

## Monoclonal Antibody Specific to a Major Fish Allergen: Parvalbumin

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### ABSTRACT

The major fish allergen, parvalbumin, is a low-molecular-weight (10 to 13 kDa), heat-stable protein. Monoclonal antibody (MAb) 3E1, developed against heat-treated catfish sarcoplasmic protein extract, recognizes a thermal-stable protein with the molecular-weight range of parvalbumin in fish extracts. We further investigated the antigen-binding characteristics of this antibody by comparing its immunoreactivity against various fish and other animal species, with a commercially available anti-parvalbumin antibody, MAb PARV-19. Soluble proteins were extracted from 67 cooked (100°C for 20 min) finfish, shellfish, meat, and poultry species. Indirect enzyme-linked immunosorbent assay (ELISA) was performed to examine the immunoreactivity of both MAb 3E1 and MAb PARV-19 with sample extracts. Western blot was performed to compare the antigenic protein banding patterns in cooked fish extracts by using these two MAbs. The ELISA results revealed that both MAbs had identical reaction patterns to the fish species tested. Removal of Ca<sup>2+</sup> from the fish extracts increased the overall immunoreactivity of both MAbs. Western blot results confirmed that the antigenic protein banding pattern in various fish species blotted by MAb 3E1 corresponded to the molecular weights of parvalbumins recognized by PARV-19. However, screening with non-fish extracts revealed MAb 3E1 to be strictly finfish specific, while PARV-19 cross-reacted with frog, rat, and rabbit extracts. Based on the heat stability, molecular weight, immunoreactivity, and Ca<sup>2+</sup>-dependent binding of the antigenic proteins, MAb 3E1 is specific to fish parvalbumin. It would therefore be a useful probe for investigating the major fish allergen in both raw and processed food.

Fish is becoming an increasingly important food source, due to its high consumption and its dietary values, namely high-quality proteins, beneficial polyunsaturated fatty acids, and lipid-soluble vitamins. However, together with wheat, soy, cow's milk, peanuts, tree nuts, shellfish, and eggs, fish is one of the eight major allergenic foods or food types that cause immunoglobulin E (IgE)-mediated food allergy in humans (2, 21, 36, 40). Food allergies have been a common problem in industrialized countries. The prevalence of fish allergy in the United States was determined to be 0.4% of the population, or 1.1 million Americans, and this number is increasing (33, 35). Clinical symptoms in fish-allergic patients can comprise acute urticaria, atopic dermatitis, asthma, gastrointestinal disorders (diarrhea, vomiting), and in some cases, even life-threatening anaphylaxis (30, 37, 41).

Major allergens generally are defined as proteins for which 50% or more of the allergic patients tested have specific IgE (20). Although fish contains a wide variety of proteins, only a few of these cause allergic reactions (25). The first purified and characterized fish allergen was Gad c 1, a codfish parvalbumin (1, 9). Parvalbumins are considered the major allergen in fish because more than 95% of fish-allergic patients have been found to have specific IgE to this protein, and many of the IgE-binding epitopes on this allergen are present in various fish species (4, 7).

Parvalbumins are a family of Ca<sup>2+</sup>-binding proteins that play an important role in muscle relaxation (16, 28). They have low molecular weights of approximately 10 to 13 kDa and acidic pI values, and are water soluble and resistant to heat treatment (1, 8).

Parvalbumins are present in relatively high quantities in the muscles of lower vertebrates, such as fish, and in lesser amounts in higher vertebrates, including humans (11). The quantity of parvalbumin also varies in different types of fish muscles; white muscle generally contains more parvalbumin than does dark muscle, which makes the dark muscle tissue of fish much less allergenic than the white muscle tissue (21, 32). Based on a comparison of their amino acid sequences, parvalbumins are subdivided into phylogenetic lineages  $\alpha$  and  $\beta$ . The  $\alpha$ -parvalbumins have pI values at or above 5.0, while the  $\beta$ -parvalbumins, containing more acidic amino acids, have a pI value of 4.5 or lower (12, 31). Members of both lineages have been identified in a number of fish species, including thornback ray, carp, mackerel, cod, salmon, and Alaska pollock (13, 27, 37, 38, 41).

Although IgE obtained from sensitized human blood can be used to detect fish allergen, the source is very limited. Unlike IgE, the quantity of IgG antibodies can be obtained from immunized animal antiserum (polyclonal antibodies) or developed by hybridoma techniques (monoclonal antibodies; MAb) for convenient detection of the fish allergen. However, currently there is no IgG antibody available for convenient detection of fish allergen. A commercial

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anti-frog parvalbumin MAb PARV-19 (originally raised against frog parvalbumins) has also been reported to bind fish parvalbumin (5, 17). However, its specificity to parvalbumin from a wide range of fish species is limited according to the literature. We intended to study further the usefulness of this antibody by examining its reactivity against an extended number of popular food fish species as well as other animal species.

In addition, a monoclonal antibody (MAb 3E1) initially developed by our group against heat-treated catfish crude sarcoplasmic protein extract was also found to be cross-reactive with other fish species. It recognizes a thermal-stable protein with a molecular weight of 10 to 13 kDa in fish extracts, which corresponds to the molecular weight of the major fish allergen parvalbumin. We therefore speculated that MAb 3E1 could be specific to fish parvalbumin, which is a heat-stable,  $\text{Ca}^{2+}$ -binding sarcoplasmic protein. In order to verify whether MAb 3E1 does in fact bind to fish parvalbumin, the present study was also designed to investigate the antigen-binding characteristics of MAb 3E1 by comparing its immunoreactivity against a wide range of fish and non-fish animal species with that of the anti-frog parvalbumin antibody, MAb PARV-19.

## MATERIALS AND METHODS

**Materials.** Tris-buffered saline, 0.5 M Tris-HCl buffer (pH 6.8), 1.5 M Tris-HCl (pH 8.8),  $N,N,N',N'$ -tetramethylethylenediamine, Precision Plus Protein Kaleidoscope Standards, 30% acrylamide-bis solution, Tris-glycine buffer,  $10\times$  Tris-glycine-sodium dodecyl sulfate (SDS) buffer, supported nitrocellulose membrane (0.2  $\mu\text{m}$  thick), and thick blot paper were purchased from Bio-Rad Laboratories, Inc., Hercules, CA. Hydrogen peroxide, horseradish peroxidase conjugated goat anti-mouse IgG (Fc specific), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and  $\beta$ -mercaptoethanol were purchased from Sigma-Aldrich Co., St. Louis, MO. Bromophenol blue sodium salt was purchased from Allied Chemical Corp., Morristown, NJ. Sodium chloride (NaCl), sodium phosphate dibasic anhydrous ( $\text{Na}_2\text{HPO}_4$ ), sodium phosphate monobasic anhydrous ( $\text{NaH}_2\text{PO}_4$ ), bovine serum albumin, sodium bicarbonate ( $\text{NaHCO}_3$ ), sodium carbonate ( $\text{Na}_2\text{CO}_3$ ), citric acid monohydrate, SDS, Tween 20, and all other chemicals, reagents, filters (Whatman No. 1 paper), 96-well polystyrene microplate (Costar 9018) were purchased from Fisher Scientific, Fair Lawn, NJ. All solutions were prepared by using distilled, deionized pure water processed via the NANOpure DIamond Ultrapure water system (Barnstead International, Dubuque, IA). All chemicals and reagents were analytical grade.

Twenty-five authentic fish samples were obtained from the Florida Department of Agriculture and Consumer Services, Tallahassee. An additional 26 different fish samples were obtained from reliable seafood distributors and from local fish markets. Crab, shrimp, scallop, frog legs, fresh beef loin, lamb shoulder, pork loin, frozen dressed rabbit, whole turkey, and chicken were purchased from local supermarkets. Horsemeat was obtained from a private source. Rat muscles were obtained from the Biological Facilities, Florida State University. Monoclonal mouse anti-frog parvalbumin IgG (PARV-19) was obtained from Sigma-Aldrich.

**Protein extraction from fish and meat samples.** Approximately 10 g of half-thawed fish samples from each species was weighed into beakers. The beakers were covered with aluminum foil, sealed with adhesive tape, and heated in a boiling water bath

for 20 min. The cooked samples were then cooled to room temperature and mashed into fine particles by using a glass rod, after which a threefold concentration of saline (0.15 M NaCl; in milliliters) was added to the mashed samples (3:1 [vol/wt]), and the mixture was homogenized for 1 min at 11,000 rpm. The homogenized samples were allowed to stand at 4°C for 2 h, which was followed by centrifuging at  $5,000 \times g$  for 30 min at 4°C. Supernatants obtained after centrifugation were then filtered through Whatman No. 1 filter paper and stored at  $-20^\circ\text{C}$  until use. The non-fish lean muscle samples, including both poultry and other meats, were ground and then prepared in the same manner as were the fish samples.

**Production of MAb 3E1.** The cooked catfish crude extract was dialyzed (molecular-weight cutoff of 10,000) in 10 mM phosphate-buffered saline (PBS) for 24 h, with frequent changes, and the dialyzed extract was used as the immunogen. Three female BALB/c mice were immunized subcutaneously with 100  $\mu\text{g}$  per mouse of the immunogen emulsified with an equal volume of Freund's complete adjuvant. Three booster injections prepared in the same manner by using Freund's incomplete adjuvant were administered to each mouse at 4-week intervals. The mouse with the highest titer was injected intraperitoneally with 100  $\mu\text{g}$  of the immunogen in PBS 4 days prior to fusion. The immunization and the subsequent hybridoma procedures were performed in compliance with the Florida State University's Animal Welfare Guidelines.

The spleen cells from the immunized mice were fused with murine myeloma cells (P3x63.Ag8.653, ATCC CRL 1580) at a ratio of 5:1 in the presence of polyethylene glycol (molecular weight 4,000) for hybridoma production. The general procedures described by Köhler and Milstein (22) were followed with modifications and the following specific screening procedures. The initial screening of hybridomas was against the immunogen by using indirect enzyme-linked immunosorbent assay (ELISA). Those positive hybridomas were selected and expanded. For a secondary selection, the expanded positive hybridomas were screened for reactivity with extracts from six cooked meat (beef, pork, sheep, horse, chicken, and turkey) and four other fish species (tilapia, tuna, flounder, and rainbow trout). Only those hybridomas with no cross-reactivity with the six non-fish samples were cloned twice by limited dilution. Five cross-reactive, fish-specific MAbs were selected. MAb 3E1 was selected for further characterization in this study because it is fish specific, cross-reactive with other fish species, and reacts with a group of 10- to 13-kDa thermal-stable proteins (likely parvalbumin) in fish extracts. Supernatant of MAb 3E1 obtained from the propagated cell cultures was used in this study. The isotype of MAb 3E1 was determined with a mouse MAb isotyping kit (ISO-2 1 kit, Sigma) as IgG1, according to the manufacturer's protocol.

**Indirect ELISA.** Properly diluted sample protein extract in 0.06 M carbonate buffer (pH 9.6) was coated (2  $\mu\text{g}/100 \mu\text{l}$  per well) onto the wells of a 96-well polystyrene microplate (Costar 9018, Fisher Scientific) and incubated at 37°C for 2 h. The plate was then washed three times with 0.05% (vol/vol) Tween 20 in 10 mM PBS (PBST; pH 7.2) and incubated with 200  $\mu\text{l}$  per well of blocking solution (3% nonfat dried milk in PBS) at 37°C for 2 h, which was followed by another washing step. Supernatant of MAbs 3E1 or PARV-19 ascites fluid appropriately diluted in antibody buffer (1% [wt/vol] bovine serum albumin in PBST) was added to each well (100  $\mu\text{l}$ ) and incubated at 37°C for 2 h. After washing three times with PBST, 100  $\mu\text{l}$  of the secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG-Fc specific diluted 1:3,000 in antibody buffer) was added to each well

and the plate incubated at 37°C for 2 h. It was then washed five times before the addition of the substrate solution (22 mg of ABTS and 15  $\mu$ l of 30% hydrogen peroxide in 100 ml of 0.1 M phosphate-citrate buffer, pH 4.0) for color development at 37°C for 30 min. The enzyme reaction was stopped by adding 0.2 M citric acid solution, and the absorbance measured at 410 nm by using a microplate reader (model MQX200R, BioTek Instruments, Inc., Winooski, VT).

**Ca<sup>2+</sup>-dependent binding assay.** In order to add or remove Ca<sup>2+</sup> from the reaction system, 10 mM CaCl<sub>2</sub> or 10 mM ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), respectively, was added to 0.06 M carbonate buffer (pH 9.6) and the pH of the buffer adjusted back to pH 9.6. Each sample extract was then diluted in the prepared buffers to obtain a concentration of 2  $\mu$ g/100  $\mu$ l and coated on the plate to perform the indirect ELISA, as described above.

**Epitope comparison.** In order to test if the antibodies used in this study bind to the same epitope, the additivity test as described by Friguet et al. (10) was performed with modifications. The amount of the antigen and antibody was previously optimized to ensure the saturation conditions. The microplate wells were coated with 100  $\mu$ l of cooked fish extract containing 0.5  $\mu$ g of soluble proteins diluted in carbonate buffer (pH 9.6). The plate was incubated for 2 h at 37°C, which was followed by washing three times with PBST. After the washing step, each well of the microplate was blocked with 200  $\mu$ l of nonfat dry milk solution (3% [wt/vol] in 10 mM PBS) and incubated at 37°C for 2 h. During incubation, three solutions of antibody were prepared: MAb 3E1 diluted 1:3 in antibody buffer (1% [wt/vol] bovine serum albumin in PBST), MAb PARV-19 diluted 1:7,500 in antibody buffer, and both diluted antibodies together in the ratio 1:1. After two further washings with PBST, 100  $\mu$ l of each of the antibody solution was added into the microplate, which was followed by incubation at 37°C for 2 h. The rest of this assay was performed according to the indirect ELISA procedure as described above. The absorbance was read at 410 nm for MAb 3E1 alone, MAb PARV-19 alone, and for the two antibodies together.

**SDS-PAGE and Western blot.** SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to resolve the soluble proteins in different sample extracts according to the method of Laemmli (23), with modifications. Briefly, soluble proteins (3  $\mu$ g of protein in 10  $\mu$ l per lane) from the samples were loaded on 5% stacking gel (pH 6.8) and separated on 14% polyacrylamide separating gel (pH 8.8). The gel was subjected to electrophoresis at 200 V for 45 min by using a Mini-Protein 3 electrophoresis cell (1610-3301, Bio-Rad) connected to a power supply (model 3000, Bio-Rad).

Western blot assay was carried out according to the method of Towbin et al. (39), with modifications in order to determine the molecular weights of the immunogenic components that reacted with each MAb. After separation of the proteins on the polyacrylamide gel by means of SDS-PAGE, protein bands were transferred electrophoretically (1 h at 100 V) from the gel to nitrocellulose membranes by using a Mini Trans-Blot unit (Bio-Rad). On completion of the transfer, the membrane was washed with 20 mM Tris, 500 mM NaCl, and 0.05% Tween 20 (TBST; pH 7.5), and then blocked with 1% bovine serum albumin in TBS for 2 h. After another washing step, the membrane was incubated with the MAb 3E1 supernatant or PARV-19 ascites fluid diluted 1:1 and 1:5,000, respectively, in antibody buffer for 2 h at room temperature. The excess antibody reagent was removed by washing with TBST, and the membrane was then incubated for 1 h at room temperature

with the secondary antibody (goat anti-mouse IgG-alkaline phosphatase conjugate diluted 1:3,000 in antibody buffer). After washing, the membrane was incubated with 5-bromo-4-chloro-3-indolyl phosphate-*p*-nitroblue tetrazolium chloride in 0.1 M Tris buffer (pH 9.5) for about 3 min to develop the color. The color reaction was stopped by washing the membrane with distilled water. The appearance of a dark purplish band indicated an antibody-binding site. Prestained broad range protein standards (Precision Plus Protein Kaleidoscope Standards, 161-0375, Bio-Rad) were used as molecular-weight markers for both the SDS-PAGE and Western blot.

## RESULTS AND DISCUSSION

**Species specificity of MAbs 3E1 and PARV-19.** In order to compare the species specificity of MAbs 3E1 and PARV-19, non-competitive indirect ELISA was performed by using these two antibodies with fish and meat cooked extracts. The immunoreactivity of MAbs 3E1 and PARV-19 against cooked protein extracts from 51 different fish species, 16 non-fish animal samples, and four food protein additives was examined. The results are summarized in Table 1.

The ELISA results showed that MAb 3E1 had an identical reaction pattern to that of the anti-frog parvalbumin MAb, PARV-19, when reacted with all the fish species tested. Both MAbs reacted strongly with the majority of the fish species tested, with the exceptions being swordfish, yellowfin tuna, pollock, cod, Idaho rainbow trout, wild salmon, whiting, and haddock. This indicates that the antigen recognized by these two antibodies is likely to be the same.

Although there was no difference between the reaction patterns of MAbs 3E1 and PARV-19 for the fish species, screening with non-fish extracts revealed that MAb 3E1 reacted only with finfish, while PARV-19 also reacted with frog, rat, and rabbit extracts. As noted earlier, the MAb anti-frog parvalbumin (PARV-19) obtained from Sigma-Aldrich Corp. was previously reported to bind  $\alpha$  and  $\beta$  frog parvalbumins (17). According to the product datasheet, this antibody was produced by using purified frog muscle parvalbumin as the immunogen, so the reaction with frog parvalbumin was expected. PARV-19 has also been reported to cross-react with parvalbumin in different fish including Pacific and horse mackerel, red sea bream, sardine, carp, catfish, cod, and tilapia (5, 21).

The indirect ELISA experiment revealed that some fish species do not react with either MAb 3E1 or PARV-19. Eight out of the 51 fish species tested showed little or no reaction for both antibodies. Recently Chen et al. (5) reported that PARV-19 did not react with extracts from yellowfin tuna, and our result for yellowfin tuna confirms this observation. Moreover, screening with a large number of fish species showed that in addition to yellowfin tuna, several other fish species also failed to react with PARV-19. The lack of a reaction with those fish species could indicate that either skeletal muscle from those fish species does not contain parvalbumin or the amount of parvalbumin is too small to be detected. Many factors including fish species, tissue type, age, and environment may account for the differences in parvalbumin quantity among fish species. However, parvalbumin was previously found in cod and salmon.

TABLE 1. Immunoreactivity of MAbs 3E1 and PARV-19 against cooked fish, shellfish, meat, poultry, and food additive samples determined by indirect ELISA<sup>a</sup>

| Market name           | 3E1 | PARV-19 | Market name            | 3E1 | PARV-19 | Market name          | 3E1 | PARV-19 |
|-----------------------|-----|---------|------------------------|-----|---------|----------------------|-----|---------|
| Amberjack             | ++  | +       | Orange roughy          | ++  | +++     | Yellow edge grouper  | +++ | +++     |
| Basa                  | ++  | ++      | Orange spotted grouper | +++ | +++     | Yellowtail snapper   | +++ | +++     |
| Blue catfish          | +++ | +++     | Pollock                | —   | —       | Yellowfin tuna       | —   | —       |
| Black grouper         | +++ | +++     | Pompano                | ++  | +       | Crab                 | —   | —       |
| Black sea bass        | ++  | +++     | Red grouper            | +++ | +++     | Scallop              | —   | —       |
| Camouflage grouper    | ++  | ++      | Redmouth grouper       | ++  | +++     | Shrimp               | —   | —       |
| Channel catfish       | +++ | +++     | Red snapper            | ++  | +++     | Chicken breast       | —   | —       |
| Cobia                 | ++  | ++      | Idaho rainbow trout    | —   | —       | Chicken thigh        | —   | —       |
| Cod                   | —   | —       | Scamp                  | ++  | +++     | Turkey breast        | —   | —       |
| Caribbean red snapper | ++  | +++     | Striped bass           | +++ | +++     | Turkey thigh         | —   | —       |
| Cubera snapper        | ++  | +++     | Southern flounder      | ++  | ++      | Beef                 | —   | —       |
| Coral trout           | ++  | ++      | Squaretail grouper     | ++  | +++     | Deer                 | —   | —       |
| Dusky tail grouper    | ++  | ++      | Sheephead              | ++  | +++     | Elk                  | —   | —       |
| Farm salmon           | —   | —       | Sunfish                | +++ | +       | Frog legs            | —   | +       |
| Gag grouper           | +++ | +++     | Vermilion snapper      | ++  | +++     | Horse                | —   | —       |
| Gray snapper          | +++ | +++     | Swordfish              | —   | —       | Lamb                 | —   | —       |
| Hybrid catfish        | +++ | +++     | Tra                    | +++ | +++     | Pork                 | —   | —       |
| Haddock               | —   | —       | Trout cod              | ++  | +++     | Rabbit               | —   | +/-     |
| Hog snapper           | +   | +       | Tomato hind            | +   | ++      | Rat thigh            | —   | +++     |
| Lane snapper          | ++  | +++     | Tilapia                | +++ | +++     | Bovine serum albumin | —   | ?       |
| Mahi-mahi             | ++  | +       | Wahoo                  | +   | +/-     | Egg albumin          | —   | ?       |
| Mullet                | +++ | +++     | Whiting                | —   | —       | Nonfat dry milk      | —   | ?       |
| Mangrove snapper      | +++ | +++     | Wavy-lined grouper     | ++  | ++      | Soy concentrate      | —   | ?       |
| Ocean perch           | +++ | +++     | Wild salmon            | —   | —       |                      |     |         |

<sup>a</sup> Absorbance readings at 410 nm. —, <0.15; +/-, 0.15–0.199; +, 0.2–0.499; ++, 0.5–0.999; +++, >1; ?, not tested.

It was unclear why both 3E1 and PARV-19 did not react with these extracts in this study. It is possible that minor differences in the structure of parvalbumin among various fish species can affect the binding characteristics of the anti-parvalbumin antibody. However, an analysis of the variations in the concentration of parvalbumin among different

fish species, as well as an investigation of parvalbumin’s structure, is beyond the scope of the present study.

Heat-stable protein profile in cooked fish extracts.

SDS-PAGE was performed to obtain the thermal-stable protein banding pattern of cooked fish extracts. The representative SDS-PAGE protein profiles of 20 fish species are shown in Figure 1. Two major groups of proteins (appearing as 36- and 10- to 13-kDa bands on the gel) remained soluble after heat treatment of the fish samples. The 36-kDa protein presented in all the extracts of cooked fish species tested, whereas the 10- to 13-kDa band appeared in the majority of the fish species tested, but was missing in 3 species assayed on the gel—namely pollock, yellowfin tuna, and farm-raised salmon—indicating the lack of a detectable quantity of the 10- to 13-kDa protein in these 3 fish species. This result agrees with that of the indirect ELISA, which found a negative reaction with anti-parvalbumin PARV-19 in these species. This provides further evidence that the 10- to 13-kDa proteins are parvalbumins. Either one or two distinct bands of this group of 10- to 13-kDa proteins appeared on the gel for most of the species was observed. The presence of more than one band in some fish species suggests the existence of different isoforms of this protein. Van Do et al. (41) reported that more than one parvalbumin isotype are generally present in the muscle tissue of fish. Some fish species have been shown to display two to five isoforms of parvalbumin (41). The differential expression of this protein is most probably related to the

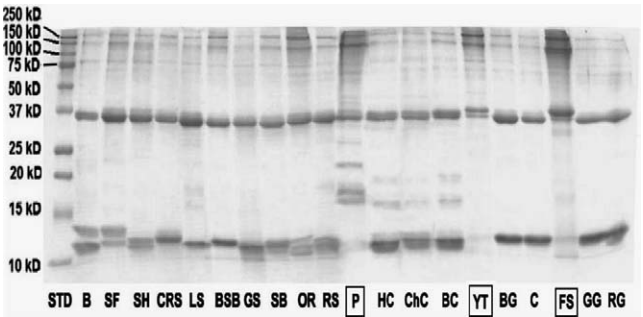


FIGURE 1. SDS-PAGE profiles of 20 cooked fish species. There are two groups of major protein bands (36, and 10 to 13 kDa) shown on the gel. The 36-kDa protein appears in all extracts of cooked fish species tested, whereas the 10- to 13-kDa band (parvalbumin) is missing in three species: pollock, yellowfin tuna, and farm-raised salmon. B, basa; SF, southern flounder; SH, sheephead; CRS, Caribbean red snapper; LS, lane snapper, BSB, black sea bass; GS, gray snapper; SB, striped bass; OR, orange roughy; RS, red snapper; P, pollock; HC, hybrid catfish; ChC, channel catfish; BC, blue catfish; YT, yellowfin tuna; BG, black grouper; C, cobia; FS, farm salmon; GG, gag grouper; RG, red grouper; □, very low amount or no parvalbumin.

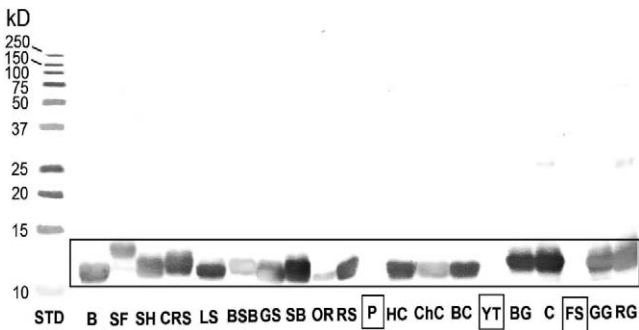


FIGURE 2. Antigenic protein banding patterns in cooked fish extracts via Western blot analysis, using MABs 3E1. This MAb reacts with the 10- to 13-kDa protein in all fish species except these three species: pollock, yellowfin tuna, and farm-raised salmon. B, basa; SF, southern flounder; SH, sheephead; CRS, Caribbean red snapper; LS, lane snapper; BSB, black sea bass; GS, gray snapper; SB, striped bass; OR, orange roughy; RS, red snapper; P, pollock; HC, hybrid catfish; ChC, channel catfish; BC, blue catfish; YT, yellowfin tuna; BG, black grouper; C, cobia; FS, farm salmon; GG, gag grouper; RG, red grouper; □, very low amount or no parvalbumin.

physiological requirements of the developmental stages of the growing fish (18). Considering the quantity present in fish muscle, the low molecular weight, the thermal stability, the existence of different isoforms, and the immunoreactivity with the anti-parvalbumin antibody of the 10- to 13-kDa protein, it is reasonable to assume that this group of proteins shown in the gel is fish parvalbumin.

The 36-kDa protein that appeared in all the fish species tested corresponds to the molecular weight of fish tropomyosin. Because tropomyosin has also been reported as a heat-stable protein (29), it is reasonable to assume this 36-kDa protein to be tropomyosin. Tropomyosin, one of the striated muscle regulatory proteins, forms a family of highly conserved actin-binding proteins (6). Fish tropomyosin has been reported in several studies, in which the existence of multiple isoforms of this protein was also observed (14, 15). Tropomyosin has been identified as the major allergen in many shellfish, especially crustaceans and mollusks, but to date there have been no reports that fish tropomyosin could cause allergic reactions (24, 26). Because MAb 3E1 does not recognize this 36-kDa protein, the further characterization of this protein is not relevant to this study.

**Antigenic protein for MAB 3E1.** After the SDS-PAGE analysis, protein bands were further characterized by Western blot utilizing MAb 3E1 in order to reveal the specific antigenic protein reacting with the antibody. In this experiment, MAb 3E1 was tested against cooked extracts of the same 20 fish species (Fig. 2). All protein bands in the molecular-weight range of 10 to 13 kDa were recognized by MAb 3E1. However, no bands in the range of 10 to 13 kDa appeared in pollock, yellowfin tuna, and farm-raised salmon. The immunoreactivity of MAb 3E1 against these 20 fish species—as determined by both indirect ELISA and Western blot—agreed closely with each other. These results provide further evidence that MAb 3E1 binds fish parvalbumin.

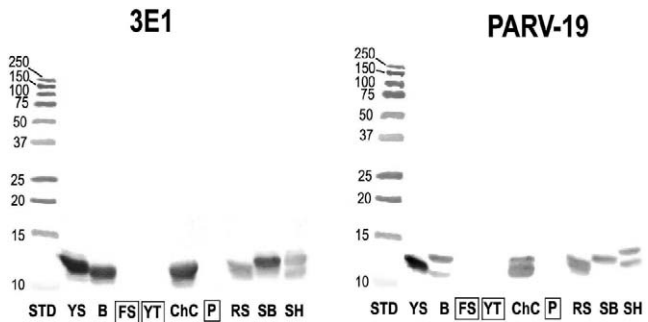


FIGURE 3. Comparison of the antigenic protein banding patterns in cooked fish extracts by using MABs 3E1 and PARV-19 in Western blot analysis. YS, yellowtail snapper; B, basa; FS, farm salmon; YT, yellowfin tuna; ChC, channel catfish; P, pollock; RS, red snapper; SB, striped bass; SH, sheephead; □, very low amount or no parvalbumin.

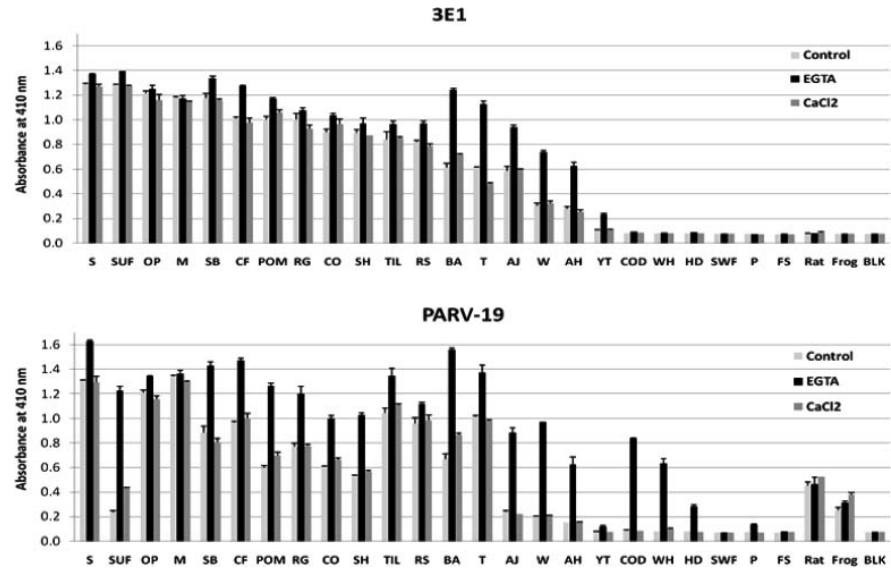
To confirm the above observation that the antigen recognized by MAb 3E1 is fish parvalbumin, a side-by-side comparison of the antigenic proteins profile recognized by MAb 3E1 and MAb PARV-19 in nine randomly selected cooked fish extracts was also made (Fig. 3). Both MABs tested in this experiment had almost identical reaction patterns with all the fish samples tested. The molecular weight of parvalbumin recognized by PARV-19 matched the size of the 10- to 13-kDa protein bound by MAb 3E1. The results from the Western blot, together with the results from SDS-PAGE and indirect ELISA, demonstrate clearly that the protein recognized by MAb 3E1 is indeed fish parvalbumin.

**Ca<sup>2+</sup>-sensitive epitopes.** The anti-parvalbumin MAB PARV-19 has been reported to bind parvalbumin in a Ca<sup>2+</sup>-dependent manner (17). To investigate whether the binding of the antigen with MAB 3E1 is also Ca<sup>2+</sup> dependent, indirect ELISA was performed with both MABs, against cooked fish and meat extracts that had been diluted in coating buffer (the control), or coating buffer containing either the Ca<sup>2+</sup>-chelating reagent EGTA (to remove Ca<sup>2+</sup>) or CaCl<sub>2</sub> (to add Ca<sup>2+</sup>). The indirect ELISA results for cooked extracts from rat, frog, and 24 representative fish species in the presence and absence of Ca<sup>2+</sup> by using MABs 3E1 and PARV-19 are shown in Figure 4.

Comparing the readings for the control samples, there were no observable changes in the immunoreactivity of both antibodies against all the samples tested with added CaCl<sub>2</sub>. However, an increase was observed in the immunoreactivity of all the samples reacted with MAB 3E1 after removing Ca<sup>2+</sup> by adding EGTA. Similar results, but with slightly enhanced reaction signals, were observed in samples reacted with the MAB PARV-19. Three fish species (cod, whiting, and haddock), none of which reacted with PARV-19 in the control samples, showed a dramatic increase in ELISA absorbance under Ca<sup>2+</sup>-depleted conditions; removal of Ca<sup>2+</sup> from these three fish extracts, however, had no effect on their immunoreactivity when the samples were reacted with MAB 3E1. In general, MAB 3E1 appeared to be less Ca<sup>2+</sup> sensitive than was PARV-19.

Parvalbumins belong to a family of Ca<sup>2+</sup>-binding pro-

FIGURE 4. Effect of presence and absence of  $\text{Ca}^{2+}$  on the immunoreactivity of anti-parvalbumin-specific MAb 3E1 and PARV-19 with fish and meat extracts determined by indirect ELISA. Absorbance readings at 410 nm. S, scamp; SUF, sunfish; OP, ocean perch; M, mullet; SB, striped bass; CF, catfish; POM, pompano; RG, red grouper; CO, cobia; SH, sheephead; TIL, tilapia; RS, red snapper; BA, basa; T, tra; AJ, amberjack; W, wahoo; AH, Alaskan halibut; YT, yellowfin tuna; COD, codfish; WH, whiting; HD, haddock; SWF, swordfish; P, pollock; FS, farm salmon; Rat, rat thigh; Frog, frog leg; BLK, blank.



teins and are characterized by the presence of three typical helix-loop-helix  $\text{Ca}^{2+}$ -binding domains, termed “EF hands” (3, 19). Seiberler and others (34) reported that the IgE recognition of EF-hand pollen allergens could be modulated by the presence or absence of protein-bound  $\text{Ca}^{2+}$  (34). A number of studies have been conducted to investigate the effect of  $\text{Ca}^{2+}$  on the immunoreactivity of parvalbumins. The IgE binding to carp parvalbumin was greatly reduced after the removal of  $\text{Ca}^{2+}$  in a Western blot experiment (4, 37). MAb PARV-19 was also reported to bind parvalbumin in a  $\text{Ca}^{2+}$ -dependent manner. The recognition of frog parvalbumin by mouse monoclonal anti-frog parvalbumin antibody PARV-19 has been reported to decrease for samples treated with EGTA (17).

Our ELISA result showed an increase in the immunoreactivity of MAb PARV-19 with fish extracts after the removal of  $\text{Ca}^{2+}$ , which disagrees with previously reported studies investigating the  $\text{Ca}^{2+}$  dependence of parvalbumin with IgE. Several factors should be considered when comparing these seemingly contradictory results, however. First, the antibody-binding characteristics are determined by the location and shape (linear or conformational) of the epitope, and the presence or absence of  $\text{Ca}^{2+}$  may or may not affect the epitope binding of the antigen. Secondly, the effect of  $\text{Ca}^{2+}$  depletion has predominately been studied for IgE antibodies. IgE and IgG are two different classes of antibodies. IgE binding deals with allergenicity, whereas IgG binding deals with antigenicity. The allergen-binding sites recognized by the IgE antibodies naturally found in fish-sensitive patients’ serum are unlikely to be the same as the IgG antibodies that are produced by immunizing animals with an antigen. The form of antigen preparation (native or heat-treated), as well as the methods of hybridoma selection in the case of MAb development, also greatly affects the conformational response of the antigen as well as the binding characteristics of the antibodies. Moreover, all previous studies employed Western blot analysis to study the  $\text{Ca}^{2+}$  dependency of the antibody, while in the current study we have tested the effect of  $\text{Ca}^{2+}$  by using ELISA, in which the antigen is not denatured by the SDS used in

the Western blot. Swoboda et al. (37) suggested that removal of free  $\text{Ca}^{2+}$  ions can cause changes in the conformation of IgE epitopes and/or unfolding of the antigenic protein, both of which would result in a reduction of IgE-binding in the sera of fish allergic patients. The same conformational changes may also take place after removal of  $\text{Ca}^{2+}$  in our experiment, but in contrast to the previously reported results for IgE-epitopes, removal of  $\text{Ca}^{2+}$  may expose more hidden IgG epitopes and lead to an increase in the epitopes of MAb 3E1 and PARV-19 for fish parvalbumin.

**Epitope comparison.** If MAb 3E1 and PARV-19 both bind to parvalbumin, then binding sites may or may not be overlapping. The epitope comparison was therefore performed to determine whether the MAb 3E1 and PARV-19 bind to the same epitope on the antigenic protein parvalbumin. The additivity index (AI) was calculated to be 36.27%, based on the equation by Friguet et al. (10):

$$\text{AI} = \frac{A_{1+2} - \left( \frac{A_1 + A_2}{2} \right)}{A_1 + A_2 - \left( \frac{A_1 + A_2}{2} \right)} \times 100$$
$$= \left[ \left( \frac{2A_{1+2}}{A_1 + A_2} \right) - 1 \right] \times 100$$

where  $A_1$ ,  $A_2$ , and  $A_{1+2}$  are the absorbance readings reached, in the additivity test, with the first antibody alone, the second antibody alone, and the two antibodies together, respectively. The absorbance readings for MAb 3E1 alone ( $A_1$ ), the MAb PARV-19 alone ( $A_2$ ), and the two antibodies together ( $A_{1+2}$ ) are shown in Figure 5.

If the antibodies bind to the same epitope of the common antigenic protein,  $A_{1+2}$  should be equal to the mean value of  $A_1$  and  $A_2$ , and AI will be equal to zero. If, on the contrary the two antibodies bind to different, non-overlapping epitopes on the antigenic protein,  $A_{1+2}$  should be the sum of  $A_1$  and  $A_2$ , and AI will be equal to 100%. Generally, it is considered that the antibodies share the same binding

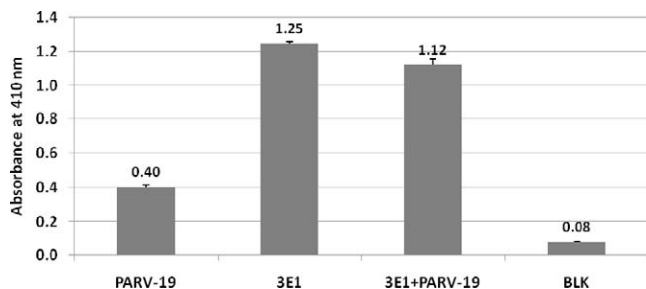


FIGURE 5. Epitope comparison of MAb 3E1 and PARV-19. Absorbance readings at 410 nm of immunoreactivity of cooked tra extract containing 0.5  $\mu$ l of soluble proteins, against MAb PARV-19, MAb 3E1, and the two antibodies together (3E1 plus PARV-19) in the ratio 1:1. BLK, blank.

side or their binding sides overlap to some degree if AI is below 50%.

For the tested antibodies, it appeared that the binding of the first MAb inhibited the binding of the second MAb. The AI value obtained in this experiment was below 50%; thus, we can conclude that both antibodies, MAb 3E1 and MAb PARV-19, recognize exactly the same protein (parvalbumin), but their epitopes (binding sites), although different, overlap to an extent, causing inhibitive binding to each other on the parvalbumin. These two different but partially overlapped epitopes may explain the similar immunoreactivity patterns of these two antibodies with most of the fish species but with a slight difference in their species specificity. Apparently, the epitope with MAb 3E1 is monospecific to fish, whereas the epitope with MAb PARV-19 covers the conserved region of amino acid sequence on the parvalbumin not only from fish, but also from frog and rat. Although the nonoverlapping region of these two epitopes causes the difference in the immunoreactivity of these two MAbs, the overlapping region of their epitopes has excluded them to be used as a pair of capture and detection antibodies in a sandwich type of immunoassay. Knowing that the antigenic protein for MAb PARV-19 is parvalbumin, the result of epitope comparison further supports the hypothesis that MAb 3E1 is parvalbumin specific.

In conclusion, MAb 3E1 recognizes a group of heat-stable proteins with a molecular weight between 10 and 13 kDa in fish samples, with each antigenic protein corresponding to the molecular weight of each parvalbumin recognized by the anti-frog parvalbumin MAb PARV-19. MAb 3E1 and MAb PARV-19 have almost identical positive and negative immunoreaction patterns to all the finfish species tested, using both indirect ELISA and Western blot. The antibody binding of the antigen was  $\text{Ca}^{2+}$  dependent for both MAbs, and the ELISA response was enhanced with the removal of  $\text{Ca}^{2+}$  in the reaction system. Unlike MAb PARV-19, which reacts with frog, rat, and rabbit, MAb 3E1 only reacts only with finfish. Based on the heat stability, molecular weight of the antigen,  $\text{Ca}^{2+}$ -dependent binding characteristics, competitive binding sites with anti-parvalbumin antibody, and immunoreactivity with various fish species, it is clear that MAb 3E1 is specific to fish parvalbumin. This antibody would be a useful tool in the detection and investigation of the major fish allergen, parval-

bumin, in majority of fish species. Further investigation of the content and molecular isoforms of parvalbumin in fish species that showed negative results in this study with both PARV-19 and MAb 3E1 is undergoing in our laboratory.

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