Development of Monoclonal Antibody for Rock Shrimp Identification Using Enzyme-Linked Immunosorbent Assay

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Rock shrimp specific protein M, determined from a comparison of the SDS–PAGE protein banding pattern of rock shrimp with those of 23 other seafood and meat samples, was isolated from 12% polyacrylamide gels and used to develop monoclonal antibodies (McAb). The highly specific McAb (4H2-10D3) recognized the antigenic determinant(s) present on the 17.7-kDa component of protein M in both the native and heat-denatured rock shrimp extract. Specificity of the McAb was confirmed by testing against pink, white, and rock shrimp protein extracts using Western blotting technique and also against 23 commonly consumed seafood and meat samples by immunodot blotting and ELISA. The McAb was also applied in ELISA under optimized test conditions to detect the presence of rock shrimp in 26 various seafood and meat mixture samples. The McAb correctly detected rock shrimp in all these samples. Heat treatment of rock shrimp extract enhanced the reactivity with this McAb. Furthermore, the presence of rock shrimp as low as 4.3 ng on the average in sample mixtures containing various seafood or meat samples could be detected.

INTRODUCTION

There has been a tremendous growth in seafood consumption in this country due to changes in consumer attitudes toward health and nutrition. With the increased demands for higher quality seafood products, consumers may have already encountered the willful or unintentional adulteration by dealers who substitute higher quality and priced seafood with lower quality and less expensive products.

Identification of seafood species can be difficult. The use of morphological characteristics for identification requires a great deal of experience; unintentional fraud can occur when identification is done by untrained personnel, and the resulting products may be declared misbranded and/or adulterated by regulatory agencies (Vondruska et al., 1988). Several electrophoretic methods are used officially to differentiate the species of seafood or seafood products (AOAC, 1984). However, these methods are laborious and time-consuming and require substantial equipment. A large data bank of various seafood species is also needed for effective protein profile comparison. These methods are therefore limited for field test applications. In addition, morphological characteristics can be lost and protein denaturation will result during processing. Therefore, morphological and electrophoretic methods may be less effective for seafood identification. There has been a great need to develop a fast and reliable method to identify seafood species and to quantify their levels in seafood products to assure product quality and thus to protect consumers' value.

Immunological assays, as an alternative, can be used to reduce the test time and cost, as well as to increase the sensitivity in detecting food components (Hayden, 1977, 1978, 1981; Hitchcock et al., 1981; Karpas et al., 1970; Skerritt, 1985). Lundstrom (1984, 1985), using hybridoma technology, developed the enzyme-linked immunosorbent assay (ELISA) to identify fish species and to differentiate fish stock. Crude protein extracts from heat-denatured fish muscle were used to immunize mice for the preparation of monoclonal antibodies (McAbs). However, high cross-reactivity of the antibodies was noted between unrelated fish species, and species specificity was not found when McAbs were tested against numerous fish species. This might have been due to the fact that most of the antibodies produced against the crude extracts were specific for most of the prevalent proteins such as glyceraldehyde phosphate dehydrogenase (Scopes, 1970), rather than the less abundant species-specific proteins in the extracts. Similar results were also reported by Tracy et al. (1983), who tried to produce McAbs using a human urinary protein extract. Most of the positive hybridomas produced antibodies reactive to albumin, an abundant urinary protein in renal disease, rather than the protein of interest, PC-30. However, after the separated protein was used as antigen following two-dimensional gel electrophoresis, PC-30-specific McAb was developed as confirmed by autoradiography.

ELISA has been widely used to differentiate fish species and unprocessed beef, pig, horse, sheep, kangaroo, camel, goat, and buffalo meat (Kang'ethe et al., 1982; Lundstrom, 1984, 1985; Patterson et al., 1984; Skerritt, 1985; Whittaker et al., 1983). ELISA offers great advantages in that (1) the test can be performed in a short period of time, (2) a small volume of species-specific antisera is required, (3) the antisera can be mixed for multispecificity in a screening procedure, (4) equipment is available to semiautomate the assay, and (5) the increased sensitivity of the assay allows the use of simple sampling techniques (Whittaker et al., 1983).

The present study was carried out to develop rock shrimp (*Sicyonia brevirostris*) specific McAb by using isolated rock shrimp proteins as antigen and to use this specific McAb as a model to increase the specificity of the ELISA test for species identification. The applicability of the ELISA test to identify rock shrimp among seafood and meat samples and quantify rock shrimp content in various protein mixtures was also investigated.

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MATERIALS AND METHODS

Preparation of Water-Soluble Proteins. Water-soluble proteins were extracted from 23 different seafood and meat samples, including rock shrimp, as previously reported (An et al., 1988). Samples were chopped and mixed at a ratio of 1:3 (w/v) with an aqueous solution containing 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 10 mM EDTA, and 0.01% (w/v) sodium azide. The samples were homogenized by using a Polytron instrument (setting 5.5, Brinkmann Instrument Co., Westbury, NY) at room temperature for 1 min and then centrifuged at 48000*g* for 20 min at 20 °C. The supernatants were collected, and the protein contents were determined (Lowry et al., 1951).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). SDS–PAGE was performed according to the method of An et al. (1988) using a Protean II (vertical slab) unit (Bio-Rad, Richmond, CA) with slab gels consisting of a running gel (12%) and a stacking gel (3%). Protein samples (35 μg/20 μL) were run initially at a constant current of 15 mA/slab and then increased to 30 mA/slab when the marker front reached the running gel. Following electrophoresis, the proteins were stained with 0.125% (w/v) Coomassie Brilliant Blue R-250 (Bio-Rad) in 40% ethanol and 7% acetic acid and then destained in 7% acetic acid. Electrophoretic patterns were recorded by developing the positive image using Kodak electrophoresis duplicating paper (Eastman Kodak, Rochester, NY). A low molecular weight protein kit (Pharmacia, Piscataway, NJ) was used as protein standards, which contained phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (21 100), and α-lactalbumin (14 400).

Purification of Protein M. Rock shrimp SDS–PAGE protein bands with molecular weights of 17 700 and 18 500, referred to as protein M, were shown to be specific for rock shrimp when compared with pink or white shrimp protein profiles (An et al., 1988). After electrophoresis, protein M was eluted from the 12% SDS–PAGE slab gels of the water extract of rock shrimp proteins. The gels were homogenized in water for 1 min by using a Polytron instrument, and the mixtures were centrifuged at 2000*g* for 20 min at room temperature. The eluted proteins in the supernatant were pooled and dialyzed overnight at 4 °C in a membrane tubing (molecular weight cutoff 6000–8000, Spectrum Medical Industries, Inc., Los Angeles, CA) against an excess amount (about 100× in volume) of phosphate-buffered saline (PBS, pH 7.4) containing 1.4 M NaCl, 0.1 M Na_2HPO_4, and 0.3 M KH_2PO_4, with constant stirring to remove residual SDS. The dialyzed protein M was analyzed for protein content and purity by using SDS–PAGE following the previously described procedures (An et al., 1988).

Two-Dimensional Gel Electrophoresis of Rock Shrimp Water Extract. Two-dimensional electrophoresis using urea IEF in the first dimension and SDS–PAGE in the second dimension was performed as previously described (An et al., 1988, 1989). The first dimension using rock shrimp protein extract at 150 μg was done with a tube gel (11.5 cm × 1.7 mm) and a Pharmacia electrophoresis apparatus GE-4II unit. After the focused gel was removed and equilibrated in 0.125 M Tris-HCl buffer (pH 6.8) for 1 h, the gel was placed on the top of the SDA–PAGE slab gel consisting of the stacking gel (3% acrylamide, w/v) and the separating gel (12% acrylamide, w/v) and maintained in position with 1% agarose. The slab gel was run until bromophenol blue reached the bottom of the gel (An et al., 1988, 1989). The proteins were stained with Coomassie Blue R-250 and destained.

Immunization Procedures. Four 6-week-old Balb/cBYJ female mice (Jackson Laboratories, Bar Harbor, ME) were each immunized with 100 μg of protein M freshly emulsified in RIBI adjuvant (monophosphoryl lipid A–trehalose dimycolate, RIBI Immunochem Research, Inc., Hamilton, MT). 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, in 150-μL volume into the intraperitoneal cavity, and in 150-μL volume into one anterior dorsal subcutaneous site. The injection process was repeated three times, each at 2-week intervals. The titer of the serum against protein M was determined by ELISA, as described below, 1 week after the second and third booster injections. A final boost was given intraperitoneally 26 days after the third booster and 4 days prior to the fusion.

Development of Monoclonal Anti-Protein M Antibodies. Monoclonal antibodies against protein M were produced according to previously established protocols of Kao and Klein (1986). Spleen cells harvested from immunized mice were fused with SP2/0 myeloma cells at a ratio of 7.5:1 (spleen cells/myeloma cells) by using 50% polyethylene glycol 1540. The fused cells were suspended in hypoxanthine–aminopterin–thymidine (HAT) selective medium and seeded into 96-well flat-bottom culture plates. Ten to 14 days later, the supernatants of growth-positive wells were screened for production of anti-protein M antibodies by testing with protein M and rock shrimp protein extracts by ELISA and immunodot blot. Hybridomas initially producing anti-protein M were expanded and rescreened. Hybridomas that showed continued production of anti-protein M antibodies were cloned by the limiting dilution method (Zola and Brooks, 1982). McAb isotypes were determined with ScreenType (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Three cloned hybridomas (designated as 2E8-2B10, 4H1-8F11, and 4H2-10D3) that produced anti-protein M McAb with high reactivity for rock shrimp extract only were propagated intraperitoneally in three or four male Balb/cBYJ mice to produce McAb-containing ascites. Anti-protein M McAbs from these ascites were purified by using the protein A–Sepharose 4B (Sigma Chemical Co., St. Louis, MO) column chromatography as described by Ey et al. (1978).

Enzyme-Linked Immunosorbent Assay for Protein M. Each well of a 96-well Nunc immunoplate was coated overnight at 4 °C with 100 μL (5 μg/mL) of protein M or water protein extracts in PBS containing 0.02% azide, pH 7.4 (PBS-az). Each well was then washed three times with PBS-az containing 0.5% Tween 20 (PBS-T) and incubated with 1% bovine serum albumin (BSA) in PBS for 1 h at 4 °C. The wells were washed three times again with PBS-T and incubated at room temperature for 1 h with supernatants of the hybridoma cultures or purified McAb (4H2-10D3) in 100 μL/well. After three washes with PBS-T, the wells were added with the 1:1000 diluted rabbit anti-mouse IgG conjugated with alkaline phosphatase (Sigma) with PBS containing 1% BSA and incubated for 1 h. After three additional washes with PBS-T, *p*-nitrophenyl phosphate (1 mg/mL, Sigma) in 0.1 M carbonate buffer (pH 9.6) was added to each well, and the plate was incubated for 45 min in the dark. Absorbance was read at 405 nm by using an ELISA plate reader (Molecular Devices, Palo Alto, CA).

Immunodot Blotting. Aqueous shrimp protein extracts (20 μg/20 μL) were dotted onto nitrocellulose membrane paper (BA85, Schleicher and Schuell, Keene, NH) assembled in a Minifold I dot blot apparatus with an incubation plate (Schleicher and Schuell) and incubated for 1 h. The nitrocellulose membrane was then blocked by incubating with PBS-az containing 5% nonfat milk for 1 h at room temperature (Johnson et al., 1984). Following washing with PBS-T for 5 min, the membrane was incubated in the purified McAb (100 μg/mL) in PBS-az containing 1% BSA for 30 min. After washing for 5 min with PBS-T, the membrane was incubated with rabbit anti-mouse IgG (1 μg/mL) conjugated with alkaline phosphatase for 30 min. Following additional washing with PBS-T, the membrane was incubated in nitroblue tetrazolium (0.1 mg/mL) and 5-bromo-4-chloro-3-indolyl phosphate (0.05 mg/mL) solution in 0.1 M Tris buffer containing 1 mM $MgCl_2$ (pH 8.8) until color development. Positive samples containing protein M or rock shrimp were identified by the development of a blue color at the site of protein immobilization.

Western Blotting. Crude protein extracts of pink, white, and rock shrimp after electrophoresis on 12% SDS–PAGE gels (An et al., 1988), as well as the two-dimensional gel profiles of rock shrimp protein extract, were electrophoretically blotted onto Immobilon membrane paper (Millipore Corp., Bedford, MA) at 4 °C using a Trans-Blot device (Bio-Rad) at 50 mA for 2 h. After proteins were transferred onto the membrane, they were processed by the same techniques used for immunodot blotting with only slight modifications. The membrane paper

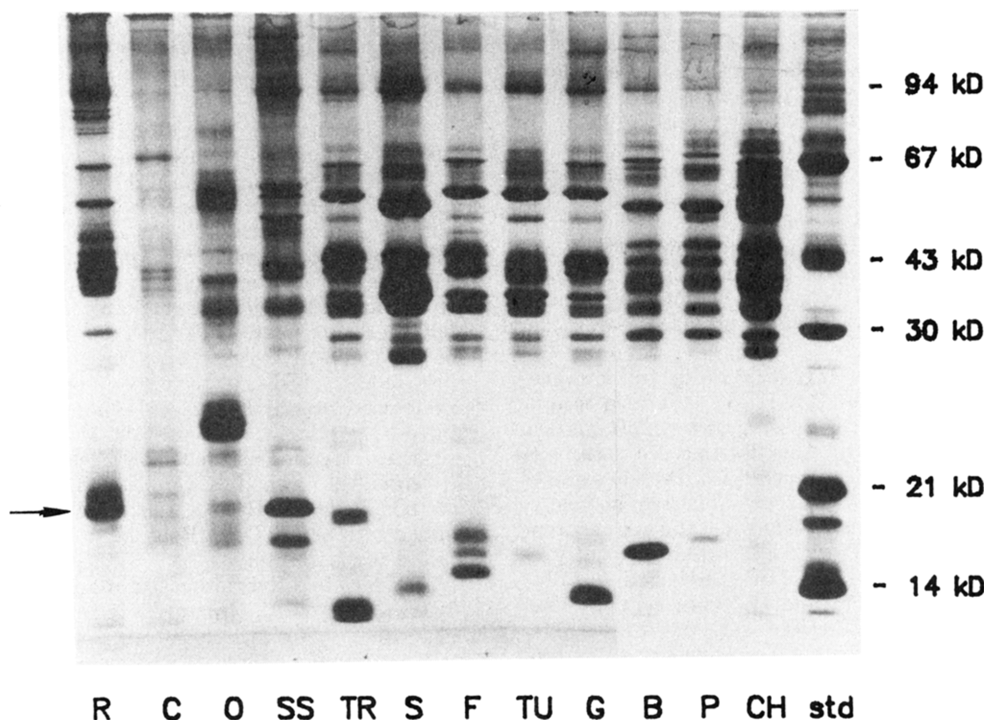

Figure 1. SDS–PAGE profiles of water-soluble proteins from various seafood and meat (cathode on top). The arrow designates protein M. R, rock shrimp from Cedar Key, FL; C, clam; O, oyster; SS, sea scallop; TR, trout; S, salmon; F, flounder; TU, tuna; G, grouper; B, beef; P, pork; CH, chicken; std, low molecular weight protein standards with molecular weight marked on the side.

was blocked with 5% nonfat milk in PBS-az for 2 h, followed by 2- and 1-h incubations in the respective solution containing the purified McAb and the alkaline phosphatase conjugate.

Pretreatment of Antigens. The water extracts of rock, pink, and white shrimp were either heat-treated or maintained as the native state. Heat-treated samples were prepared by boiling the extracts in water for 5 min followed by centrifugation at 2000*g* for 5 min to remove protein aggregates. Both the native and heat-treated samples were analyzed for protein concentration by using the Lowry method, diluted to 5 μg/mL, and then added with or without SDS [final concentration of SDS, 0.1% (w/v)]. The samples were each reacted with the 4H2-10D3 McAb at 100 μg of protein/mL on immunodot blot or by ELISA to determine if heat treatment or the addition of SDS would increase the reactivity of rock shrimp proteins with this McAb.

Optimization of McAb and Antigen Concentrations for ELISA. *Antibody Concentration.* McAb at 0.5, 1, 5, 10, 50, 100, or 200 μg of protein/mL was tested by ELISA to determine the optimal concentration needed to achieve highly specific reactivity for rock shrimp proteins. Only the heat-treated water extracts of rock, pink, or white shrimp at 5 μg of protein/mL were used to coat the plate wells.

Antigen Concentration. Heat-treated water extracts of pink, white, and rock shrimp at 0.5, 1, 5, 10, 50, 100, or 500 μg of protein/mL were used to coat the plate wells (0.1 mL/well). 4H2-10D3 McAb at 50 or 100 μg of protein/mL was tested on these plates to determine the optimal concentration of antigen needed for maximum reactivity in the ELISA test.

Blind Study To Detect and Quantitate Rock Shrimp. A single blind test using ELISA was employed to determine the specificity of the 4H2-10D3 McAb for rock shrimp protein. The reactivity of rock shrimp was tested along with 23 other species of seafoods including oyster, scallop, lobster, clam, various fish, and shrimp obtained from various locations and meat samples including chicken, pork, and beef. The water-soluble protein extracts of these diverse food samples were heat-treated for 5 min in boiling water followed by centrifugation at 2000*g* for 5 min. After protein content was determined, the protein concentration of each sample was adjusted to 10 μg/mL and a 100-μL aliquot was analyzed by ELISA. Experiments were performed in two replications.

The sensitivity of the 4H2-10D3 McAb in detecting rock shrimp in sample mixtures containing seafood and meat samples was conducted in a double-blind study using the ELISA test. The water extracts of the 26 seafood and meat samples (including 3 rock shrimp samples), after heat treatment and centrifugation, were each adjusted to a protein concentration of 500 μg/mL. They were combined randomly with each other or with PBS in various ratios unknown to the author to yield 24 sample mixtures. The sample mixtures were serially diluted with PBS (1:5 v/v) to 5^{-7} of the original protein content. Each of the serially diluted samples at 100 μL was then subjected to the above-mentioned ELISA test to determine the lowest amount of rock shrimp protein that reacted with the McAb. The most diluted samples which gave absorbance values greater than 0.2 were marked. The experiment was repeated once. The rock shrimp protein content in the marked samples was calculated by using the equation

$$\text{rock shrimp content (ng)} = (\text{protein content (ng)}) \times (\text{rock shrimp percentage}) \times (\text{dilution factor})$$

RESULTS AND DISCUSSION

SDS–PAGE Banding Patterns of Various Seafood and Meat Samples. Seafood species and meat samples each showed a distinct SDS–PAGE protein pattern useful for species identification (Figure 1). The protein profile of rock shrimp was clearly different from those of the other seafoods and meats including clam, oyster, sea scallop, fish, beef, pork, and chicken. The most significant profile variations occurred among samples in the lower one-third section of the gel (anodic region). Protein bands (molecular weight <25 000) in this section were unique for each sample in terms of band number and molecular weights. The two major bands unique for rock shrimp in this section were those with molecular weights of 17 700 and 18 500, and these were referred to as protein M.

Protein profiles from various shrimp samples and lobsters collected worldwide were also compared with those of rock shrimp (Figure 2). In general, profiles for pink, white, blue, and brown shrimp were relatively similar; the movements of the two major bands found in the lower one-third section of the gel were almost identical. When

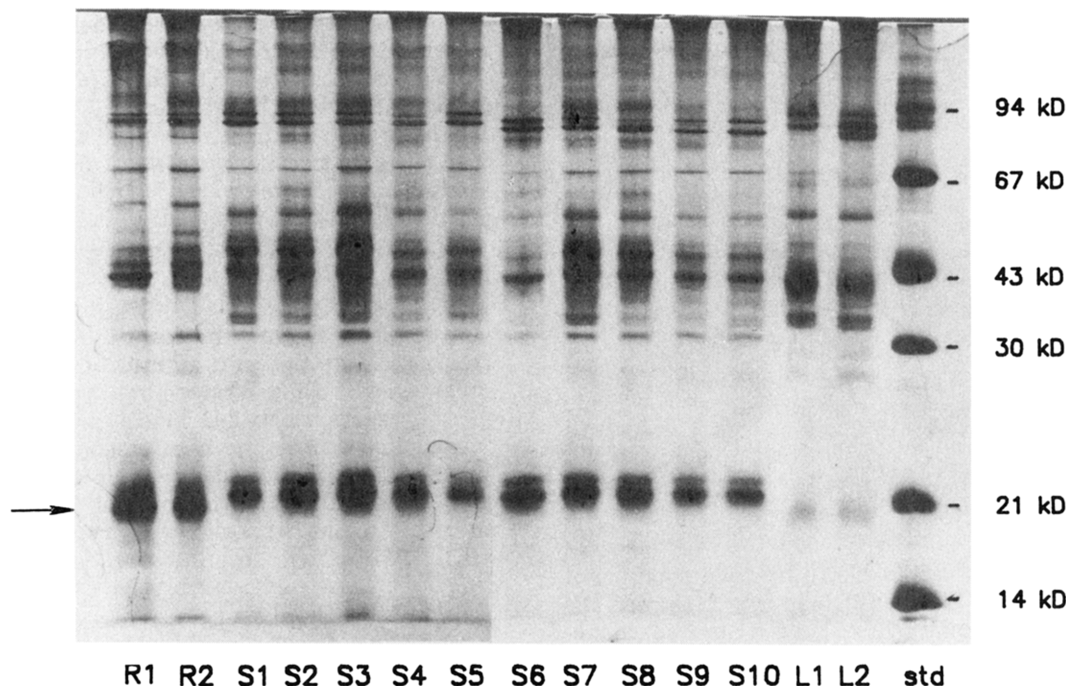

Figure 2. SDS-PAGE profiles of water-soluble proteins from various shrimp and lobsters (cathode on top). R1, rock shrimp from Port Canaveral, FL; R2, rock shrimp from Cedar Key, FL; S1, white shrimp from Ecuador; S2, Argentine-red shrimp; S3, blue shrimp from Ecuador; S4, pink shrimp from Tampa, FL; S5, white shrimp from Peru; S6, brown shrimp from North Carolina; S7, white shrimp from Colombia; S8, white shrimp from Georgia; S9, white shrimp from Honduras; S10, blue shrimp from Honduras; L1, lobster from Australia; L2, lobster from Florida; std, low molecular weight protein standards with molecular weight marked on the side. Rock shrimp protein M also marked with an arrow.

compared to rock shrimp protein M, these two bands had slightly lower R_f values and thus higher molecular weights. An et al. (1988) also reported that the SDS-PAGE protein profiles of the water extracts of pink and white shrimp were very similar. The protein pattern of lobster was different from those of shrimp samples.

Two-dimensional electrophoretic analysis showed that protein M was actually two proteins (data not shown). Protein M comprised 19.7% of the total water-soluble rock shrimp proteins as estimated from the intensity of the protein bands shown on the gel (data not shown).

Screening of Monoclonal Anti-Protein M Antibodies. During the screening process, eight of the original 400 seeded cultures were found to produce antibodies that showed 3–10 times higher reactivity for rock shrimp protein than for pink or white shrimp protein as determined by ELISA. The supernatants of these eight cultures also showed high reactivity for protein M; the absorbance readings were >0.4.

The results of the immunodot blot tests indicated that antibodies produced by these eight cultures failed to react with the water extracts of pink and white shrimp (data not shown); the antibodies only showed weak reaction with rock shrimp extract as indicated by the faint blue color developed. However, antibodies from the 2E8, 4H1, and 4H2 cultures showed positive reactions with rock shrimp extracts containing 0.1, 0.5, or 1.0% SDS. On the basis of color intensity, rock shrimp extract containing 0.1% SDS showed the highest reactivity, which was followed by 0.5 and 1.0% SDS (data not shown). Dimitriadis (1979) has previously demonstrated that SDS, at concentrations greater than 0.2%, inhibits antigen-antibody reactions to a significant degree in immunodiffusion and immunoprecipitation tests.

The binding of proteins to nitrocellulose membrane is affected by several factors, such as pH, molecular charge, and the presence of competing substances (Wallis et al., 1979). SDS at high concentration is believed to reduce the ionic interaction of proteins to the anionic nitrocellulose membrane by contributing negative charges to protein molecules. Thus, fewer protein molecules are bound to the membrane for reaction with antibody. SDS at a higher concentration also inhibits antigen-antibody reactions by causing denaturation of the protein molecules (Dimitriadis, 1979). The presence of only 0.1% SDS in rock shrimp extract, however, may cause slight changes in protein M configuration and enhance reactivity with the antibodies produced by the three cultures. On the basis of the ELISA and immunodot blot test results, these three cultures were subjected to cloning.

Again, the screening process was based on the reactivity of the supernatants with rock shrimp extract and protein M using ELISA and immunodot blot tests. The clones only showed high reactivity with rock shrimp extract containing 0.1% SDS by immunodot blot test. No reactivity occurred with pink or white shrimp extracts by both tests (data not shown).

Only one clone from each culture showing the highest reactivity for rock shrimp extract (2E8-2B10, 4H1-8F11, and 4H2-10D3) was chosen to propagate intraperitoneally in mice to produce McAb-containing ascites. All McAbs belonged to the IgG_1(k) isotype as determined by the isotyping kit (data not shown). In the presence of β-mercaptoethanol, the McAbs only showed two distinct bands (light and heavy chains) on the 12% polyacrylamide gels after SDS-PAGE. After purification of IgG molecules by affinity chromatography using a protein A column, the McAbs were assessed again for reactivity with rock, pink, and white shrimp extracts by immunodot blot; the 4H2-10D3 McAb showed the highest specific reactivity for rock shrimp extract (data not shown). This McAb was therefore used to develop immunochemical assays to detect rock shrimp.

This study illustrates the importance of using both protein M and rock shrimp extract for screening the desirable hybridoma clone. This is especially important

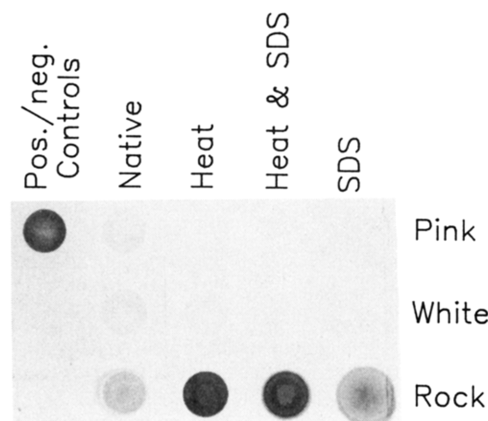

Figure 3. Effect of heat treatment, addition of SDS, or the combination of both on the reactivity of rock shrimp extract with 4H2-10D3 McAb as analyzed by immunodot blot. Mouse IgG and BSA were used as positive and negative controls.

for the development of the McAb for food component identification. The 4H1-9E12 clone, which showed the highest reactivity among all the clones for protein M (absorbance reading 2.057), showed only a weak reactivity for rock shrimp extract at 0.210. On the other hand, the 4H2-10D3 clone, which had an absorbance reading of only 0.706 for protein M, had an increased reading of 0.907 for rock shrimp protein extract. Clone 4H1-9E12 would have been selected if only the result with protein M was used. However, the 4H2-10D3 clone became the choice when the reactivity of this McAb with both protein M and rock shrimp extract was considered.

The use of different screening methods could affect the reactivity of McAbs with a specific antigen. The 4H2-12B12 McAb showed the highest reactivity for rock shrimp extract by ELISA (absorbance reading 0.965). However, the reactivity of this McAb with the same antigen on immunodot blot was not as strong as that of the 4H2-10D3 McAb as judged by the intensity of the developed color (data not shown). The different protein binding mechanism to immunosorbent materials, such as microtitration plates and nitrocellulose membranes, could contribute to this variation. Protein binding to nitrocellulose membranes can induce partial denaturation of the protein due to ionic interaction with the surface negative charge (Towbin and Gordon, 1984; Van Oss et al., 1987). Thus, McAbs specific for denatured proteins will be more reactive with the partially denatured proteins present on nitrocellulose membranes than with the same proteins in the native state bound on ELISA plates.

Pretreatment of Proteins for Reaction Using Immunodot Blot Test. 4H2-10D3 McAb was shown by immunodot blot test to react with rock shrimp extract in the native state. However, heat treatment of the extract in boiling water for 5 min greatly enhanced the reactivity with this McAb (Figure 3). Since the protein M used to immunize mice had been heat-denatured before the SDS–PAGE run, it is not surprising that 4H2-10D3 McAb is more reactive to heat-treated rock shrimp extract than to nonheated extract. Heat treatment of proteins may cause conformational changes to expose more buried epitopes for reaction with this McAb. The high specificity of this McAb for heat-denatured proteins enables its use to detect the presence of rock shrimp in processed seafood products.

The addition of SDS to heat-treated or nontreated rock shrimp extract also increased antigen reactivity with the 4H2-10D3 McAb on the immunodot blot, but to a lesser extent than heat treatment of the antigen alone (Figure 3). The increase in protein reactivity in the presence of low concentrations of SDS was believed to be related to protein conformational changes to reveal hidden epitopes as previously discussed. SDS might have an adverse effect on McAb reactivity by affecting the binding of the heat-denatured proteins to the nitrocellulose membrane. It has been reported that the presence of low concentrations of SDS did not affect antibody production or their specificity but decreased the nonspecific binding of antibody to unrelated antigen (Dimitriadis, 1979; Stumph et al., 1974; Tjian et al., 1974). Therefore, protein samples were heat-treated for immunodot blot tests in later studies.

Specificity Determination of 4H2-10D3 McAb by Western Blotting and Immunodot Blotting. The advantages of using Western blotting to determine the specific reaction between McAb and proteins are well documented (Burnette, 1981; Gershoni and Palade, 1983). McAb 4H2-10D3 showed only one positive reaction area that corresponded to protein M of rock shrimp on immunostained blot, while no reaction occurred for pink and white shrimp. On immunostained blot of the two-dimensional electrophoretic profile of rock shrimp extract, the 4H2-10D3 McAb was shown to react only with the 17.7-kDa protein of the protein M (data not shown). This result indicated that the 4H2-10D3 McAb reacted only with the 17.7-kDa component of rock shrimp protein M and not with proteins from pink or white shrimp or with the protein standards.

Cross-reaction of McAbs may occur with unrelated proteins sharing a structural similarity with the immunizing antigen (Goding, 1983). To determine if any cross-reactivity occurred, the 4H2-10D3 McAb was tested by immunodot blot against 23 commonly consumed unrelated species of seafood and meat (clam, oyster, sea scallops, chicken, port, beef, fishes, lobsters, 10 different shrimp from various regions) as well as the three rock shrimp harvested from the east and west coasts of Florida (Port Canaveral and Cedar Key) or at two different seasons (February and December). The McAb showed similar reactivity with rock shrimp samples harvested from different locations (dots A2 and A3 in Figure 4a and dots C1 and D1 in Figure 4b) and during different seasons (dots A3 and C7 in Figure 4a), indicating that this McAb was highly specific for rock shrimp regardless of the harvest location and season. No cross-reactivity was observed with the other shrimp species or lobsters. This rock shrimp specific McAb can thus be used for effective shrimp differentiation purposes.

The 4H2-10D3 McAb reacted slightly with the distant species including tuna, beef, pork, and chicken, as indicated by the light color developed. Protein M like molecules may thus be present in these distant species. However, the intense blue color developed for rock shrimp was easily differentiated from the less reactive species. The 4H2-10D3 McAb is thus highly specific for rock shrimp.

Optimization of ELISA Test. *Pretreatment of Antigens.* Heat treatment of rock shrimp extract in boiling water for 5 min enhanced the reactivity with the 4H2-10D3 McAb using ELISA test (Figure 5). However, the addition of SDS to heat-treated or nontreated rock shrimp extract decreased the antigenic reactivity with the McAb (Figure 5). Protein binding to plastic microtitration plates is achieved by adsorption through hydrophobic interactions between nonpolar protein substructures and the nonpolar plastic matrix (Clark and Engvall, 1980). The presence of interfering chemicals, such as SDS, can affect this weak binding (Clark and Engvall, 1980). Therefore, the adsorption of shrimp antigen onto ELISA plates may have been reduced and resulted in a reduced reactivity with the McAb.

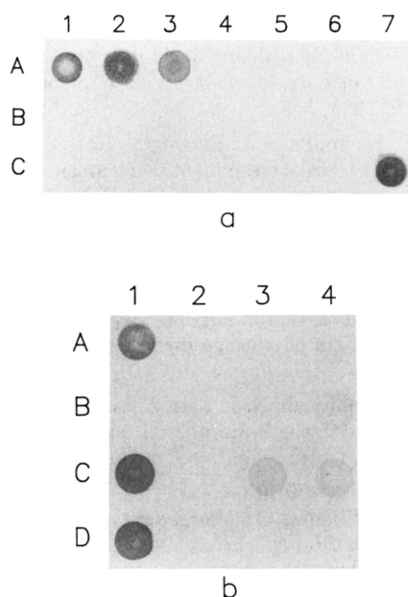

Figure 4. Determination of the specific reactivity of 4H2-10D3 McAb with rock shrimp as analyzed on immunodot blots with the water extracts in 20 μL containing 20 μg of proteins: (a) various shrimp and lobsters; (b) various seafood and meat. Samples used in test a include A1, mouse IgG; B1, 1% BSA; A2, rock shrimp from Port Canaveral, FL; A3, rock shrimp obtained in February from Cedar Key, FL; A4, white shrimp from Ecuador; A5, Argentine-red shrimp; A6, blue shrimp from Ecuador; B2, pink shrimp from Tampa, FL; B3, white shrimp from Peru; B4, brown shrimp from North Carolina; B5, white shrimp from Colombia; B6, white shrimp from Georgia; C2, white shrimp from Honduras; C3, blue shrimp from Honduras; C4, lobster from Australia; C5, lobster from Florida; and C7, rock shrimp obtained in December from Cedar Key, FL. Samples used in test b include A1, mouse IgG as positive control; B1, 1% BSA as negative control; C1, rock shrimp from Port Canaveral, FL; D1, rock shrimp from Cedar Key, FL; A2, clam; B2, oyster; C2, sea scallop, D2, trout; A3, salmon; B3, flounder; C3, tuna; D3, grouper; A4, beef; B4, pork; and C4, chicken.

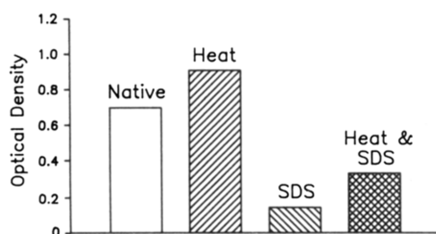

Figure 5. Effect of heat treatment, addition of SDS, or the combination of both on the increment of reactivity of rock shrimp proteins at 5 μg/mL with 4H2-10D3 McAb on ELISA.

Antibody and Antigen Concentrations. The reactivity of the 4H2-10D3 McAb with rock shrimp extract at 5 μg/mL increased as the amount of McAb used increased; it plateaued when antibody concentration reached 20 μg/mL (data not shown). In addition, it was shown that when the 4H2-10D3 McAb at 50 or 100 μg/mL was used, rock shrimp extract at 10 μg/mL provided the highest reactivity (data not shown); McAb at 50 μg/mL generally produced a lower absorbance reading than at 100 μg/mL. Thus, for a better ELISA result, rock shrimp extract as well as the unknown samples at 10 μg/mL was used to react with the McAb at 100 μg/mL.

Identification of Rock Shrimp in a Blind Study. Rock shrimp samples obtained from the east and west coasts of Florida (samples X8 and X7) and at different months (February and December; samples X7 and X22) were equally reactive with the 4H2-10D3 McAb and were correctly identified in a blind study using 24 various seafood and meat samples (Table I). The reactivity of this McAb for rock shrimp samples was 9 times higher than that for the other seafood and meat samples or for the other unrelated shrimp samples. The yellow color developed for rock shrimp when this McAb was used was so intense visual inspection could be used to determine the reactivity. This will facilitate the use of ELISA test for field testing.

Table I. Reactivity of the 4H2-10D3 McAb[a] with the Water Extracts[b] of the Various Seafood and Meat Samples in the Blind Study Using the ELISA Test

sample no.	unknown sample	optical density[c]
X1	trout	0.111 ± 0.012
X2	sea scallops	0.095 ± 0.009
X3	Argentine-red shrimp	0.095 ± 0.011
X4	beef	0.097 ± 0.004
X5	salmon	0.126 ± 0.002
X6	flounder	0.102 ± 0.008
X7	rock shrimp, Cedar Key, FL (Feb)	1.508 ± 0.068
X8	rock shrimp, Port Canaveral, FL	1.125 ± 0.071
X9	white shrimp, Colombia	0.112 ± 0.016
X10	white shrimp, Honduras	0.109 ± 0.006
X11	oyster	0.098 ± 0.012
X12	tuna	0.117 ± 0.002
X13	blue shrimp, Honduras	0.093 ± 0.010
X14	lobster, Australia	0.094 ± 0.011
X15	white shrimp, Peru	0.099 ± 0.015
X16	clam	0.096 ± 0.004
X17	grouper	0.102 ± 0.008
X18	pork	0.098 ± 0.007
X19	blue shrimp, Ecuador	0.097 ± 0.011
X20	lobster, FL	0.094 ± 0.011
X21	brown shrimp, NC	0.097 ± 0.010
X22	rock shrimp, Cedar Key, FL (Dec)	1.303 ± 0.059
X23	pink shrimp, FL	0.096 ± 0.012
X24	chicken	0.104 ± 0.019
X25	white shrimp, Ecuador	0.112 ± 0.013
X26	white shrimp, GA	0.106 ± 0.014

[a] The McAb used was 10 μg of protein/well. [b] The protein extract used was 1 μg/well. [c] Data are means ± standard deviation.

Detectability of the ELISA Test for Rock Shrimp in Mixtures in a Blind Study. Among the 24 unknown samples prepared by randomly mixing the various seafood, fish, or meat samples in various ratios with rock shrimp, all 13 sample mixtures containing rock shrimp (even as low as 17%) were correctly identified by using the test conditions developed for ELISA. These positive samples all had an absorbance reading greater than 1.0 (data not shown).

The reactivity of the McAb to detect the lowest amount of rock shrimp present in these 13 positive mixture samples was determined by testing the McAb with the mixture samples subjected to 5-fold serial dilutions to 5^{-7}. The detectability was interpreted as the minimal amount of rock shrimp protein present in the most diluted samples that, after reacting with McAb, had an absorbance reading greater than 0.2 which was at least 2 times greater than the readings for background and negative controls. The detectability was found to range from 0.19 to 13.6 ng with an average of 4.3 ng (data not shown). The variation of the values was partially attributed to the numerical variation of the absorbance readings that were used for the determination of the rock shrimp levels in these sample mixtures. Rock shrimp proteins in highly diluted samples bound better to the microtitration plates, perhaps due to the reduced competition by other proteins for the same binding sites. The composition of the sample mixtures containing seafood and/or meat proteins also could affect the reactivity of rock shrimp extract with the McAb.

Protein M accounted for 19.7% of rock shrimp soluble proteins; therefore, the sensitivity of using the 4H2-10D3 McAb in detecting protein M in ELISA test was 0.84

ng (4.3 ng × 19.7%). This ELISA assay could be used to reveal the presence of rock shrimp at nanogram levels in mixtures containing unrelated species of seafood or meat.

CONCLUSION

In summary, the 4H2-10D3 McAb developed in this laboratory was proven to be rock shrimp specific by ELISA, immunodot blot, and Western blot tests. This McAb was reactive to heat-treated rock shrimp proteins and was shown to be very effective and sensitive in blind studies to identify rock shrimp and to detect the presence of rock shrimp in samples containing mixtures of diverse seafoods or other nonseafood meats by using the ELISA test. The detection of rock shrimp proteins in various seafood and meat sample mixtures can be achieved at a level as low as 4.3 ng on the average by using the optimal ELISA testing conditions. The ELISA procedure using this McAb is fast, sensitive, and reproducible and thus can be used for fast screening of species used in food products.

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