

Discrimination of South African Commercial Fish Species (*Merluccius capensis* and *Merluccius paradoxus*) by LC-MS/MS Analysis of the Protein Aldolase

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Analysis of the protein aldolase using proteomic methodologies allowed the discrimination of the Cape hakes, Merluccius capensis and Merluccius paradoxus. A classical bottom-up proteomics approach, consisting of two-dimensional gel electrophoresis, tryptic in-gel digestion, and LC-MS/MS analyses, was performed for the characterization and de novo sequencing of the aldolase spots. The sequences discovered presented a high degree of homology with the aldolase A sequence from the teleostei Tetraodon nigroviridis. De novo sequencing of several MS/MS spectra from the aldolase A spots allowed the characterization of two species-specific peptide biomarkers. One of them was unique to Merluccius capensis while the other was present only in Merluccius paradoxus. These peptide biomarkers can be used to discriminate between both species of Cape hakes.

KEYWORDS *proteomics, aldolase, hake, two-dimensional gel electrophoresis, mass spectrometry, de novo sequencing*

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INTRODUCTION

Consumers increasingly demand knowledge about the quality and origin of seafood products and commercial regulations imposed by many countries (Directive 2000/13/EC, 2000; EU Commission Regulation No. 2065/2001, 2001), making it necessary to develop new analytical tools to distinguish between closely related fish species. This is the case for Cape hakes, *Merluccius capensis* Castelnau (1861) and *Merluccius paradoxus* Franca (1960), two different species belonging to the Merlucciidae family with similar morphological features and which coexist in the same catch area, overlapping their geographical distribution in the South African coast. Due to this overlapping distribution, the species are caught and managed jointly and no distinction is made in stock management. Since the meat quality is very different between these species, which in turn results in significant differences in their market prices, it is important in some instances to authenticate the commercial product (Pérez et al., 2005). To assure a correct discrimination between both hake species, DNA-based (Pérez et al., 2005; Chapela et al., 2007) and protein identification methodologies (Mackie and Jones, 1978; Piñeiro et al., 2001) have been used.

Recently, proteomic tools have been successfully applied to the identification and authentication of hake species, focusing the analysis on parvalbumins (PRVBs; Carrera et al., 2006; Jorge et al., 2007) and nucleoside diphosphate kinases (NDKs; Carrera et al., 2007). Using two-dimensional gel electrophoresis (2-DE) and by analyzing certain protein spots (PRVBs or NDKs) by MALDI-TOF-MS and LC-ESI-IT-MS/MS, several peptide biomarkers were discovered, allowing the differential classification and identification of all the commercial, closely related species belonging to the family Merlucciidae. In this sense, important efforts have been made in many labs searching for new potential peptide or protein biomarkers.

Fructose-1,6-bisphosphate aldolase (E.C. 4.1.2.13) catalyses the reversible cleavage of D-fructose-1,6-bisphosphate in the glycolytic pathway (Horecker, 1972), leading to the formation of D-glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Three related isoforms (A, B and C), each encoded by a separate gene (Lebherz and Rutter, 1969), are present in almost every vertebrate. Aldolase A is found mostly in muscle and is associated with glycolysis; aldolase B is present in liver, kidney, and intestine and is involved in gluconeogenesis and the metabolism of fructose; and aldolase C is found in the neural tissues. Aldolase is a highly abundant protein in the white, fast-contracting, muscle of most fishes (Nakagawa and Nagayama, 1989), and has been considered in other organisms as a potential enzyme marker to distinguish trematode species (Saijuntha et al., 2006). Thus, it may be a good candidate for an authenticity biomarker of fresh or processed seafood products.

The main goal of the present work is to characterize species-specific peptides from aldolase in order to differentiate both species of Cape hakes, using a classical bottom-up proteomics approach, mainly by means 2-DE and LC-ESI-IT-MS/MS.

MATERIALS AND METHODS

Fish Material

Two different hake species, *Merluccius capensis* (Cape hake) and *Merluccius paradoxus* (Deep-water Cape hake), were employed in this study. Specimens were caught on the coast of South Africa, frozen onboard at -30°C with special care in keeping their morphological characters in good shape, and shipped by plane to our laboratory for the analyses. The weight of the specimens studied was in the range of 3 to 6 kg.

At least 10 fish specimens from each of the hake species were subjected to taxonomical study according to their anatomical and morphological features by an expert marine biologist. Genetic identification was performed at the Food Biochemistry laboratory of the Marine Research Institute (IIM-CSIC, Vigo, Pontevedra, Spain), using the fishID kit based on the amplification of specific mitochondrial DNA sequences (Bionostra S.L., Madrid, Spain). Three samples from each of the species were selected as representative for this study.

Extraction of Sarcoplasmic Proteins

Sarcoplasmic protein extraction was carried out by a 30 s homogenization of raw white muscle (5 g) in 10 mL of a pH 7.2, 10 mM Tris-HCl buffer, supplemented with 5 mM PMFS, using an Ultra-Turrax device (IKA-Werke, Staufen, Germany). The extracts were centrifuged at 40000 g for 20 min at 4°C (J221-M centrifuge, Beckman, Palo Alto, CA) and the supernatants recovered and maintained at -80°C until used. Protein concentration in the extracts was determined by the bicinchoninic acid method (Sigma Chemical Col., USA).

Two-Dimensional Gel Electrophoresis (2-DE)

Isoelectric focusing (IEF) was performed at 10°C in a Multiphor II electrophoresis unit (Amersham Biosciences, Sweden). Forty μL of protein extracts (4 mg/mL) were loaded on gels by duplicate on IEF strips with a narrow range of pH 4.0–6.5 (Amersham Biosciences) using a sample applicator paper. A mixture of protein standards in the 2.5–6.5 pH range (low pI standard from Amersham) was included. IEF conditions were as follows: 1500 V, 50 mA, 30 W until at least 4000 Vh were reached. IEF strips corresponding to individual

lanes were cut immediately after the run was completed and kept frozen at -80°C until the second dimension by SDS-PAGE was run. A duplicate IEF strip was stained with 0.1% Coomassie Brilliant Blue (Sigma Chemical) according to the manufacturer's staining protocol.

Equilibration of IEF gel strips was carried out at room temperature. The strips were placed in sample buffer (Laemmli, 1970) containing 0.75% dithiothreitol for 10 min and then for another 10 min in sample buffer containing 4.5% iodoacetamide. Once equilibrated, the strips were subjected to the second dimension using preparative vertical SDS-PAGE (10%T and 3%C) gels with Tris-Tricine buffer, at 15°C using the Multiphor II system (Amersham Biosciences) as previously described (Carrera et al., 2006). A low molecular weight protein standard (14–97 kDa; LMW Calibration kit, Amersham Biosciences) was employed as reference. The running conditions were: 100 V, 40 mA, and 150 W, during 16–18 h. Protein spots were visualized by the Coomassie Brilliant Blue (CBB) staining (Amersham Biosciences) according to the manufacturer's instructions. PDQuest software (Bio-Rad, Hercules, CA) was used for image analyses.

Peptide Fragmentation by LC-MS/MS

Protein spots were excised from 2-DE gels maximizing the protein-to-gel ratio and subjected to in-gel digestion with trypsin (Promega, Madison, WI, USA) as described elsewhere (Jensen et al., 1999). Peptide digest were analyzed online by LC-MS/MS using a LC system model SpectraSystem P4000 (Thermo Fisher, San Jose, CA) coupled to an ion trap mass spectrometer model LCQ Deca XP Plus (Thermo Fisher) as described previously (Carrera et al., 2007). The separation was performed on a $0.18\text{ mm} \times 150\text{ mm}$ BioBasic-18 RP column (ThermoHypersil-Keystone), using 0.5% acetic acid in water and in 80% acetonitrile as mobile phases A and B, respectively. A 90 min linear gradient from 5 to 60% B at a flow rate of $1.5\text{--}1.7\text{ }\mu\text{L/min}$ was used. Peptides were detected using survey scans from 400 to 1600 amu (3 μscans), followed by a data-dependent ZoomScan (5 μscans) and MS/MS scan (5 μscans), using an isolation width of 3 amu and a normalized collision energy of 35%. Fragmented masses were set in dynamic exclusion for 3 min after the second fragmentation event and singly charged ions were excluded from MS/MS analysis.

Mass Spectrometry Data Processing

MS/MS spectra were searched using SEQUEST (Bioworks 3.1 package, Thermo Fisher) against the nr.fasta database (NCBI Resources, NIH, Bethesda, MD). The following constraints were used for the searches: tryptic cleavages after Arg and Lys, up to two missed cleavage sites, and tolerances $\pm 1.8\text{ Da}$ for precursor ions and $\pm 0.8\text{ Da}$ for MS/MS fragments

ions. The variable modifications allowed were methionine oxidation, carbamidomethylation of Cys, and acetylation of the N-terminus of the protein.

De novo sequencing was performed by manual interpretation of the ion series of the spectra with the aid of the software package DeNovoX (Thermo Fisher). Homology between *de novo* obtained sequences and those in the NCBI database was analyzed using BLAST.

RESULTS AND DISCUSSION

Two-Dimensional Gel Electrophoresis

Sarcoplasmic protein extracts of three samples from each of the species studied were subjected to 2-DE using gradient gels (12–14%) which were stained with CBB. To ensure the reproducibility of the experiments, three gels per individual were run. 2-DE patterns for both hake species are shown in Figure 1. Aldolase spots were localized according to the M_r and pI data previously described for enzyme in muscle, ordinarily about 40 kDa and 6.45 units (Jaillon et al., 2004). M_r and pI values for *Merluccius capensis* and *Merluccius paradoxus* were 43 kDa, 6.5 units and 42 kDa, 6.55 units, respectively. These values were close enough to preclude species discrimination using only 2-DE, and further characterization of aldolase spots by mass spectrometry was needed.

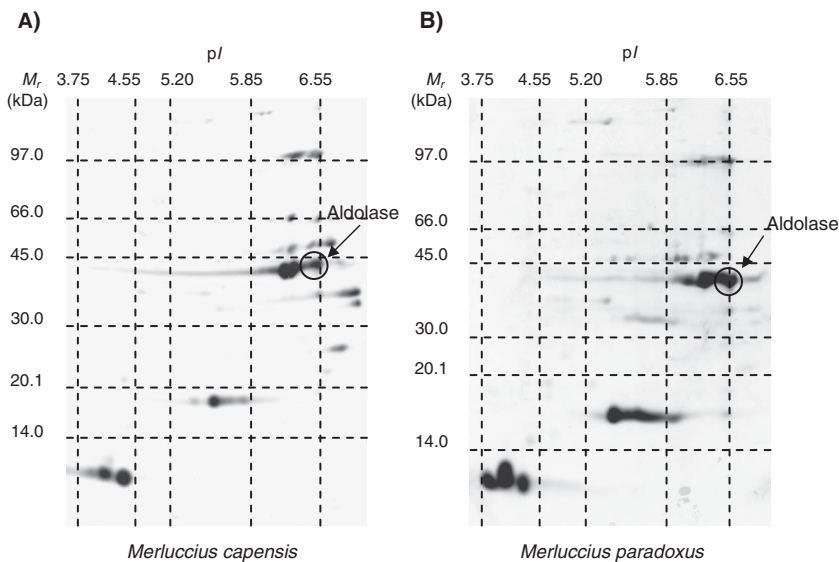


FIGURE 1 Two-dimensional gel electrophoresis maps of the sarcoplasmic fraction from *Merluccius capensis* (A) and *Merluccius paradoxus* (B) where the aldolase spots are indicated.

LC-MS/MS Analysis

Aldolase spots were digested *in situ* with trypsin, and LC-ESI-IT-MS/MS analysis was performed on the resulting digests. The spectra were analyzed using SEQUEST against the NCBI nr database. The produced amino acid sequences were considered as correct when XCorr values were higher than 2.5 for peptides with charge ≥ 3 , higher than 2 for peptides with charge 2, and higher than 1.5 for peptides with charge 1; ΔCn had to be equal or higher than 0.1 for all the peptides. Table 1 shows a compilation of 18 amino acid sequences that were considered to be correct using these parameters. Fifteen of them were exactly coincident with the aldolase A sequences from the teleostei *Tetraodon nigroviridis* (Q4RVI9_TETNG, UniProtKB; Jaillon et al., 2004), 2 with the aldolase A from teleostei *Danio rerio* (Q803Q7_DANRE, UniProtKB; Song et al., 2004), and 1 with the aldolase A from *Cephaloscyllium umbratile* (Q76BC5_9CH0N UniProtKB; Kikugawa et al., 2004). These results confirm that the protein spots analyzed correspond to the aldolase A isoform.

Several MS/MS spectra were subjected to *de novo* sequencing using the DeNovoX software (Table 2) with the results verified by manual interpretation of their respective fragment, b and y ions (Figure 2). The sequences obtained by both software and manual procedures are indicated in Table 2. The doubly-charged ion with m/z 834.42 corresponds to the peptide YTNQ-DIAMATVTALR, which is only present in the Cape hake, *Merluccius capensis*; 842.73 corresponds to a methionine residue oxidation. Conversely, the doubly-charged ion at m/z 849.52, corresponding to the sequence YTNQE-IAM*ATVTALR, was exclusively found in *Merluccius paradoxus*. A substitution of an Asp for a Glu in the fifth amino acid position in the sequence is responsible for the mass difference (Figure 2). This substitution may therefore be used as a criterion to differentiate individuals belonging to each of the species.

A comparative analysis was performed using all the aldolase entries present in the UniProtKB database. It could be proved, by homology searching using BLAST, that although all these sequences were highly homologous, the *de novo* sequenced species-specific peptides were unique and, consequently, usable as biomarkers. Figure 3 shows the aldolase amino acid sequences from both Cape hakes, aligned with the aldolase A from the teleostei *Tetraodon nigroviridis* (Q4RVI9_TETNG, UniProtKB), which presents the highest homology with those found in the present work: 32.2% with the sequence from *Merluccius capensis* and 25.0% with that from *Merluccius paradoxus*.

The potential use of aldolase A for identification purposes has been demonstrated in this work. In addition, the development of the specific antibodies for both peptides biomarkers provides a simple, fast, and straightforward method for the routine species discrimination of both species.

TABLE 1 Aldolase Peptide Sequences from *Merluccius capensis* and *Merluccius paradoxus* Identified by SEQUEST

m/z (z)	(M+H) ⁺	Peptide sequence	Protein reference	Missed ¹	Position (P') ²	C ³	p ³
530.68 (2+)	1059.58	KELSDIAQR	Aldolase, <i>Tetraodon nigroviridis</i> (Q4RV19_TETNG)	1	13–21		
466.53 (2+)	931.48	ELSDIAQR	Aldolase, <i>Tetraodon nigroviridis</i> (Q4RV19_TETNG)	0	14–21		
628.72 (3+)	1884.04	IVAPGKGILAADESTGSVAK	Aldolase, <i>Danio rerio</i> (Q803Q7_DANRE)	1	22–41		
943.12 (2+)	1884.04	IVAPGKGILAADESTGSVAK	Aldolase, <i>Danio rerio</i> (Q803Q7_DANRE)	1	22–41		
660.35 (2+)	1318.68	GILAADESTGSVAK	Aldolase, <i>Tetraodon nigroviridis</i> (Q4RV19_TETNG)	0	28–41		
440.26 (3+)	1318.68	GILAADESTGSVAK	Aldolase, <i>Tetraodon nigroviridis</i> (Q4RV19_TETNG)	0	28–41		
738.36 (2+)	1474.78	GILAADESTGSVAKR	Aldolase, <i>Tetraodon nigroviridis</i> (Q4RV19_TETNG)	1	28–41		
447.52 (2+)	893.42	DESTGSVAK	Aldolase, <i>Tetraodon nigroviridis</i> (Q4RV19_TETNG)	0	33–41		
933.06 (2+)	1863.90	RFQSINAENTENRR	Aldolase, <i>Tetraodon nigroviridis</i> (Q4RV19_TETNG)	2	42–56		
776.85 (2+)	1551.70	FQSINAENTENR	Aldolase, <i>Tetraodon nigroviridis</i> (Q4RV19_TETNG)	0	43–55		
854.95 (2+)	1707.80	FQSINAENTENRR	Aldolase, <i>Tetraodon nigroviridis</i> (Q4RV19_TETNG)	1	43–56		
540.21 (2+)	1078.55	QLLFTADDR	Aldolase, <i>Tetraodon nigroviridis</i> (Q4RV19_TETNG)	0	60–68		
352.52 (2+)	703.41	GMVVGIK	Aldolase, <i>Tetraodon nigroviridis</i> (Q4RV19_TETNG)	0	101–107		
649.30 (2+)	1296.76	LAILENANVLAR	Aldolase, <i>Tetraodon nigroviridis</i> (Q4RV19_TETNG)	0	160–171		
398.25 (2+)	795.44	VLAAMYK	Aldolase <i>Cephaloscyllium umbratile</i> (Q76BC5_9CH0N)	0	207–213		
848.52 (2+)	1695.90	ALSDHHVYLEGTLIK	Aldolase, <i>Tetraodon nigroviridis</i> (Q4RV19_TETNG)	0	214–228		
599.75 (2+)	1197.60	PWALTFSYGR	Aldolase, <i>Tetraodon nigroviridis</i> (Q4RV19_TETNG)	0	293–302		
401.52 (2+)	801.48	ALQASALK	Aldolase, <i>Tetraodon nigroviridis</i> (Q4RV19_TETNG)	0	303–310		

¹Missed: noncleavage sites.

²Position according to Schechter's nomenclature (Schechter and Bergen, 1967).

³C: *Merluccius capensis*; P: *Merluccius paradoxus*; (■) denotes the presence of a peptide and (□) the absence.

TABLE 2 Aldolase Peptide Sequences from *M. capensis* and *M. paradoxus* Identified by *de novo* Sequencing

<i>m/z</i> (z)	(M+H) ⁺	Peptide sequence				Position (P) ³	C ⁴	P ⁴
		DeNovoX (Relative probability) ¹	Manual interpretation	Missed ²				
834.42 (2+)	1667.84	[TY]LQDIAMATVTANR (24.2%)	YTN QDI AMATV T TALR	0	242–256			
842.73 (2+)	1683.83	[TY]LEDIAM*ATVTANR (11.2%)	YTN QDI AM*ATV T TALR	0	242–256			
849.52 (2+)	1697.85	[TY]NQEIAM*ATVTALR (28%)	YTN QEI AM*ATV T TALR	0	242–256			

¹] amino acid order indistinguishable; *oxidation.

²Missed: noncleavage sites.

³Position according to Schechter's nomenclature (Schechter and Bergen, 1967).

⁴C: *Merluccius capensis*; P: *Merluccius paradoxus*; (■) denotes the presence of a peptide and (□) the absence.

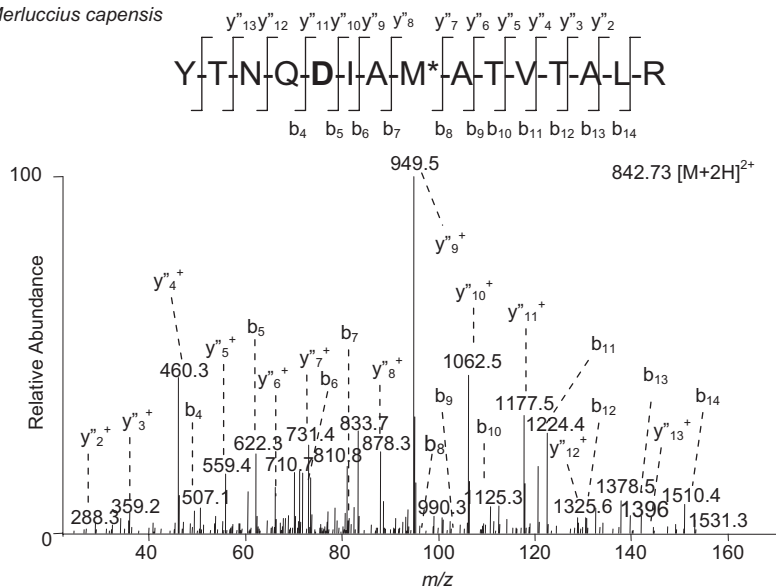
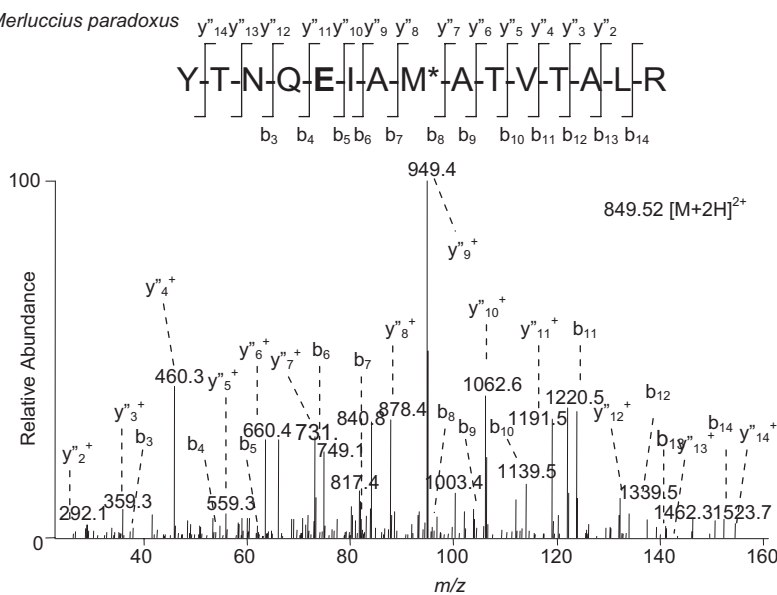
Merluccius capensis*Merluccius paradoxus*

FIGURE 2 *De novo* MS/MS sequencing of the differential peptides YTNQDIAM*ATVTALR, YTNQEIAM*ATVTALR, which are present in Aldolase A from *Merluccius capensis* and *Merluccius paradoxus*, respectively; *oxidation.

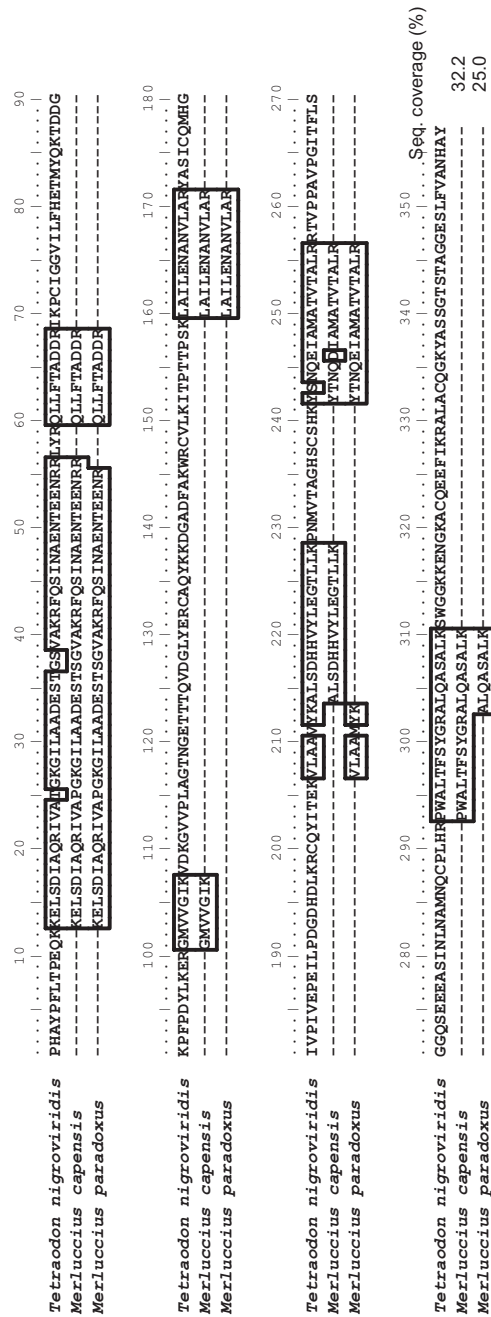


FIGURE 3 Alignment of aldolase amino acid sequences for both Cape hakes with the sequence from *Tetraodon nigroviridis* (accession number Q4RV19_TETNG, UniProtKB); (-) not determined amino acid.

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