

Development of a Monoclonal Antibody Detection Assay for Species-Specific Identification of Abalone

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Abstract: Species identification based on biochemical and molecular techniques has a broad range of applications. These include compliance enforcement, the management and conservation of marine organisms, and commercial quality control. Abalone poaching worldwide and illegal trade in abalone products have increased mainly because of the attractive prices obtained and caused a sharp decline in stocks. Alleged poachers have been acquitted because of lack of evidence to correctly identify species. Therefore, a robust method is required that would identify tissue of abalone origin to species level. The aim of this study was to develop immunologic techniques, using monoclonal and polyclonal antibodies, to identify 10 different abalone species and subspecies from South Africa, the United States, Australia, and Japan. The combination of 3 developed monoclonal antibodies to South African abalone (*Haliotis midae*) enabled differentiation between most of the 10 species including the subspecies *H. diversicolor supertexta* and *H. diversicolor diversicolor*. In a novel approach, using antibodies of patients with allergy to abalone, the differentiation of additional subspecies, *H. discus discus* and *H. discus hannai*, was possible. A field-based immunoassay was developed to identify confiscated tissue of abalone origin.

Key words: abalone, mollusks, antigens, Western blot, allergens, species identification.

INTRODUCTION

The trend to a healthier eating style has dramatically increased the consumption of marine products. However, the opening of international food markets has also resulted in overexploitation of marine resources worldwide, especially of species with high market values (Sotelo et al., 1993).

The South African abalone (Gastropoda; *Haliotis* sp.) is one example of such overexploitation. It is believed that up to twice the annual commercial quota is removed by poachers. The escalation of poaching in South Africa not only poses a significant economic threat to the abalone industry, but also has a critical impact on existing abalone stocks (Sweijd et al., 1998).

Because their shell is removed before the animals are processed, the identification of abalone tissues or, specifically, abalone of South African origin, is extremely difficult. The biochemical characterization of abalone species and in

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particular subspecies is, therefore, of growing importance in the management and conservation of abalone stocks, compliance enforcement, and commercial quality control.

Individuals of every species are characterized by a specific genetic composition that determines, in turn, the amino acid sequence of the proteins. Therefore, many of the methods used in species identification rely on methods of protein analysis such as isoelectric focusing or gel electrophoresis. Immunologic techniques have also been used for species identification, which presents many advantages such as relative simplicity, low cost, and rapid results (Sweijd et al., 2000).

However immunologic studies relating to marine organisms are scarce, probably because of close relationships among species. Detailed studies are documented for several fish species (Huang et al., 1995; Carrera et al., 1996; Shepard and Hartmann, 1996) and one crustacean species (An et al., 1990), but to our knowledge, no mollusk species have been immunologically analyzed.

In general, experimental animals are immunized with proteins (antigens) in order to raise suitable antibodies. Polyclonal antibodies that bind to many parts of the same proteins are generally raised in rabbits, whereas monoclonal antibodies, directed only to one specific part of a protein, are generated in mice. It has been reported that a subject who developed a food allergy to abalone also produced polyclonal antibodies to various proteins (allergens) (Lopata et al., 1997). Antibodies are subsequently used to detect particular antigens in tissue or other products using a variety of techniques.

For the generation of abalone-specific antibodies, we intended to utilize proteins that withstand denaturation and can be detected also in heat-treated products. Our group has previously demonstrated that abalone proteins can be potent allergens and are exceptionally stable (Lopata et al., 1997). We therefore used these identified proteins to produce monoclonal and polyclonal antibodies.

The present study reports the first successful attempt to identify different closely related abalone species and to differentiate between abalone tissue and that from other mollusks (Table 1) using immunologic methods. We generated 3 different monoclonal antibodies against heat-stable proteins from abalone (*Haliotis midae*), which have previously been described (Lopata et al., 1997). Ten different abalone species from South Africa, Japan, and Australia were characterized on Western blots using the specific monoclonal antibodies and polyclonal antibodies generated in rabbits. In a novel approach, serum antibodies were used

Table 1. Monoclonal Antibody Reactivity to Different Abalone Species on Western blot*

MoAb	MW (kDa)	<i>H. midae</i>	<i>H. spadicea</i>	<i>H. rubra</i>	<i>H. discus discus</i>	<i>H. discus hamai</i>	<i>H. sieboldii</i>	<i>H. diversicolor supertexta</i>	<i>H. diversicolor diversicolor</i>	<i>H. gigantea</i>	<i>H. laevigata</i>
1.4	45	+	+	+	+	+	+	+	+	+	+
2.11	35	-	+	+	-	-	-	-	+	-	-
2.12	45	+	+	+	-	-	-	+	-	-	-
	35	-	+	+	-	-	-	-	-	-	-
	45	+	+	+	+	+	+	+	+	+	+

*A plus (+) indicates binding, (-), no binding.

from subjects with abalone allergy, to identify abalone species. The development of a field-portable immunoassay, using monoclonal antibodies with the immunostick method, is described as a means of identifying abalone tissue.

MATERIALS AND METHODS

Preparation of Extracts

Protein extracts were prepared from 10 abalone species, from South Africa (*Haliotis midae* and *H. spadicea*), Australia (*H. rubra*), and Japan (*H. diversicolor supertexta*, *H. diversicolor diversicolor*, *H. sieboldii*, *H. discus discus*, *H. discus hannai*, *H. gigantea*, and *H. laevigata*). Extracts were also prepared from 6 other mollusk species (Limpet, *Patella granularis*, snail, *Helix aspersa*, periwinkle *Oxystele* spp., black mussel *Choromytilus meridionalis*, white mussel *Donax serra*, and squid *Loligo vulgaris reynaudii*, Crustacean species shrimp *Metapenaeus monoceros*, tiger prawn *Panaeus monodon*, rock lobster—West Coast *Jasus lalandii*, rock lobster—East Coast *Panulirus hamarus*, rock lobster—South Coast *Palinurus gilchristii*, and langoustine *Palinurus* spp., and 1 fish species hake *Merluccius paradoxus*). Some of the species were, in addition, heated for 10 minutes and extracts were generated from these tissues. In brief, edible portions were removed, cut into small pieces, and extracted into phosphate-buffered saline (PBS) overnight at 4°C. The extracts were sterile filtered (0.45 µm, Millipore, Bedford, Mass.), the protein concentration was determined (BCA-Protein Assay, Kit, Pierce, Rockford, Ill.), and aliquots were stored at -80°C.

Gel Electrophoresis and Western Blotting

To verify anybody binding to different antigens in abalone and related mollusk species, the proteins were separated by molecular weights using sodium dodecylsulfate (SDS) gel electrophoresis and the separated proteins were transferred to membranes (Western blotting).

Abalone extracts and control extracts of limpet and snail were separated on gradient gels (4%–16%) under reducing conditions. Molecular weight markers (BDH, Electrian molecular weight marker) were included to estimate the molecular weights of the separated proteins. For staining of the gels the different extracts were adjusted to 20 µg of total protein for each sample and about 2 µg of protein for Western blotting. Gels were stained with Co-

massie Brilliant Blue (Sigma, St. Louis, Mo.) to display the protein banding patterns.

For Western blotting, proteins were transferred by electroblotting onto polyvinylidene difluoride membrane (Hybond-PVDF, Amersham, Piscataway, N.J.) employing the semi-dry method. The protein-bound membranes were blocked for 1 hour in 1% blocking reagent (Chemiluminescence Assay, Boehringer Mannheim, Germany) and washed twice in PBS containing Tween-20 (PBS-T).

To analyze the human serum for antibody binding, serum samples (1:30) were incubated overnight. The blots were then washed in PBS-T and incubated for 2 hours with a mouse antihuman IgE antibody (DAKO [Carpinteria, Calif.] Clone 1; 1:5000), washed and finally incubated with rabbit antimouse-horseradish peroxidase (HRP) antibody (DAKO; 1:5000) for another 30 minutes. After an overnight wash, the blots were developed using the chemiluminescence detection system (Boehringer Mannheim) and exposed to Kodak x-ray film.

For the detection of monoclonal antibody binding, the membrane was incubated with condition medium for 2 hours followed by 2 washes with PBS-T. Rabbit antimouse-biotin antibody (DAKO; 1:5000) was added for 30 minutes, followed by incubation with HRP-streptavidin (DAKO; 1:30,000). Subsequent steps were identical to the method mentioned above.

Purification of Abalone Allergens for Immunization

Three proteins (including 2 previously identified allergens) of abalone extract were purified by preparative SDS gel electrophoresis. The gels were soaked in KCl solution (0.02 M) for 15 minutes followed by a 5-minute rinse with distilled water, which produced white bands. Because one protein was near the 38-kDa and 45-kDa allergens, 3 protein bands were cut out, squeezed through a syringe, and left in extraction buffer (50 mM NH₄HCO₃; 0.1% SDS) overnight at room temperature. Subsequently, the extracted proteins were analyzed for purity by immunoblotting with patient serum or staining with Coomassie Blue and the proteins were numbered as fraction 1, 2, and 3, with the approximate molecular weights of 38, 42, and 45 kDa, respectively.

Production of Monoclonal Antibodies

BALB/c mice were immunized twice at day 0 and 17 with 50 µg each of purified proteins 1, 2, and 3 in complete

Freund's adjuvant (Difco). Two weeks after the second immunization, the sera were tested for antibody production using a direct enzyme-linked immunosorbent assay (ELISA). A final booster (with incomplete Freund's adjuvant) was given intraperitoneally three days before the fusion. The spleen cells of the immunized mice were fused with SP2 mouse myeloma cells using the PEG fusion technique (Harlow and Lane, 1988). The produced hybridomas were screened by Western blotting and cloned further by limiting dilution.

Hybridoma culture medium (condition medium) containing monoclonal antibodies was used to determine the isotype of the antibodies by a standard ELISA procedure (Coligan et al., 1994).

Production of Polyclonal Antibodies in Rabbits

For the production of polyclonal antibody, 50 µg of each of the 3 protein fractions (antigens) was used to immunize one New Zealand rabbit. In addition, polyclonal antibodies were raised against another South African abalone species (*H. spadicea*) by using 450 µg of whole protein extract. The 2 New Zealand rabbits received the extracted proteins in complete Freund's adjuvant subcutaneously injected at several sites. Subsequent immunizations were performed 2, 4, and 8 weeks after the first immunization with the same protein concentration in incomplete Freund's adjuvant.

Patient Sera

Sera were collected from 5 subjects with history of allergy after ingestion of abalone (*Haliotis midae*). These subjects illustrated specific antibody binding to several proteins in the abalone extract by immunoblotting (Western blotting).

Immunostick Assay

For the development of a field test, the Nunc-Immuno Stick assay (MaxiSorp; Nalge Nunc Int., Rochester, N.Y.) was used. In brief, tissue was cut with a scalpel into small pieces and incubated in 1 ml of PBS for 1 hour in tubes with the paddles inserted. All the following incubations were completed at 37°C, and between each step the paddle and tube were washed in running tap water. After antigen binding, the free binding sites were blocked with milk powder (2%) for an additional hour and incubated with the detecting monoclonal antibody 1.4 for 30 minutes. The secondary antibody, rabbit antimouse (DAKO; 1:1000),

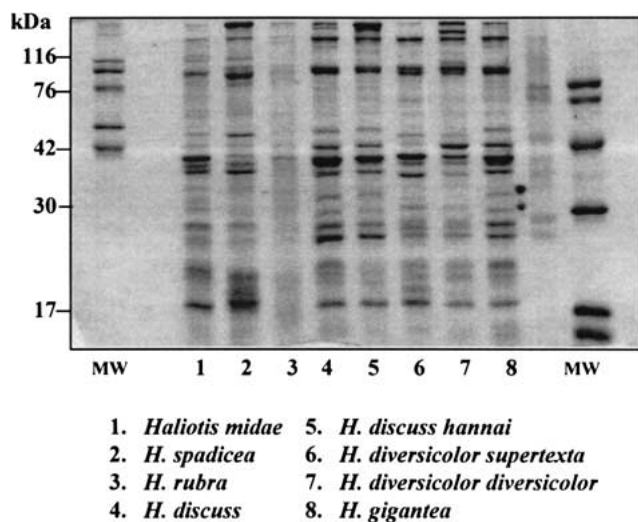


Figure 1. SDS gel electrophoresis of extracts of 8 different abalone species from South Africa (*H. midae* and *H. spadicea*), Australia, and Japan. The species names are labeled from 1 to 8. The molecular weights (MW) are indicated on the left side in kilodaltons, kDa.

was incubated for 20 minutes, and finally the substrate 3,3',5,5'-tetramethylbenzidine (TBM, 1%) was added. The blue color of the substrate develops after about 10 to 20 minutes.

RESULTS

Protein extracts and SDS Gel Electrophoresis

All the prepared extracts were separated by reducing SDS gel electrophoresis to verify the extraction of soluble proteins and identify species-specific protein patterns. The SDS gel electrophoresis of the different abalone species illustrated proteins with molecular weights ranging from 17 to over 200 kDa (Figure 1), with most of the proteins in the molecular range of 25 to 50 kDa. Some of the protein banding patterns make species identification possible. A strong 35-kDa band in the first species and 45-kDa and 200-kDa protein bands in the later species distinguished the 2 South African species *H. midae* and *H. spadicea*. Furthermore, the 2 subspecies *H. diversicolor supertexta* and *H. diversicolor diversicolor* were clearly distinguished by the presence of a strong 35-kDa band in the first species and a 42-kDa and about 180-kDa and 200-kDa protein bands in the latter species.

Protein extracts of cooked and dried abalone were also separated by SDS gel electrophoresis (data not shown) and demonstrated a striking loss of most of the protein bands except in the range of 35 to 50 kDa, which has recently been demonstrated by (Lopata et al., 1997).

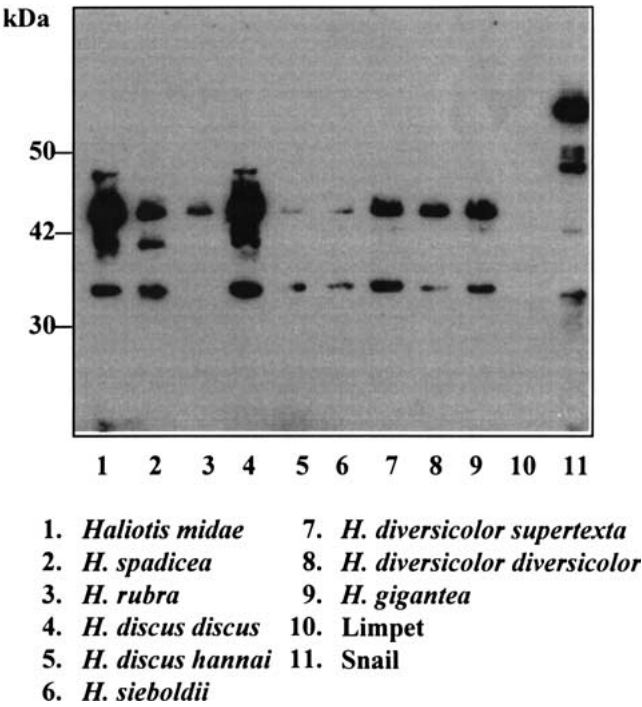


Figure 2. Western blot of IgE antibody reactivity of an abalone-sensitive subject to different abalone and mollusk species. The species names are labeled from 1 to 11. The molecular weights are indicated on the left side in kilodaltons.

Human Serum Binding

The IgE binding components of the separated protein extracts were analyzed by Western blotting with serum of one abalone-allergic subject. Two major allergens were demonstrated by immunoprinting in most of the analyzed mollusks (Figure 2) with molecular weights of about 38 kDa and 45 kDa. The IgE binding patterns of the 2 South African abalone species (*H. midae* and *H. spadicea*) were almost identical to those of the Japanese species *H. discus discus*, however, *H. midae* and *H. discus discus* demonstrated an additional protein band of about 49 kDa. These 3 species differed from the other 6 species analyzed, which were very similar to each other. There was an IgE binding pattern similar to that of snail (lane 11, *Helix aspersa*) with additional bands in the higher molecular weight range, but there was no binding observed to limpet.

Rabbit Serum Binding

Polyclonal antibodies were produced in rabbits against a mixture of 3 purified proteins (38, 42, and 45 kDa) from *H. midae* and total protein extract of *H. spadicea*. Ana-

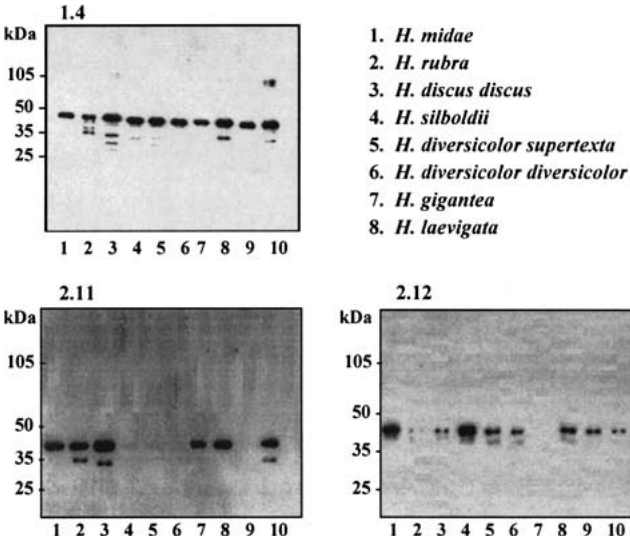


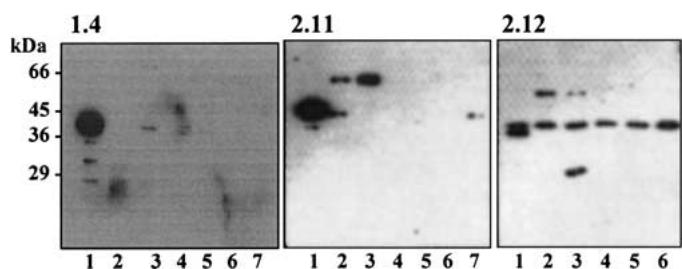
Figure 3. Western blot of antibody reactivity of monoclonal antibodies 1.4, 2.11, and 2.12 to different abalone species. The species names are labelled from 1 to 10; species 1 and 2 are from South Africa. The molecular weights are indicated on the left side in kilodaltons.

lyzing the 2 sera for antibody binding to different abalone species by Western blot demonstrated a qualitatively different binding profile (data not shown). The rabbit sera to the 3 extracted *H. midae* proteins demonstrated, as expected, binding in the 40-kDa range. However, in the 2 South African species, binding to several proteins was observed, whereas in the other 7 abalone species analyzed only one major band of about 42 kDa appeared. The limpet extract showed no binding whatsoever, and the snail extract bound only to very high molecular weight proteins.

The binding profile of the antiserum developed to whole extract of *H. spadicea* was quite different. There were 2 major proteins recognized with molecular weights of about 90 kDa and 120 kDa in all abalone species analyzed. No binding was observed to the limpet extract.

Monoclonal Antibody Binding

Positive clones were selected using Western blot and ELISA for specific binding to the 3 purified proteins. Of the 18 positive clones identified, 3 were selected because of their strong binding on Western blots and analyzed in more detail. The 3 monoclonal antibodies, 1.4, 2.11, and 2.12, are of the IgG1 subtype.



- 1. Abalone
- 2. Black Mussel
- 3. White Mussel
- 4. Snail
- 5. Limpet
- 6. Squid
- 7. Periwinkle

Figure 4. Western blot of antibody reactivity of monoclonal antibodies 1.4, 2.11, and 2.12 to different mollusk species. The species names are labeled from 1 to 7. The molecular weights are indicated on the left side in kilodaltons.

To assess possible cross-reactions of the generated monoclonal antibodies to proteins in other abalone species and related seafood, antibody binding was analyzed by Western blot studies. Figure 3 demonstrates that the 3 analyzed Monoclonal antibodies bound to proteins with similar molecular weights in the 10 different abalone species from South Africa, Australia, and Japan. Monoclonal antibody 1.4 recognized predominately the same protein was observed in 45 kDa in all analyzed species. In addition, binding to a 35-kDa protein was observed in 4 species (*H. spadicea*, *H. rubra*, *H. diversicolor diversicolor*, and *H. laevigata*). Monoclonal antibody 2.11 demonstrated binding to a protien with a molecular weight of about 42 kDa, and 2.12 to 45 kDa protein. Monoclonal antibody 2.11 showed additional binding to a 35-kDa protein in some species. However, 2.11 did not show any binding whatsoever to 4 of the 10 species analyzed (*H. discus discus*, *H. discus hannai*, *H. silboldii*, and *H. gigantea*). A similar lack of protein recognition was observed for monoclonal antibody 2.12 for abalone species *H. spadicea* and *H. diversicolor supertexta*. The binding profiles of the 3 monoclonal antibodies are summarized in Table 1.

The produced antibodies also demonstrated a distinct binding profile when binding to various mollusk species (Figure 4). Monoclonal antibody 1.4 recognized only abalone proteins and a very weak band in white mussel, 2.11 showed binding only to proteins in abalone, black mussel, and white mussel and a very weak signal to periwinkle, but no binding to snail, limpet, or squid; in contrast, 2.12 recognized the same protein of about 45-kDa in all analyzed mollusk species with additional bands in abalone, black mussel, and white mussel.

A direct comparison of monoclonal antibody binding with antibody binding of the 4 allergic subjects is demonstrated in Figure 5. The human serum demonstrates binding to several protein bands, whereas monoclonal antibodies 1.4, 2.11, and 2.12 bind only to single protein bands with molecular weights of about 35 and 45 kDa.

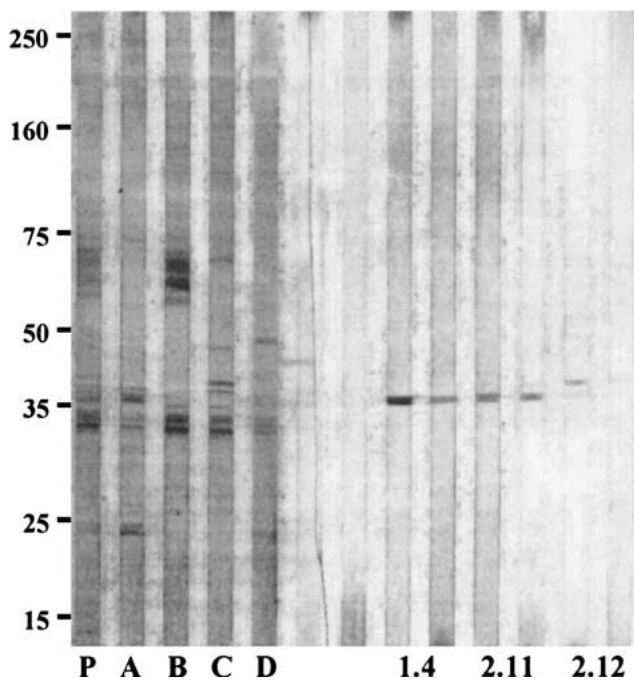


Figure 5. Western blot of IgE antibody reactivity of 4 abalone-sensitive subjects (lanes A to D) and a serum pool of the same subjects (lane P) to *H. midae*. IgE reactivity is compared with monoclonal antibody reactivity of 1.4, 2.11, and 2.12. The molecular weights are indicated on the left side in kilodaltons.

Monoclonal Antibodies and Heated Extracts

To investigate the stability of the detected cross-reacting proteins, tissue of the different species was heated before protein extraction. Monoclonal antibody 1.4 demonstrated binding only to the heat-treated periwinkle (*Oxystele* spp.) extract (data not shown). Monoclonal antibodies 2.11 and 2.12 recognized major proteins of 42 and 45 kDa, respectively, in all mollusk species, except squid and octopus for 2.11. However, 1.4 and 2.11 seemed to lose antibody reactivity to heat-treated abalone. No binding was observed for any of the 6 local crustacean species analyzed.

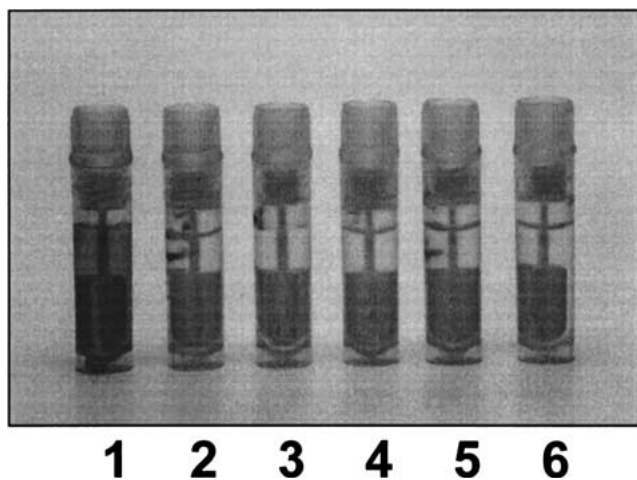


Figure 6. ImmunoStick assay for positive identification of abalone (*H. midae*) tissue. Lane 1, abalone (*H. midae*); lane 2, black mussel; lane 3, oyster; lane 4, limpet; lane 5, rock lobster; lane 6, hake.

ImmunoStick Assay

For the positive identification of tissue as being of abalone origin in a portable assay, we compared the antibody reactivity of monoclonal antibody 1.4 to different tissue sources (Figure 6). Of 6 different tissues we tested, only abalone (*H. midae*) was positive as seen by a blue color that developed after about 10 minutes. The other tissues from black mussel, oyster, limpet, rock lobster, and hake demonstrated a negative response.

DISCUSSION

Increased consumption of seafood has led to increased harvesting and farming of seafood. Approximately 72% of harvested fish and shellfish worldwide is utilized for human consumption. It is estimated that between 1985 and 1989, world harvests of all seafood species increased by 15%, while shellfish increased at a rate of 22% (Moody et al., 1993). Current global seafood consumption yearly is approximately 14 kg per capita (Leung and Chu, 1998). Furthermore, aquaculture of seafood is becoming a major source of income in many countries.

High market prices create the risk of overexploitation of marine organisms and fraudulent substitution of seafood produce. An increase in abalone poaching worldwide and, in particular, in South Africa, is affecting the natural abalone stocks as well as the legitimate abalone industry (Sweijd et al., 1998). For these reasons, a method was

required to unequivocally identify abalone tissue to the species level and differentiate this tissue from other mollusk tissues.

Electrophoretic techniques have been used for many years to identify marine organisms. This has been successfully applied in the identification of various fish species (Rehbein, 1995) and also crayfish species (Powell et al., 1995) using isoelectric focusing. Yet the identification of closely related mollusk species has not been attempted. In our study, using 1-dimensional electrophoresis, we were able to distinguish the two South African abalone species as well as the 2 *H. diversicolor* subspecies by comparing proteins in the higher molecular weight range. However, the variability of the species-specific proteins was too low to differentiate convincingly all 10 analyzed abalone species from one another.

Because of the low degree of differentiation, we embarked on using immunologic techniques, which present many advantages over electrophoretic or genetic methods of species identification (Sweijd et al., 2000). Immunologic techniques are inexpensive, specific, and relatively easy to perform and lend themselves to the development of portable, non-laboratory-based kits, which are helpful for compliance officials in the field. Serum antibodies from seafood-sensitive subjects have been shown to bind to major antigens of 14-kDa in fish (Hansen et al., 1997) and 38 kDa in crustaceans (Leung et al., 1996). However, because these major antigens are very similar among the species, they could not be used to differentiate among species. In contrast to these findings, we could demonstrate in our study that the utilization of serum antibodies of abalone-sensitive subjects allowed differentiation of the 2 South African species from each other and 6 other abalone species.

The stability of some abalone proteins is comparable with a similar property of an allergen found in shrimp (Lehrer et al., 1990). Leung and colleagues have described a 38-kDa cross-reacting allergen in crustacean and mollusk species including *H. diversicolor* (Leung et al., 1996) and cloned tropomyosin of oyster (*Crassostrea gigas*), a major allergen in this mollusk species (Leung and Chu, 2001). We therefore propose that the 35-kDa IgE-binding protein identified in our study is abalone tropomyosin, which explains the thermostability of this protein. Nevertheless, the stable 45-kDa abalone protein is a novel mollusk allergen only recently identified (Lopata et al., 1997).

The presence of these stable allergens in the different abalone species confirmed that these proteins were good candidates for the development of monoclonal antibodies.

The selection of different antibodies identified 3 suitable clones with different binding patterns. These results demonstrate that certain antibody-binding epitopes on the same protein can be species-specific whereas other epitopes seem to be common and can be found in a variety of species. In a study with monoclonal antibodies generated to a 39-kDa protein from the Taiwan shrimp (*Parapenaeus fissures*), it was shown that 6 isoforms existed for this allergen, which had no marked difference in their amino acid composition (Lin et al., 1993). Isoproteins are identical with respect to molecular size, but when analyzed more closely, they may differ at up to 25% of the amino acid residues. Consequently, one cannot exclude the existence of isoproteins in abalone.

To obtain another tool in identifying various species, polyclonal antibodies were generated to the purified abalone proteins. However, the polyclonal antibodies from rabbits were only able to identify reliably the 2 South African species. A report on the development of an ELISA for the detection of fish species also used polyclonal antibodies (Cespedes et al., 1999). However, the generated antisera were only rendered species-specific after blocking the cross-reacting antibodies with heterologous protein extracts. Therefore, the use of monoclonal antibodies, as demonstrated in our study, has the advantage of being species-specific without preabsorption of cross-reacting antibodies. In addition, monoclonal antibodies can even be specific to a whole group of related species as in our study, where no monoclonal antibody binding activity was observed to representatives of the class Bivalvia or Cephalopoda.

The immunostick assay developed in our study is easy to perform, is specific, and does not require the use of sophisticated apparatus. The ability to identify minute abalone tissue samples from other marine organisms could equip fisheries and compliance officials with a diagnostic tool to help control poaching.

In summary, the use of the specific monoclonal antibodies could allow differentiation between the 2 South African abalone species as well as the 10 analyzed abalone species. However, for an immunoassay technique to be used in various parts of the world on different abalone species, comparison with standard proteins or reference samples would be required. Future research into the identity of the 35-kDa and 45-kDa antibody-binding proteins will determine the amino acid sequence and presence of these proteins in other mollusk species. Furthermore, the possible use of the generated specific monoclonal antibodies on other mollusk species will be investigated where

immunologic detection to species level is necessary for management or conservation.

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