustered together. Because each fish species displayed its own specific protein pattern, this IEF system could be used to identify these fish species. The results also showed that each fish species possessed distinct proteins. For example, the p*I* 6.18 protein was unique for VS, the p*I* 5.44 protein was unique for HF, and the p*I* 5.0 and 5.13 proteins were unique for GS. Fish species also shared common proteins among themselves. The p*I* 5.93 protein (protein *a*), which was present in larger quantities in red snapper, was a minor constituent in VS and LS. The p*I* 5.36 protein was present in red snapper and VS and, to a lesser degree, in HF, while the p*I* 5.18 protein was detected in red snapper, VS, and LS.

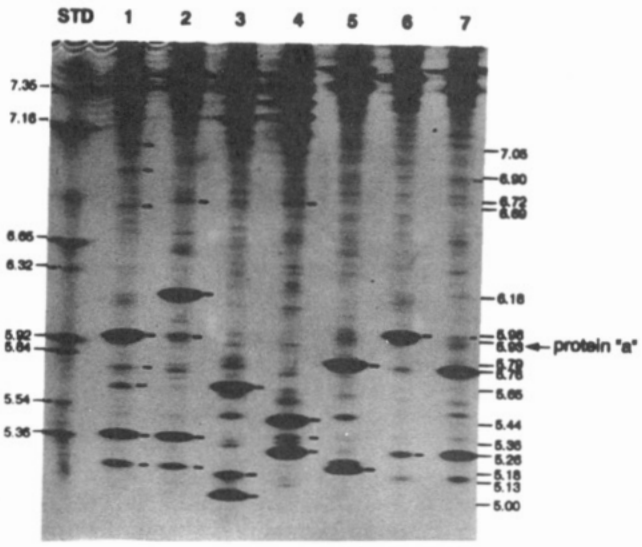

Figure 1. Profiles of water-soluble sarcoplasmic protein extracts from (1) red snapper, (2) vermilion snapper, (3) gray snapper, (4) hogfish, (5) lane snapper, (6) mutton snapper, and (7) yellowtail snapper on an IEF gel containing 2% ampholyte mixture (20% pH 3−10 and 80% pH 4−6.5). The numerical designations indicate apparent p*I* values of the protein bands. Protein *a* with p*I* value of 5.93 was used to immunize mice.

This IEF system was also used to compare protein profiles of authentic red snapper and red snappers obtained from the Texas and Florida coasts off the Gulf of Mexico with those of OS, BS, LL, PG, and WG. Red snappers from these two different locations showed no difference in IEF protein profiles from authentic red snapper (data not shown). The findings were similar to previously reported results that red snapper could be differentiated from the other species by comparison of the IEF protein patterns (Huang et al., 1995).

Screening of Monoclonal Antibodies against Red Snapper Protein *a*. Sera from immunized mice, when tested against the seven authentic fish extracts, showed a selected reactivity with red snapper and VS but not with the other five non-red snapper species (data not shown). Therefore, the spleen cells from these immunized mice were used for production of hybridomas.

Of 464 growth positive hybridomas, 28 showed positive reactivity (absorbance readings >0.4; background absorbance 0.12) with red snapper extract as deter-

Table 1. Reactivities of Four Cloned Cultures with Various Fish Extracts Using ELISA

	absorbance at 405 nm									
culture	1[a]	2	3	4	5	6	7	8	9	10
C1C1	1.41[b]	1.29	0.15	0.11	0.91	0.81	0.86	0.15	0.15	0.18
A1B2	0.93	0.52	0.35	0.45	0.33	0.46	0.56	0.36	0.34	0.31
A2C3	0.67	0.33	0.24	0.35	0.40	0.41	0.46	0.29	0.32	0.43
C2A2	0.75	0.37	0.44	0.57	0.44	0.53	0.43	0.55	0.55	0.47

[a] Fish extracts were tested at 50 μg/mL. Fish samples are (1) red snapper, (2) vermilion snapper, (3) gray snapper, (4) hogfish, (5) lane snapper, (6) mutton snapper, (7) yellowtail snapper, (8) onespot snapper, (9) black snapper, and (10) Madras snapper. [b] Absorbance was recorded 1.5 h after reaction with C1C1 McAb and after 7 h with the other three McAbs. Absorbance values were the means of triplicate tests. The standard deviations ranged from 0.01 to 0.08. The background readings were 0.12–0.16.

mined by ELISA. They were then tested for specificity with the extracts of 10 fish species (the seven authentic species plus OS, BS, and LL) using ELISA after further propagation. Only four of these 28 hybridomas showed satisfactory reactivity with red snapper. They also showed cross-reactivity with VS, LS, MS, and YS. These four hybridomas were then subjected to single cell cloning, and the four best clones (C1C1, A1B2, A2C3, and C2A2) were obtained.

Table 1 demonstrates that antibodies from these four clones showed greater reactivity with red snapper than with the other nine species. C1C1 McAb had the highest reactivity to red snapper according to absorbance. It also reacted with VS, LS, MS, and YS. McAbs A1B2, A2C3, and C2A2 had lower reactivities than C1C1 to red snapper. However, these three McAbs showed various degrees of selectivity with the other nine species. These four McAbs were produced in large quantities via ascites. The isotypes of these four McAbs were IgG1.

Specificity Determination of McAbs by Western Blotting, Immunodot Blotting, and ELISA. Binding of the four McAbs to individual fish protein components was studied using Western blotting of IEF gels (Burnette, 1981; Gershoni and Palade, 1983). C1C1 McAb reacted with one major protein from red snapper, VS, MS, and YS and with two proteins from LS (data not shown). These fish proteins apparently shared a similar epitope for C1C1 McAb. Similarly, A1B2 McAb reacted with one protein from red snapper, VS, LS, MS, and YS, while A2C3 and C2A2 McAbs each reacted with only one protein from red snapper, VS, and LS. Thus, it is obvious that the four McAbs are different from each other. Only McAbs C1C1 and C2A2 were used for further studies.

Cross-reactivity of these two McAbs with raw fish proteins was further tested by immunodot blotting against 22 fish species and by ELISA with 36 commonly consumed seafood and meat samples. From the color development of the immunodot blotting assay, it was evident that C1C1 McAb reacted more strongly with red snapper and VS than with LS, MS, and YS (Figure 2). The remaining 17 fish species either reacted weakly or not at all with C1C1 McAb. The C2A2 McAb also showed a stronger specific reactivity with red snapper, but it did not react with VS (Figure 2). Therefore, from the dark color intensity of the immunodot blotting assay using McAb C1C1, red snapper and VS could be differentiated from the other 20 fish species. Red snapper and VS could then be distinguished using C2A2 McAb, which reacted strongly with red snapper but not with VS.

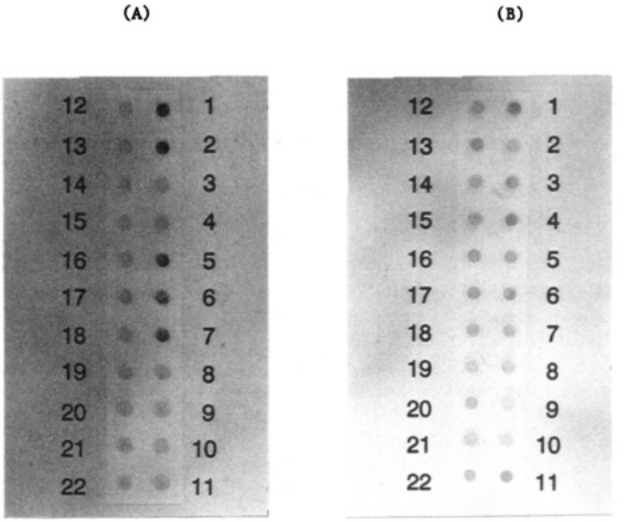

Figure 2. Reactivity of (A) C1C1 and (B) C2A2 McAbs with 22 fish samples using immunodot blotting assay. Samples are (1) red snapper, (2) vermilion snapper, (3) gray snapper, (4) hogfish, (5) lane snapper, (6) mutton snapper, (7) yellowtail snapper, (8) onespot snapper, (9) blackspot snapper, (10) Madras snapper, (11) pink porgy, (12) white grunt, (13) Atlantic salmon, (14) greater amberjack, (15) Gag grouper, (16) flounder, (17) bigeye tuna, (18) swordfish, (19) mahi-mahi, (20) spot, (21) shark, and (22) gray tilefish.

ELISA testing confirmed the immunodot blotting studies (Table 2). C1C1 McAb showed stronger reactivity with red snapper than with VS, LS, MS, and YS. Although C2A2, in general, showed a stronger reactivity with red snapper, it cross-reacted with pink porgy, white grunt, OS, bigeye tuna, mahi-mahi, Atlantic sturgeon, blue crab, and chicken (Table 2). The results also showed that red snapper could be identified by reacting raw fish extracts with these two McAbs in ELISA. The reaction of C1C1 McAb with 36 seafood and meat samples would reduce the sample pool to 5 reactive species: red snapper, VS, LS, MS, and YS. Since C2A2 reacted well with red snapper but not as well with VS, LS, MS, and YS, the sequential use of C1C1 followed by C2A2 in ELISA can differentiate red snapper from these four species.

The reactivities of these two McAbs against protein extracts from 36 cooked seafood and meat samples are also shown in Table 2. McAb C1C1 still showed strong reactivities with red snapper, VS, LS, MS, and YS. White grunt was the only other fish sample that reacted with C1C1. McAb C2A2 also showed moderate reactivity with red snapper, MS, BS, LL, channel catfish, and oyster. Compared to C1C1, the reactivities of C2A2 with cooked samples of the five fish species, red snapper, VS, MS, LS, and YS, were greatly reduced (absorbance readings 0.29–0.54). The reactivities of C2A2 with cooked samples of these five fish species were also greatly reduced when compared to those of raw samples (absorbance readings 0.44–0.83). Since each of these two McAbs showed unique reaction patterns with the extracts of different fish species, red snapper could also be identified by a combination of cooking and using C1C1 and C2A2. Thus, these two McAbs could facilitate and improve the accuracy of red snapper identification. For example, the differentiation of red snapper from VS or LS will be difficult if only McAb C1C1 is used to react with raw extracts (Table 2). However, because the extract of cooked red snapper reacted strongly with C1C1 and moderately with C2A2, while the cooked VS and LS reacted only with McAb C1C1 but not with C2A2

Table 2. Reactivities of Two Monoclonal Antibodies with Water Extracts of Various Raw and Cooked Fish and Meat Samples Using ELISA

	absorbance at 405 nm			
	raw		cooked	
sample[a]	C1C1[b]	C2A2[c]	C1C1	C2A2
red snapper	1.94	0.89	1.71	0.54
vermilion snapper (VS)	1.75	0.42	0.84	0.35
gray snapper (GS)	0.21	0.32	0.33	0.39
hogfish (HF)	0.21	0.45	0.22	0.35
lane snapper (LS)	1.58	0.43	1.66	0.29
mutton snapper (MS)	1.31	0.61	0.96	0.49
yellowtail snapper (YS)	1.57	0.61	1.29	0.30
pink porgy (PG)	0.25	0.71	0.20	0.29
white grunt (WG)	0.41	0.84	0.57	0.27
onespot snapper (OS)	0.23	0.72	0.27	0.27
blackspot snapper (BS)	0.35	0.41	0.24	0.43
Madras snapper (LL)	0.24	0.48	0.22	0.44
black snapper	0.22	0.58	0.33	0.26
Atlantic salmon	0.17	0.45	0.33	0.32
greater amberjack	0.26	0.81	ND[d]	ND
Gag grouper	0.23	0.48	0.20	0.28
flounder	0.16	0.42	0.21	0.26
bigeye tuna	0.27	0.74	0.28	0.36
swordfish	0.25	0.66	ND	ND
mahi-mahi	0.18	0.70	0.23	0.34
spot	0.32	0.57	ND	ND
shark	0.31	0.45	ND	ND
gray tilefish	0.21	0.46	ND	ND
spotted seatrout	0.23	0.39	0.20	0.31
sheepshead	0.16	0.56	0.21	0.29
gray triggerfish	0.24	0.52	0.29	0.36
channel catfish	0.22	0.34	0.17	0.43
Atlantic sturgeon	0.22	0.63	0.36	0.42
white shrimp	0.22	0.54	0.21	0.29
oyster	0.18	0.62	0.34	0.50
scallop	0.31	0.41	0.18	0.35
blue crab	0.20	0.63	0.16	0.24
pork	0.24	0.61	0.25	0.31
beef	0.19	0.53	0.15	0.18
chicken	0.24	0.78	0.17	0.28
turkey	0.24	0.45	0.19	0.19

[a] Protein extracts at 5 μg/well were used. [b] C1C1 McAb at 5 μg/well was used. Absorbance readings were recorded 1.5 h after reaction. [c] McAbs C2A2 at 10 μg/well were used. Absorbance readings were recorded 7 h after reaction. All absorbance values were the means of triplicate tests. The standard deviations ranged from 0.02 to 0.08. The background readings were 0.12–0.16. [d] Not determined.

(Table 2), red snapper could be accurately differentiated from these species.

Optimization of Antibody and Antigen Concentrations for ELISA Test. McAb C1C1 showed a dose-related increase in reactivity with red snapper extract at 5 or 10 μg/mL; the reactivity plateaued when McAb was at 50 μg/mL (data not shown). Furthermore, C1C1 McAb at 25 or 50 μg/mL showed the highest reactivity with red snapper extract at 50 μg/mL (data not shown). A similar approach for C2A2 found that red snapper extract at 50 μg/mL provided the most satisfactory reactivity with C2A2 at 100 μg/mL. Therefore, McAbs at 50 μg/mL for C1C1 and 100 μg/mL for C2A2 were used in ELISA to ensure greater sensitivity for detection of red snapper.

Use of McAbs in ELISA To Identify Red Snapper in a Blind Study. A blind study conducted separately by the author and another operator on 24 fish and meat samples using ELISA showed that seven samples (6, 10, 12, 14, 18, 21, and 24) consistently gave strong reactivity with C1C1 McAb (absorbance >1.26). The remaining 17 samples had an absorbance value of less than 0.31 (data not shown). These seven samples were then tested for reactivity with McAb C2A2 in ELISA as above. Three of them (12, 23, and 24) consistently showed higher absorbance readings than others in reacting with C2A2 (Table 3). They were later revealed to be red snapper obtained from both the Texas and Florida coasts off the Gulf of Mexico and from the Food Laboratory of the Florida Department of Agriculture and Consumer Services. Red snapper obtained from these three different locations therefore showed no difference in reactivity with C1C1 McAb (data not shown) or C2A2 (Table 3). Red snapper can thus be identified by its higher reactivity with these two McAbs using ELISA.

Table 3. Reactivities of C2A2 McAb[a] with Water Extracts of Seven Various Samples Showing Strong Reactivity with C1C1 McAb in a Blind Study Using ELISA

sample no.	sample identity	absorbance at 405 nm: operator I[b]	operator II[c]
6	vermilion snapper	0.43	0.48
10	yellowtail snapper	0.61	0.66
12	red snapper	1.03	1.12
14	lane snapper	0.41	0.48
18	mutton snapper	0.59	0.63
23	red snapper (west coast of gulf)	1.04	1.25
24	red snapper (east coast of gulf)	1.06	1.21

[a] McAb C2C2 at 10 μg/well was reacted with test samples at 5 μg/well. Absorbance readings were recorded 7 h after reaction. All absorbance values were the means of triplicate tests. The standard deviations ranged from 0.01 to 0.09. The background readings were 0.12–0.16. [b] Test was performed by another operator. [c] Test was performed by the author.

The other four samples showing strong reactivity with C1C1 McAb (absorbance readings between 1.27 and 1.62, data not shown) were VS, LS, MS, and YS. Similar to the results shown in Table 2, these four fish samples reacted differently with McAb C2A2 (Table 3).

On the basis of the results of these studies, McAb C1C1 could be used in ELISA to identify red snapper, VS, LS, MS, and YS from a pool of samples on the basis of their strong reactivities with this McAb. Red snapper could then be differentiated from the other four fish species on the basis of their differences in reactivity with McAb C2A2. Red snapper, in general, had greater reactivity with these two McAbs than VS, LS, MS, or YS.

Verification of Fish Species Using IEF and McAbs. A comparison of the McAbs with IEF to identify commercial fish species claimed by seafood retailers was performed. Twenty-four commercial fish samples collected by the Food Laboratory, Florida Department of Agriculture and Consumer Services, from statewide seafood retail stores and industries were tested by the authors using IEF gel electrophoresis (20% pH 3–10 and 80% pH 4–6.5 ampholyte mixture) and ELISA procedures. The IEF protein profiles of these samples were identified by comparing them to authentic red snapper, VS, LS, MS, and YS. Identities of these fish samples had also been determined separately by the Food Laboratory using thin-layer IEF gels. The results are summarized in Table 4.

Four claimed MS samples (11092, 13258, 13262, and 13402) by retailers were confirmed by thin-layer (by the Food Laboratory) and slab (by the author) IEF gel electrophoresis to be MS (Table 4). They all reacted strongly with McAb C1C1 but less strongly with C2A2. Therefore, the ELISA results confirmed their claimed identity as MS by the retailers.

However, this was not the case for those samples claimed to be red snapper. Only 1 (14738) of the 15 claimed red snapper samples was confirmed by gel

Table 4. Confirmation of Commercial Fish Sample Identities by Thin-Layer and Slab IEF Gels and from Reactivities with McAbs C1C1 and C2A2 Using ELISA

sample no.[a]	claimed fish species[b,g]	identity by IEF		reactivity with McAb[e]	
		thin layer[c]	slab[d]	C1C1	C2A2
11092	mutton snapper	mutton snapper	mutton snapper	1.79	0.65
13258	mutton snapper	mutton snapper	mutton snapper	1.70	0.71
13262	mutton snapper	mutton snapper	mutton snapper	1.34	0.64
13402	mutton snapper	mutton snapper	mutton snapper	1.29	0.64
13404	red snapper	lane snapper	lane snapper	1.65	0.44
13407	scarlet red snapper	no[f]	no	0.15	
13408	red snapper	no	no	0.16	
13409	red snapper	no	no	0.17	
13454	red snapper	mutton snapper	mutton snapper	0.82	0.65
13455	red snapper	no	no	0.17	
13456	red snapper	no	no	0.20	
13458	malabar red snapper	no	no	0.15	
13831	red snapper	no	no	0.18	
14737	Caribbean red snapper	no	red snapper	1.71	0.89
14738	red snapper	red snapper	red snapper	1.81	1.01
14763	red snapper	no	no	0.41	
15930	Pacific snapper	no	no	0.20	
15931	red snapper	no	no	0.21	
17223	red snapper	no	no	0.17	
17224	red snapper	no	no	0.19	
17225	imported red snapper	no	no	0.15	
17378	red snapper	no	vermilion snapper	1.75	0.50
17479	red snapper	no	no	0.17	
17579	red snapper	no	no	0.25	

[a] Fish samples were obtained from the Food Laboratory, Florida Department of Agriculture and Consumer Services, Tallahassee. [b] Identity of fish samples as claimed by seafood retailers. [c] Identity of fish samples as determined by the Food Laboratory using thin-layer IEF gel electrophoresis. [d] Identity of fish samples as determined by the author using slab IEF gels. [e] Absorbance readings at 405 nm were recorded 7 h after reaction. The values were means of triplicate tests. The standard deviations ranged from 0.02 to 0.12. The background readings were 0.12–0.16. [f] No: Identity of fish samples was confirmed by thin-layer IEF or slab IEF gels not to be the same as what was claimed by seafood retailers. Identification of these fish samples could not be performed due to the lack of authentic samples. [g] Authentic red snapper was used for comparison. Its reactivities with McAbs C1C1 and C2A2 were 1.85 and 0.95, respectively.

electrophoresis to be genuine red snapper. When reacting with McAbs C1C1 and C2A2, it gave high absorbance readings (Table 4). Samples 13404 and 13454 were identified by gel electrophoresis to be LS and MS, respectively. They also reacted strongly with McAbs C1C1 but less strongly with C2A2 (Table 4). The claimed red snapper sample 17378 gave high ELISA absorbance readings when reacted with McAbs C1C1 and to a lesser degree with C2A2 (Table 4), which indicated that this was not red snapper. This sample also failed to produce protein profiles similar to that of red snapper and was obviously not red snapper as claimed by the retailers. On the basis of the reactivity with these two McAbs using ELISA, the identification of this fish sample could be VS, MS, LS, or YS. This fish sample was identified by our slab IEF gel to be VS. The Food Laboratory could only confirm that it was not red snapper using thin-layer IEF electrophoresis.

The remaining 11 red snapper samples (13408, 13409, 13455, 13456, 13831, 14763, 15931, 17223, 17224, 17479, and 17579) all showed very weak reactivity with C1C1 McAb (absorbance readings <0.41, Table 4). These results indicated the samples were not red snapper. This conclusion was further substantiated by either IEF technique.

Sample 14737, which was claimed by retailers to be Caribbean red snapper, reacted strongly with McAb C1C1 (Table 4). The protein profile of this sample was shown by slab IEF to be similar to that of red snapper, although thin-layer IEF failed to confirm this sample as being red snapper. This sample also reacted strongly with McAb C2A2 (Table 4); therefore, this sample could very possibly be red snapper. The other four samples, claimed by retailers to be scarlet red snapper (13407), malabar red snapper (13458), Pacific snapper (15930), and imported red snapper (17225), all failed to react with McAb C1C1 (absorbance readings <0.2, Table 4). The results confirmed these samples were not red snapper. Again, this conclusion was supported by the IEF protein profiles (Table 4).

CONCLUSION

Red snapper could be identified from the water-soluble protein extracts of raw or cooked samples using two McAb-based ELISA. Because of its specificity, McAb C1C1 can be used first to separate red snapper, VS, LS, MS, and YS from other test fish samples. McAb C2A2, which shows higher reactivity with red snapper than the other four fish species, can then be used to further differentiate red snapper from these four non-red snapper species. This approach is workable and practical as indicated from the blind study to differentiate red snapper from 24 seafood and meat samples.

Water-extractable fish proteins from cooked samples also react with these two McAbs, providing additional differentiation of red snapper because the reactivity patterns of cooked red snapper, VS, LS, MS, and YS with these two McAbs are different from those of raw samples. This approach using cooked samples can be performed separately or in combination with raw samples. Running a parallel cooked and raw sample simultaneously revealed a greater sensitivity for identifying red snapper from other fish species than merely sampling a raw or cooked sample alone.

ELISA tests using these two McAbs can also be employed together with thin-layer or slab IEF gel electrophoresis to differentiate red snapper. Using McAb C1C1 in ELISA can eliminate many fish species except red snapper, VS, LS, MS, and YS. These can be further differentiated by their distinctive IEF protein profiles. This approach has been shown to be effective

in our verification study with the claimed "red snapper" samples obtained from the Food Laboratory.

Immunodot blotting can also be used as a rapid field screening method to identify red snapper. Fish extracts, following homogenization of fish meat in a Waring blender and centrifugation of the homogenates in a portable personal microcentrifuge, can be diluted properly and subjected to dot blotting followed by treatment with McAbs C1C1 and C2A2. Since only red snapper reacts strongly with these two McAbs and shows dark colored spots, this fish species can be identified. Further confirmation can be achieved in the laboratory using ELISA or IEF gel electrophoresis.

Efforts can also be made in the future to further characterize the protein profiles of red snapper, VS, LS, MS, and YS so that a red snapper-specific protein can be identified for McAb preparation. This would be essential if assay kits were to be developed for field testing of fish extracts. The combined use of C1C1 McAb for quick screening to reduce sample size and then this newly developed McAb will reduce testing time and cost and improve the accuracy of the results. However, further development to produce red snapper-specific McAbs for differentiation of red snapper from VS, MS, and YS may not be necessary if one considers the retail price of these three fish species. The market price (about $7/lb) for VS, MS, and YS is close to that of red snapper. Therefore, retailers do not have to mislabel these species as red snapper and risk violating labeling regulations.

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