MARINE BIOTECHNOLOGY

Proteomic Approach to Probe for Larval Proteins of the Mussel *Mytilus galloprovincialis*

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Abstract

A proteomic approach was used to search for larval proteins specific to the mussel *Mytilus galloprovincialis* from Galicia in northwest Spain. The study included both a comparative analysis, through two-dimensional electrophoresis, of protein expression maps of the larvae of the mussel and of 5 abundant and commercially important bivalve species from the region (*Ostrea edulis*, *Cerastoderma edule*, *Pecten maximus*, *Tapes decussatus*, and *Venarupis pullastra*) and subsequent mass spectrometric analysis of some of the protein spots. A total of 18 spots were selected and isolated from gels of *M. galloprovincialis* larvae. From their relative position on the electrophoresis gels, 6 of these were clearly exclusive to the mussel species. However, it was not clear whether the other spots were shared by other species. To overcome this ambiguity, first an analysis using matrix assisted laser desorption ionization with time-of-flight (MALDI-TOF) was conducted on the 6 spots of *Mytilus* that could possibly be shared with only one species. The peptide mass fingerprinting was completely different for the proteins compared. This result confirmed that the 6 proteins were exclusively mussel proteins, but demonstrated the utility of this approach when working with species that are poorly represented at the protein level in databases.

Key words: *Mytilus* — two-dimensional electrophoresis — mass spectrometry — proteomics — bivalve species — protein expression

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Introduction

The lack of appropriate tools for rapid and unambiguous identification of larval stages of marine invertebrate species is one of the main bottlenecks in many studies of larval ecology and distribution, which usually require handling numerous and complex plankton samples.

In bivalve mollusks, early larval stages (D-shaped or straight-hinged) of different species are similar in external shape (Lutz et al., 1992). At present bivalve larvae identification relies on the analysis, by scanning electron microscopy, of several diagnostic morphologic traits of the larval hinge such as the denticulate structures of the provinculum and the form of the lateral hinge system (Lutz, 1985). Unfortunately, this technique is tedious and requires careful disarticulation, cleaning, and mounting of the valves (Hu et al., 1992), making it impractical in extensive field studies, which require rapid identification of larvae present in numerous plankton samples. Therefore alternative methods that combine accuracy, precision, and rapidity are needed.

In the last decade the incorporation of molecular technology into the fields of marine biology and ecology (Burton, 1996) has permitted the use of novel approaches to solve this problem. These new approaches have mainly focused on searching for species-specific molecular markers, more stable and precise than the traditional morphologic characteristics (Garland and Zimmer, 2002). Following the present trends and the unquestionable advantages of the use of the polymerase chain reaction (PCR), most of these studies have been designed to produce oligonucleotide probes by means of different DNA-based approaches (Garland and Zimmer, 2002). On a few occasions, however, another interesting molecular approach, antibody production technology, has

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been considered and applied to clam larvae (Herrera and Córdoba, 1981; Herrera et al. 1982), and scallop larvae (Demers et al., 1993; Paugam et al., 2000). This technology is particularly attractive because it combines specificity, sensitivity, speed in sample analysis, and, very importantly, the possibility of being applied to whole larvae (Hare et al., 2000). However, an important drawback is the potential risk of antibody cross-reactions among species, which are frequent when the host animal is immunized with the whole target larvae rather than with a species-specific antigen (Demers et al., 1993). To overcome this problem and thus obtain a more specific antibody response, highly specific proteins should be detected first, isolated from the target larvae, and then used as antigens to inoculate the animal host.

The work presented here uses a proteomic approach to probe for specific proteins of 2-day-old larvae of *Mytilus galloprovincialis* from Galicia in northwest Spain, which is the only species of mussel present in this region (Coustau et al., 1991; Sanjuan et al., 1994).

The study includes both comparative analysis, through two-dimensional electrophoresis (2-DE), of protein expression maps of mussel larvae and 5 other commercially important bivalve species from the region (*Ostrea edulis*, *Cerastoderma edule*, *Pecten maximus*, *Tapes decussatus*, and *Venarupis pullastra*) and subsequent analysis, by mass spectrometry, of some of the protein spots. 2-DE is a unique method for large-scale protein characterization, which combines the separation of complex protein mixtures using two independent parameters: isoelectric focusing (IEF) gel electrophoresis in the first dimension and sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) in the second dimension. This technique combined with mass spectrometry allows the detection and identification of the protein repertoire of specific tissues, cells, or organisms (Wilkins et al., 1996). Thus the complexity of a biological system can be approached in its entirety given that proteomics allows the study of many proteins simultaneously (Williams, 1999). Hence this approach may be quite useful and can be recommended even in difficult-to-diagnose organisms such as bivalve species, which present a high degree of intraspecific polymorphism at the genetic and protein levels, and are poorly characterized at the genome and proteome levels in databases. The present work demonstrates the utility of this approach in characterizing a repertoire of proteins specific and exclusive to a species.

This study is especially relevant for the economically and socially important Galician mussel cultivation, for which fast and accurate methods of identification of the first larval stages are urgently needed, in order to be integrated into the monitoring programs of spatfall prediction already in progress.

Materials and Methods

Obtaining Larvae. Two-day-old D-shaped larvae of the different bivalve species analyzed in this study were obtained in our laboratory by the induction of spawning of mature individuals sampled in several cultivation areas of the Galician coast (*M. galloprovincialis* and *C. edule*) and also supplied from public and private hatcheries (*O. edulis*, *P. maximus*, *T. decussatus* and *V. pullastra*). The age of the larvae (48 hours) was precisely controlled because protein expression of the organisms is known to vary throughout development. A detailed description of the methods of induction, fertilization, and larval culture is given elsewhere (Abalde et al., 2003). Larvae were kept frozen until use.

Protein Extraction. From lyophilized larvae proteins were extracted according to the method described by. López et al. (2002a). Protein concentration was measured according to the method of Ramagli and Rodríguez (1985).

High-resolution Two-dimensional Electrophoresis. Total protein samples of 100 to 200 μg (analytical run) or 1.5 mg (preparative gel for mass spectrometry) were used for each run. The first dimension (IEF) of gel electrophoresis was carried out on immobilized pH gradients (pH 4–7, 13 cm, Amersham Pharmacia Biotech) with a horizontal electrophoresis apparatus (IPGphor, Amersham Pharmacia Biotech) according to the method described by Görg et al., (1999, 2000). The second dimension of gel electrophoresis was carried out on 12.5% SDS-polyacrylamide gels (140 mm × 160 mm × 1.5 mm) at 15 mA per gel for 15 minutes and 50 mA per gel for approximately 5 hours until the dye front reached the bottom of the gel. The protein spots in analytical gels were visualized by silver staining using a Plus One silver staining kit (Amersham Pharmacia Biotech). Preparative gels were stained with 0.1% Coomassie Brilliant Blue R-250 in 40% ethanol, 10% acetic acid, for 3 hours and destained in 30% ethanol, 10% acetic acid.

Computer Analysis of 2-DE Patterns. Computer analysis was as previously described (López et al., 2001, 2002b), with minor modifications. In brief, silver-stained 2-DE gels were digitized at 84.7 × 84.7

μm resolution using a Bio-Rad GS-700 imaging densitometer. Protein patterns were analyzed using PD Quest software (Bio-Rad). Spots detected by the program were matched between each gel.

Visual Analysis of 2-DE Gels. The gels were analyzed by visual inspection. Once the reproducibility of patterns among different gels of the same species was assessed, 5 gels from each species were selected for analysis. A synthetic map was constructed from the 5 gels of *M. galloprovincialis*, ensuring that areas giving unsatisfactory results such as ambiguous or badly defined or overlapping spots were avoided. This synthetic map included the spots that were common to all gels of *M. galloprovincialis*. Later, this map was compared with the gels of the other species to identify spots that were specific to *M. galloprovincialis*.

For a detailed study of species differences, an analysis by areas was carried out. Five areas were chosen on the two-dimensional gels using the following criteria: (1) high resolution in the area, with no overlapping spots; and (2) ease of location on the gel. Owing to differences in protein patterns among different species, it was difficult to consistently pinpoint the same randomly chosen area on all gels. Hence spot 30 (actin), common for all species and easy to find, was used as a reference. The coordinates of this spot were drawn to help in locating the same area on all the gels of the different species studied.

Peptide Mapping by MALDI-TOF Analysis. Spots selected as being potentially specific to *M. galloprovincialis* but doubtful of any other species were analyzed by mass spectrometry. matrix assisted laser desorption ionization with time-of-flight (MALDI-TOF) mass spectrometry was performed using an Autoflex instrument (Bruker) equipped with delayed extraction and operating in reflector mode. First, the gel containing the spots to be analyzed was washed and dehydrated. The spots were then cut out, and the protein was digested with trypsin at 37°C overnight. A volume of 0.5 μl of sample was applied onto the matrix of the instrument and dried. Then 0.5 μl of saturated solution of α-hydroxycinnamic acid matrix in acetonitrile and water (1:1, v/v) containing 0.1% trifluoroacetic acid was added to the sample and dried. External calibration was used by spotting peptide standards in adjacent sample positions.

Results and Discussion

Protein Expression Patterns in Bivalve Larvae. High-resolution two-dimensional electrophoresis is able to separate complex mixtures of proteins, resolve thousands of genetic products in a single gel, and detect changes in the order of 0.1 pH units in the first dimension and of 1 kDa in the second dimension (Hanash et al., 1986). In any analysis of this type, where the aim is to compare different patterns of protein expression, it is necessary to optimize the resolution of the protein maps as a function of the origin of the samples to be analyzed. In this case the technique was optimized for the separation of total proteins of bivalve samples. The study was first carried out using two-dimensional maps with strips of wide-range immobilized pH gradients (pH 3–10 NL/13 cm). Because most of the spots appeared in intermediate pH ranges (data not shown), IPG strips in the range of 4 to 7 were chosen. This resulted in a more uniform spot distribution on a single surface, improving reproducibility. Moreover, in this isoelectric point range, electroendosmotic effects that could occur during the isoelectrofocusing of alkaline proteins were avoided (Görg et al., 2000).

Figure 1 shows a representative map of each species. Quality high-resolution gels are observed. The spots are uniformly distributed throughout the gel, clear, and well-defined. Upon visual inspection overall protein expression is clearly different. Some spots overlap or are associated with streaking; however, these spots usually appear close to well-shaped spots, and this may indicate that streaks such as these were due to individual properties of the streaking proteins rather than to problems of the 2-DE technique employed (Klose and Kobalz, 1995). The absence of overlapping areas and good separation between spots made the comparative analysis easier and thus also the isolation of the proteins from the gels.

Protein maps that allow the visualization of a greater number of spots possessing the qualities mentioned above are called analytical gels (see ''Materials and Methods''). These gels are suitable for between-species comparative analyzes. The best patterns were obtained when the amount of sample applied was 150 μg. To increase the concentration of several specific proteins for later isolation and characterization, the gels were loaded with a larger amount of sample (400 μg) (preparative gels). Preparative gels were always stained with Coomassie blue, whereas analytical gels were stained with silver.

A mean of 350 spots was detected in gels of *M. galloprovincialis* stained with silver nitrate. Spot counts were obtained automatically with the assistance of computer software, and visually by comparing the patterns observed. The number of spots detected was always below that expected from the resolution capability of the technique, which has the potential of separating thousands of proteins (Klose

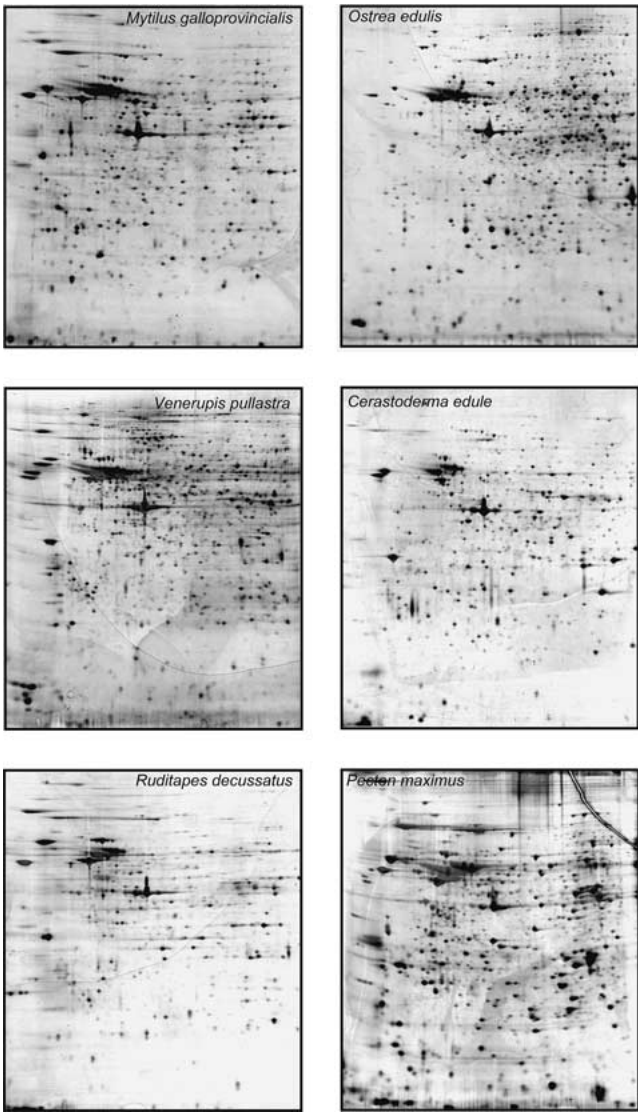

Fig. 1. Representative map of each bivalve species. Two-dimensional gels were run using strips of 13 cm and pH gradient 4 to 7.

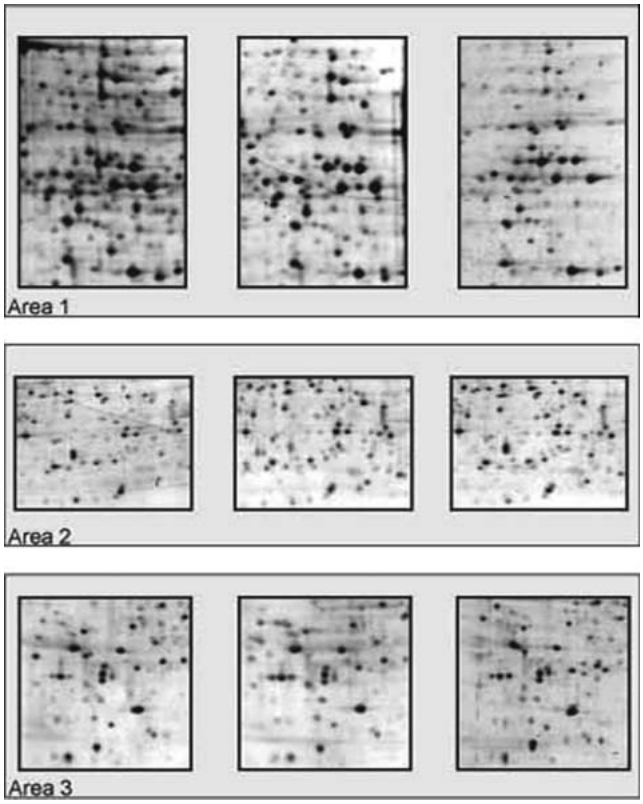

Fig. 2. Amplification of 3 areas of *M. galloprovincialis* larvae for reproducibility analysis.

and Kobalz, 1995). This apparent controversy has several possible explanations. To start with, a compromise was established between resolution and manageability, limiting the pH range and gel size. Furthermore, we used a conservative approach for the analysis of the gels, counting only clearly defined spots and not considering outermost and confusing areas. Finally, the proteins that were present in small amounts were not visible given that they were below the silver staining detection level (Jungblut et al., 1996; Corthals et al., 2000).

Reproducibility Analysis. Before comparison of the two-dimensional patterns of the different species, reproducibility of the technique was determined in our system. An intraspecific reproducibility control was used. For this 5 gels of larvae were analyzed for each species. First the entire gel was analyzed visually, and then a more detailed analysis was achieved by amplification of 3 selected areas containing no overlapping spots, which covered a large area of the gel. The 3 areas for 3 gels of *M. galloprovincialis* are shown in Figure 2. The same comparative analysis was performed for the other species (data not shown). The degree of reproducibility was high for number and position of the spots.

Protein Expression in D-shaped* Mytilus galloprovincialis *Larvae. A comparative analysis of 5 good-quality gels was carried out to obtain a two-dimensional pattern for larvae of *M. galloprovincialis* to be used as reference. The quality of these gels was determined by considering the parameters described previously: resolution, definition, homogeneous distribution, morphology and clarity of the spots, minimum background, streaks, or veined bands, and no overlapping of proteins. Using these 5 gels we constructed a synthetic two-dimensional map that included the ''spots'' that were present in all gels (Figure 3). Proteins with a molecular weight above 200 kDa could not be seen because of the limited

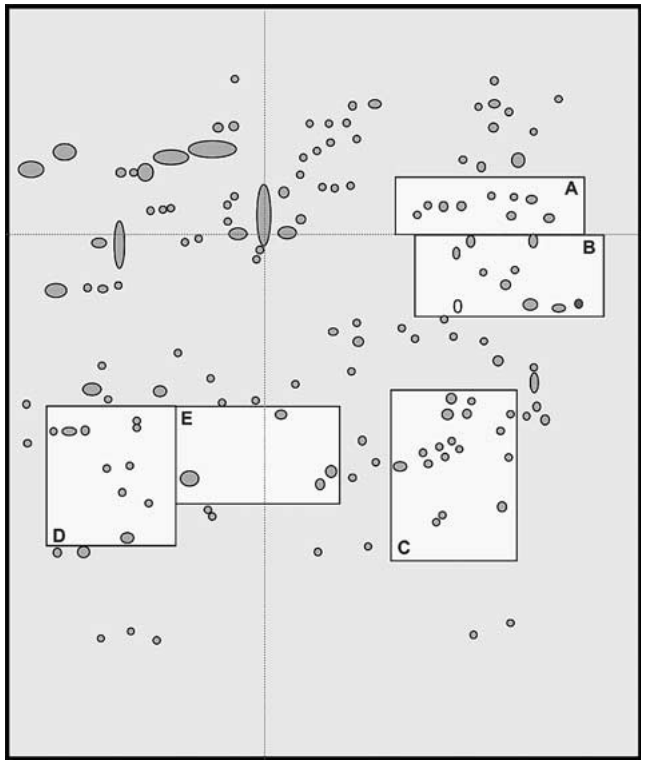

Fig. 3. Synthetic 2-dimensional gel of *M. galloprovincialis*. Five areas (white squares) were chosen for the comparative analysis following the criteria indicated in ''Results and Discussion.''

capacity of large proteins to be introduced into the gel of the first dimension (Tsuji et al., 1999). For the construction of this map, a conservative strategy was followed. Overlapping areas, areas of molecular weights or isoelectric points near the range limits analyzed and unclear or undefined spots were discarded, and only spots that were well defined in all the gels of *M. galloprovincialis* were included. The reduction in the number of spots between the real gels (350) and those included in the synthetic map (139) was due mainly to this conservative strategy.

Differential Protein Expression in Different Bivalve Species. To compare protein patterns of different bivalve species, 5 representative two-dimensional gels were chosen for each species. Each gel was compared with a synthetic map of *M. galloprovincialis*. A synthetic map was not constructed for the other species because the aim was to obtain proteins that, although always present for all *M. galloprovincialis* gels, will never appear on gels of other species. Only the presence or absence of spots was evaluated; spot intensity differences were not considered.

In general when comparing the protein patterns obtained from larvae of different bivalve species, a certain degree of similarity was observed (Figure 1).

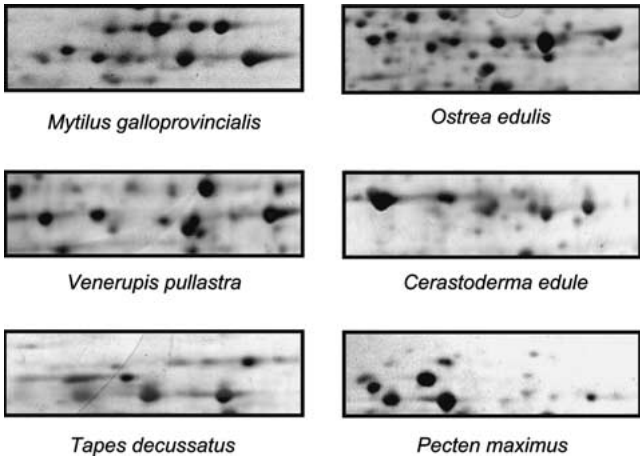

Fig. 4. For further clarity in the comparative analysis, an amplification of area A in Figure 3 in gels of all species analyzed is shown.

The number and distribution of spots were similar for all species, but each one of these presented the characteristic patterns. The spots were grouped in a particular way for each species. There were clearly common spots, like actin, the most abundant protein, and others yet unidentified. Tropomyosin, having a p*I* 4.5 and molecular weight of 33.1 kDa (López et al., 2002a, 2002b), was also present in all species, although the same form did not appear in each. For example, in *O. edulis* it appeared as a double spot. Some ambiguous spots appeared at the same location or a similar location on gels of different species, but it was not known if they represented the same protein. However, other spots were clearly species-specific.

Figure 4 shows one of the areas studied (area A, Figure 3). Spots that appeared to be exclusive of *M. galloprovincialis* were selected. When we were not sure that one of these spots could be present in another species, it was selected anyway and isolated with those of other species thought to correspond. The fact that some mussel spots were not selected for any other species was not absolute proof of specificity because this protein could perhaps be found in other species in amounts too small to be detected with staining methods, or it could appear in a bivalve species not yet analyzed. The same analysis was performed in the other areas (data not shown).

Table 1 summarizes the spots that are exclusively of mussels and those spots that probably are shared by one or more of the bivalve species studied. A total of 18 spots were selected and isolated from gels of *M. galloprovincialis* larvae (Figure 5). From their relative positions on the 2-DE gels, 6 clearly belonged to mussel exclusively. For the others it was not clear whether they also belonged to other species.

Table 1. Proteins that are Exclusive to *Mytiliis* (M) and Proteins that are Probably Shared by one, two, or four of the Bivalve Species Studied

Spots	*N*	*Spot*	*Other species*
Belonging exclusively to the mussel species	6	M **3**	
		M **10**	
		M **12**	
		M **39**	
		M **43**	
		M **48**	
Belonging to the mussel species and probably shared with one species	6	M **16**	***T. decussatus***
		M **21**	***T. decussatus***
		M **22**	***C. edule***
		M **37**	***T. decussatus***
		M **46**	***O. edulis***
		M **49**	***T. decussatus***
Belonging to the mussel species and probably shared with 2 species	5	M **1**	***T. decussatus*, *O. edulis***
		M **9**	***T. decussatus*, *C. edule***
		M **38**	***T. decussatus*, *C. edule***
		M **40**	***T. decussatus*, *P. maximus***
		M **47**	***P. maximus*, *C. edule***
Belonging to the mussel species and probably shared with 4 species	1	M 8	***T. decussatus*, *O. edulis*, *P. maximus*, *C. edule***

To overcome this ambiguity the 6 spots of *Mytilus* possibly shared with one species and those of other species were analyzed using MALDI-TOF. Figure 6 shows the mass spectra of these spots. The mass spectra (peptide mass fingerprinting) of the proteins compared were all different. This result confirmed that the 6 proteins were exclusively mussel proteins, and that this strategy is useful and appropriate for studies of species that are poorly represented at the protein level in databases.

In a future work using MALDI we would like to analyze the 6 remaining *Mytilus* proteins that show a relative position on the 2-D gels that is similar to other bivalve mollusk species studied previously in our laboratory.

Although the main focus of this article was *M. galloprovincialis*, which is the most important species in Spanish aquaculture, new comparative analysis of the basic information contained on the protein gels of the other species—all of them of high commercial importance in Galicia—will also permit us to detect specific proteins for the identification of their early larval stages.

Conclusion

Using the technique developed in this article, that is, obtaining specific proteins of *M. galloprovincialis* larvae by two-dimensional electrophoresis and mass spectrometry, we are now in the position to immunize host animals with specific antigens to avoid cross-reaction, and to obtain monoclonal antibodies for mussel larvae identification. Cross-reaction is a widely described phenomenon in immunologic responses. In bivalve identification these cross-reactions are particularly frequent when the host

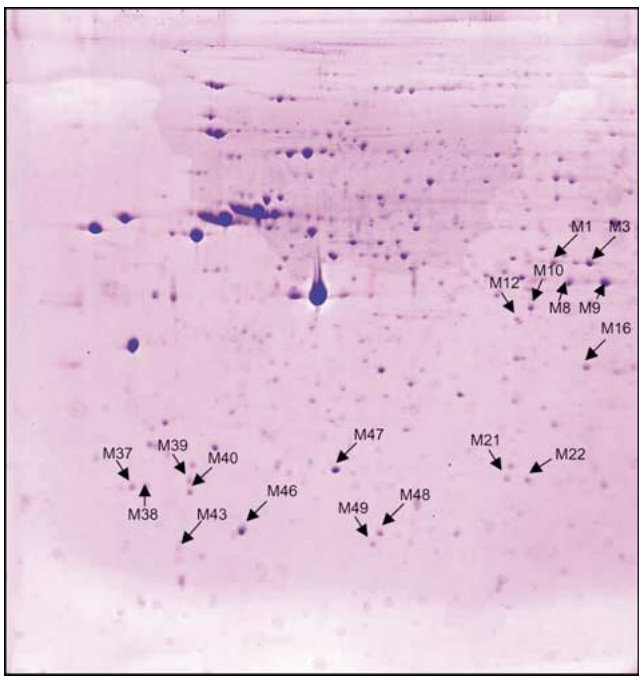

Fig. 5. Two-dimensional Coomassie blue–stained gel of *M. galloprovincialis* larvae. Labeled spots depict the 12 spots that belong exclusively to mussel (M3, M10, M12, M16, M21, M22, M37, M39, M43, M46, M48, and M49), and the 6 remaining proteins with similar relative positions are of the other bivalve species (M1, M8, M9, M38, M40, and M47).

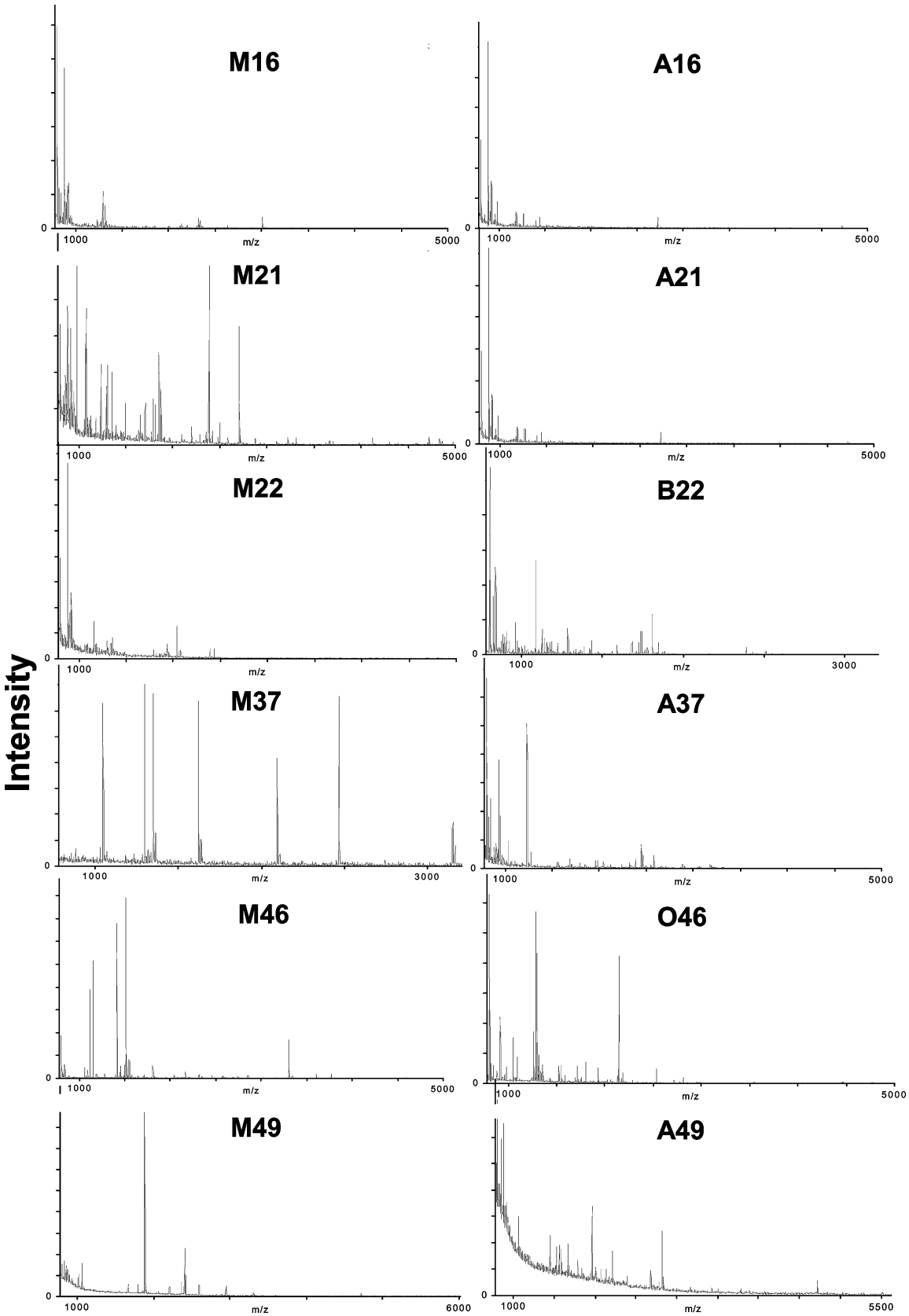

Fig. 6. Peptide mass maps by MALDI-TOF mass spectrometry analysis of spots M16, M21, M22, M37, M46, and M49 and those of the species potentially shared with *Mytilus*. M indicates *M. galloprovincialis*; A, *T. decussatus*; B, *C. edule*.

animal is immunized with the whole target larvae rather than with a species-specific antigen, such as Demers et al. (1993) described when they prepared specific monoclonal antibodies against larvae of the giant scallop *Placopecten magellanicus*. The immunization of the animals with the specific proteins here identified will favor a specific response, and although it is true that some of the antibodies obtained could cross-react with any irrelevant motif, only the antibodies that present no cross-reaction with other species of bivalves will be selected. This selection step will be carried out by indirect immunofluorescence, antibodies that recognize any other bivalve will be rejected, and therefore, immunization with specific proteins will yield specific monoclonal antibodies that only recognize the species from which the proteins were extracted. These antibodies could be used for routine assays on larvae collected from plankton samples.

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