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Characterization and partial sequencing of species-specific sarcoplasmic polypeptides from commercial hake species by mass spectrometry following two-dimensional electrophoresis

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Abbreviation: **NDKA**, nucleoside diphosphate kinase A

The *Merluccidae* family comprises marine species, some of them of high commercial value and others less appreciated, whose commercialization in Europe under the generic name of "hake" is highly remarkable. The potential of proteomics was employed in this study with the aim of achieving the differential characterization of five different hake species: *Merluccius merluccius* (European hake), *M. australis* (Southern hake), *M. hubbsi* (Argentinian hake), *M. gayi* (Chilean hake), and *M. capensis* (Cape hake), de Compostela, Escuela some of them very closely related. Species-specific polypeptides were observed for Politécnica Superior, the five hake species studied in isoelectric focusing (IEF) and/or two-dimensional elec-Laboratorio de Tecnología trophoresis (2-DE) high-resolution gels. The peptide mass maps of two polypeptide groups, previously

selected by 2-DE analysis as potentially species-specific, were obtained by “in-gel” tryptic digestion, followed by matrix assisted laser desorption/ ionization-time of flight-mass spectrometry (MALDI-TOF-MS). Analysis of group A polypeptides (with pI in the range of 5.0–5.5 and molecular mass of 17 kDa), allowed the differential classification of the hake species into two groups: the East Atlantic coast group and the West Atlantic coast group. Moreover, the peptide mass-maps from the heat-resistant parvalbumin fraction (pI below 4.5; molecular mass <12 kDa) allowed the detection of a peptide characteristic of *M. australi*s not present in the other four hake species tested. A specific 17 kDa protein from *M. merluccius* was also partially sequenced by nanospray-ion trap-tandem MS, revealing a high homology with rat nucleoside diphosphate kinase A (NDKA). This work opens the way to the application of proteomics to the differential characterization of commercial hake species at the molecular level.

Keywords: Proteomics / Two-dimensional electrophoresis / Mass spectrometry / Polypeptide / Characterization / Hake / Parvalbumin / Species identification / Nucleoside diphosphate kinase

1 **Introductio**n

Identification of fish species is gaining an increasing importance in the seafood industry because of the labelling regulations imposed by many countries all over the world [1–3]. The handling of fillets or minced fish as raw material – instead of whole fish specimens – is becoming increasingly important for the seafood industry. This fact complicates the identification of the fish species from which this material was taken, an issue that has traditionally been carried out on the basis of the analysis of the external anatomical and morphological features

In Spain, and in other European countries as well, the gadoid fish represent an important percentage of the overall fish consumption [4]. From the commercial point of view, some members of the genus *Merluccius* are more appreciated than others as fresh fish because of their excellent organoleptic features. Among them, *Merluccius merluccius*, commonly known as European hake, is the most appreciated species and deserves the highest commercial value in Spain, being preferred to other species of the genus *Merluccius* and to other gadoids.Additionally, species such as *M. capensis* deserve a

higher commercial value as frozen fish with respect to other *Merluccius* spp. New regulations concerning the labelling of fresh, cooked and canned seafood products have been recently introduced in Spain [5, 6]. These regulations do not allow the commercialization of several *Merluccius* spp. under the generic label of "hake", this making necessary the availability of robust tools able to distinguish among closely related hake species, not only for complying with such new regulations but also to prevent economical losses derived from the substitution of *M. merluccius* with other species of less commercial value.

With the aim of developing new strategies for the identification and characterization of fish species, powerful techniques based on biochemical methods are arising [7]. Several biochemical techniques have been applied to study and identify fish species of commercial interest in raw products. Among these, species identification based on protein analysis by isoelectric focusing (IEF) [8–10], polyacrylamide gel electrophoresis (PAGE) [8, 11–13], starch gel electrophoresis [14, 15], capillary electrophoresis (CE) [16], two-dimensional electrophoresis (2-DE) [17–19], and HPLC [20–22] has been carried out. During this decade, genomics have also been successfully applied to the identification of fish species [23–25], while proteomics have only but recently been considered to the identification of wild animals [26]. In this paper, we have applied for the first time the great robustness of proteomics to achieve the differential characterization of the sarcoplasmic protein fraction of fish species. The identification of species-specific proteins was carried out by combining proteomic tools such as matrix assisted laser-desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) and nanospray-ion trap-MS (nESI-ITMS) with electrophoretic techniques.

2 Material and methods

2.1 Fish material

Five different hake species (four specimens from each species) were employed in this study, these were: *Merluccius merluccius* (European hake), *M. australis* (Southern hake), *M. hubbsi* (Argentinian hake), *M. gayi* (Chilean hake), and *M. capensis* (Cape hake). Whole specimens were caught in the coast of Spain *(M. merluccius)*, South

America *(M. australis*, *M. hubbsi*, *and M. gayi*) or South Africa *(M. capensis*) and shipped in ice by overnight delivery. The whole fish specimens were identified by an expert marine biologist according to their external anatomical and morphological features.

2.2 Extraction of sarcoplasmic proteins

Samples of 5 g of white muscle were minced, mixed with bidistilled water (1:2) and homogenized by means of an Ultra-Turrax device. Afterwards, the mixture was centrifuged at 12 500 rpm/15 min in a JA20.1 rotor (J221-M centrifuge; Beckman, Palo Alto, CA) at 4ºC. The supernatants were then recovered, filtered, and maintained at –80ºC until the electrophoretic analyses were carried out.

2.3 First-dimensional electrophoresis by native IEF

Native IEF was carried out at 15ºC in a Multiphor II electrophoresis unit (Pharmacia Biotech Europe, Uppsala, Sweden) provided with a MultiTemp III refrigerated bath circulator (Pharmacia Biotech). Precast polyacrylamide 245x110x1 mm gels (Ampholine PAGplate pH 4.0-6.5; Pharmacia Biotech) for analytical IEF were employed. The anode solution was 0.1 M glutamic acid in 0.5 M phosphoric acid, and the cathode solution was 0.1 M ß-alanine. Ten to fifteen µg of total protein were loaded in each sample applicator paper. The running conditions were: 2000 V/25 mA/25 W until at least 4000 Vh were reached. First-dimensional IEF gels were stained with 0.1% Coomassie blue (Merck, Darmstadt, Germany) according to the Pharmacia Biotech staining protocol. A protein standard in the 2.5-6.5 pH range (low pI standard from Pharmacia Biotech) was included in the IEF gels. IEF strips corresponding to individual lanes were cut immediately after the running was completed for analysis in the second dimension by SDS-PAGE.

2.4 Second-dimensional electrophoresis by gradient SDS-PAGE

Equilibration of IEF gel strips was carried out at room temperature as follows. The strips were placed for 10 min in sample buffer [27] containing 0.75% dithiothreitol, and for another 10 min in sample buffer containing 4.5% iodoacetamide. Once equilibrated,

the strips were subjected to second-dimensional analysis by gradient SDS-PAGE at 15ºC in the Multiphor II electrophoresis system (Pharmacia Biotech). Precast polyacrylamide 245x180x 0.5 mm gradient gels (ExcelGel XL SDS 12-14; Pharmacia Biotech) for horizontal electrophoresis were employed. Anode and cathode buffer strips (ExcelGel SDS Buffer Strips; Pharmacia Biotech) were used. A low-molecular-mass protein standard (14–67kDa) was employed as reference. The running conditions were: 1000 V/40 mA/40 W, during 165 min. Once the bromophenol blue had reached the anode, the gels were fixed and stained by a standard silver staining protocol (Pharmacia Biotech).

2.5 Electrophoretic data processing

Mobility of the protein bands separated by IEF and SDS-PAGE was determined by using the Whole Band Analyser Software (BioImage Systems, Ann Arbor, MI, USA) in a Sun SPARCstation 5 (Sun Microsystems Inc.) equipped with a Scanmaster 3+ device (Howtek Inc.) and the corresponding pI and molecular mass calculated by comparing with the IEF and SDS-PAGE protein standards.

2.6 "In-gel" digestion of proteins, and extraction and preparation of peptides for MS analysis

Potential species-specific polypeptides (two groups for each species), as determined by 2-DE, were separated by conventional vertical SDS-PAGE (Hoefer System; Pharmacia Biotech) in polyacrylamide 20% homogeneous gels, using Tris-glycine as electrophoresis buffer [27]. With the aim of improving the tryptic digestion, and since Coomassie blue staining was in use, the protein extracts were previously mixed with Laemmli buffer (1:2). After Coomassie blue staining, the selected polypeptides were sliced from the gels and subjected to "*in situ*" tryptic digestion as described elsewhere [28]. A small aliquot (0.5 μL) of the extract was taken up and analyzed by MALDI-TOF-MS in order to obtain a peptide mass map. Gel extracts were pooled, dried down, resuspended in 10 μL of 0.1% tri-fluoroacetic acid (TFA) and subjected to automated microdesalting as previously described [29]. Briefly, the resuspended extracts were injected onto a RP-HPLC nanocolumn (Vydac C 18, 300 μm ID) at a flow of 3-5 μL/min using a Smart HPLC instrument with automatic fraction collection (Pharmacia

Biotech) equipped with a flow splitter and working at about 100 µL/min. Peptides were eluted in either a single step with methanol/ water (7:3) containing 0.1% formic acid, or applying a 10 min linear gradient and fractions of about 5 µL were collected. The fractions containing peptides were identified by MALDI-TOF-MS analysis of an 0.5 µL-aliquot of each fraction. The remainder of the fractions were used for nESI-IT-MS analysis.

2.7 Peptide mapping by MALDI-TOF-MS analysis

Analyses by MALDI-TOF-MS were performed using a Kompact Probe instrument (Kratos-Shimazdu, Manchester, UK), equipped with an extended flight tube of 1.7 m and delayed extraction, operating in linear mode. 0.5 µL of the fractions to be analysed were applied onto target and dried out. Then, 0.5 µL of saturated α-cyano-4-hydroxycinnamic acid matrix in water:acetonitrile (1:1) containing 0.1% TFA was added and dried out. Calibration was made externally by using a set of synthetic peptides.

2.8 Peptide sequencing by nESI-IT-MS

Analyses by nESI-IT MS were performed using an iontrap mass spectrometer model LCQ (Finnigan, Thermo-Quest, San Jose, CA, USA) equipped with a nanospray interface, exactly as we have described previously [30]. *De novo* sequencing of peptides not present in databases was performed by manual interpretation of the spectra. Multiple subfragmentation (MS^n) steps [30, 31] were performed in order to help determining the nature of the fragmentation series.

2.9 MS data processing

Fragmentation spectra from peptides present in data-bases were interpreted by using either the PepSearch program from the Bioworks package (ThermoQuest), or the SEQUEST program from the University of Washington, using SWISS-PROT, NR.FASTA or EST databases. Comparative analysis of peptide sequences were performed by using the programs FASTA and PILEUP from the University of

Washington.

3 Results and discussion

Protein profiles observed after the first-dimensional electrophoretic analysis by IEF revealed some differences among certain hake species (Fig. 1). Characteristic protein bands were observed either in the acidic protein fraction (pI below 4.5), this including proteins of the so-called parvalbumin group, and in the intermediate pH region (pI in the range of 5.0–5.5), especially in the case of European and Cape hakes. An apparently specific protein band with a pI value of 5.5 was observed in European hake and Cape hakes, while such protein band was not observed in the other three South American hakes (Fig. 1). In contrast, protein patterns in the cathodic region, corresponding to pI values above 6.0, resulted to be very similar in all the five hake species studied. Nevertheless, no detect able differences in the IEF protein profiles that could be helpful to distinguish European hake from Cape hake were observed (Fig. 1).

2-DE analysis (Fig. 2) of the sarcoplasmic proteins supported the results obtained by IEF (Fig. 1). However, 2-DE afforded much more valuable information with a view to achieve the differential characterization of hakes, especially in the case of closely-related species. Thus, the analysis of combined protein extracts from pairs of closely-related species had allowed to distinguish European hake from Cape hake [18]. The polypeptides that appeared to be apparently species-specific according to 2-DE were selected for further investigation by MS. The following polypeptide clusters were selected. Group A, polypeptides with pI in the range of 5.0–5.5 and 17 kDa of molecular mass; group B, the parvalbumin protein fraction, which comprises the acidic group of polypeptides (pI below 4.5) and low molecular mass (below 12 kDa) (Fig. 2).

Once the potential species-specific polypeptides had been selected by 2-DE, the protein fractions of 17 kDa (group A) and below 12 kDa (group B) were recovered in a simple and rapid manner from conventional vertical SDS-polyacrylamide preparative gels. In-gel tryptic digestion of the selected bands was carried out prior to molecular analysis by MS. As it can be seen in Fig. 3A and B, the peptide maps obtained after tryptic digestion and MALDI-TOF-MS analysis revealed significant differences among the five hake species. In the analysis of the proteins of the group A (with pI in the range of 5.0–

5.5 and 17 kDa of molecular mass), MALDI-TOF-MS was able to differentiate between the hakes from the East Atlantic coast (European hake and Cape hake) and those from the Western Atlantic coasts (Chilean hake, Argentinian hake, and Southern hake) (Fig. 3A). Thus, MALDI-TOF-MS analysis of group A polypeptides previously characterized by 2-DE allowed the differential classification of the five hake species tested into two groups. This finding confirmed the results obtained for these species with other techniques such as specific enzyme staining [32] and mitochondrial DNA analysis [24].

As stated above, peptide mass maps from the parvalbumin protein group (group B polypeptides) from each of the five hake species were also obtained by MALDI-TOF-MS. The results clearly indicated that only the Southern hake species could be distinguished from the other four species by this technique (Fig. 3B). A comparative analysis of the 2-DE patterns had revealed species-specific profiles in the group B polypeptides for all the five hake species tested, although the pI and molecular mass of the three parvalbumins present in European hake and Cape hake were almost identical. Surprisingly, the analysis of the group B polypeptides by MALDI-TOF-MS did not reveal significant differences among four of the five hake species tested. In contrast, this technique was useful in the identification of a specific peptide in Southern hake, a result that may have a significant applied interest for the molecular identification of this species with respect to the other commercial hake species tested.

Since MALDI-TOF-MS analysis of the group B polypeptides -parvalbumin fraction yielded little information valuable for differentiation among hake species – except for Southern hake – a further investigation of the polypeptide bands belonging to the group A was undertaken. Thus, the tryptic digest containing the crude peptide mixtures from group A polypeptides from European hake was desalted and subjected to additional analysis by nESI-IT-MS. As exemplified in Fig. 4, some of the peptides were fragmented, obtaining tandem mass spectra; these MS/MS spectra were then used for database searching using Pepsearch or SEQUEST programs. However, none of the spectra from group A peptides gave a significant database match, indicating that their sequence was not present in the databases. The fragment spectra were then manually interpreted (Fig. 4); in some cases, ambiguous sequence interpretations were resolved by performing multiple subfragmentation (MS^n spectra) (data not shown).

As it can be seen in Fig. 4 for European hake, this allowed us to interpret satisfactorily the fragmentation series in spectra from three peptides; the obtained sequences are depicted in Table 1. These sequences were subjected to FASTA database homology searching. As shown in Table 1, the obtained sequences showed a significant sequence homology (90–100% sequence identity in 9–11 adjacent amino acids) with nucleoside diphosphate kinase A (NDKA) from rat (EC 2.7.4.6., SWISS-PROT Q05982) (Table 1), and from a large number of other species (data not shown), indicating that the group A poly-peptides are the hake homologues of rat NDKA. For further consistence, we have also subjected to MS/MS sequencing some peptides from the other hake species having the same nominal mass than those in Table 1; the observed MS/MS spectra were essentially identical to those obtained from European hake peptides, indicating that they had the same sequence. NDK enzymes are required for the synthesis of nucleoside triphosphates (NTPs) other than ATP. Such enzymes provide NTPs for the nucleic acid biosynthesis, CTP for the synthesis of lipids, UTP for polysaccharide synthesis and GTP for protein elongation, signal transduction and microtubule polymerization. NDK enzymes are also known as metastasis inhibition factor nm23 [33]. In the case of rat, the decreased expression of the NDKa isoform is associated with metastasic potential of mammary-adenocarcinoma cells [33]. NDK from rat has a length of 152 amino acids and a molecular mass of 17 293 Da [34].

The potential use of NDKA and certain parvalbumins of hake for identification purposes has been introduced in this work. With this objective in mind, additional investigations based on the amino acid sequence of specific peptides from the hake homologue of NDKA and selected parvalbumins are currently being developed at our laboratory by MS, with the aim of developing a fast and sensitive method for the characterization and identification of commercial hake species. This work opens the way to the application of proteomic methods to fish species and seafood products for both identification and characterization purposes. Thus, and unlike the cases of milk and egg proteins, fish proteins have seldom been studied using MS by food researches up to now [35].

4 Concluding remarks

The combination of 2-DE analysis, MALDI-TOF-MS and nESI-TI-MS has been applied to the differential characterization of species specific polypeptides from commercial hakes. MALDI-TOF-MS was revealed as a useful technique for the specific detection of *M. australis*; this approach may be of applied interest in the specific identification of this species in light-heated seafood products, since the species-specific peptide map described in this work was obtained from the heat-stable protein group, the so-called parvalbumin fraction. A 17 kDa novel hake protein, potentially useful as a molecular marker for a rapid and specific differentiation between the five commercial hake species, has also been characterized. Thus, nESI-IT-MS allowed the sequencing of some peptides derived from the protein isolated from European hake, which was then identified as the hake homologue of NDKA.

In summary, the combination of high-resolution 2-DE and MS, which provides the basis for the emerging technology of proteomics, proved to be a useful, reproducible and sensitive strategy for the molecular characterization of peptides in fish products for both identification and characterization purposes. However, proteomic techniques should be automated in order to achieve rapid and reproducible results, thus making these techniques useful for the high-throughput analysis of foodstuffs.

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5 References

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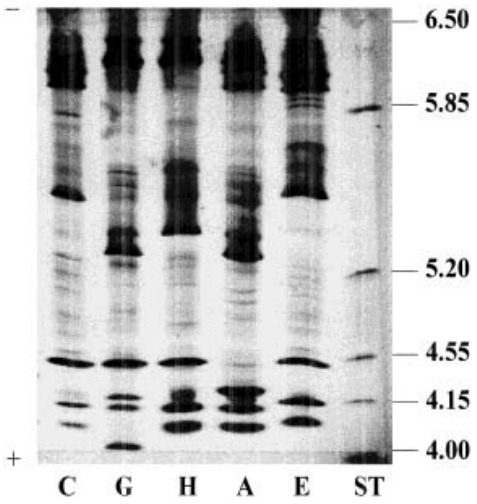


Figure **1**. IEF gel (pH range 4–6.5) showing the sarcoplasmic protein extracts corresponding to the following hake species. (C) *M. capensis*; (G) *M. gayi*; (H) *M. hubbsi*; (A) *M. australis*; (E) *M. merluccius*; (ST) low pI marker.

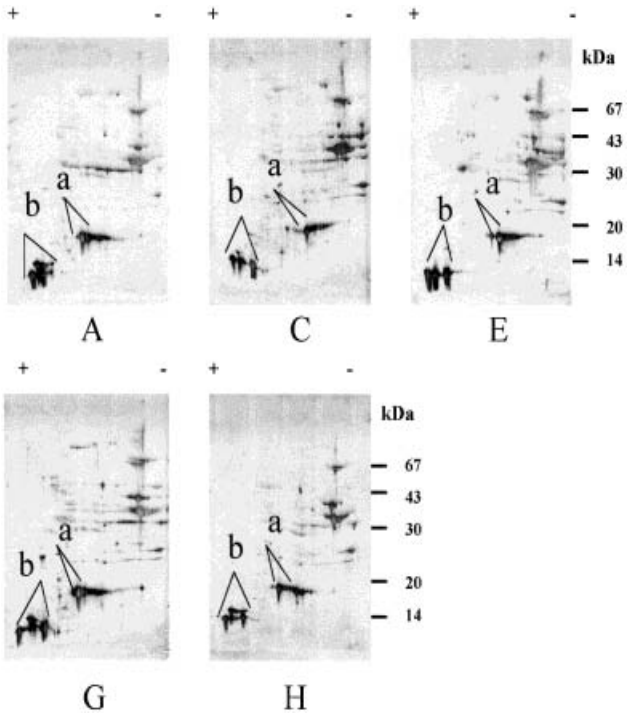
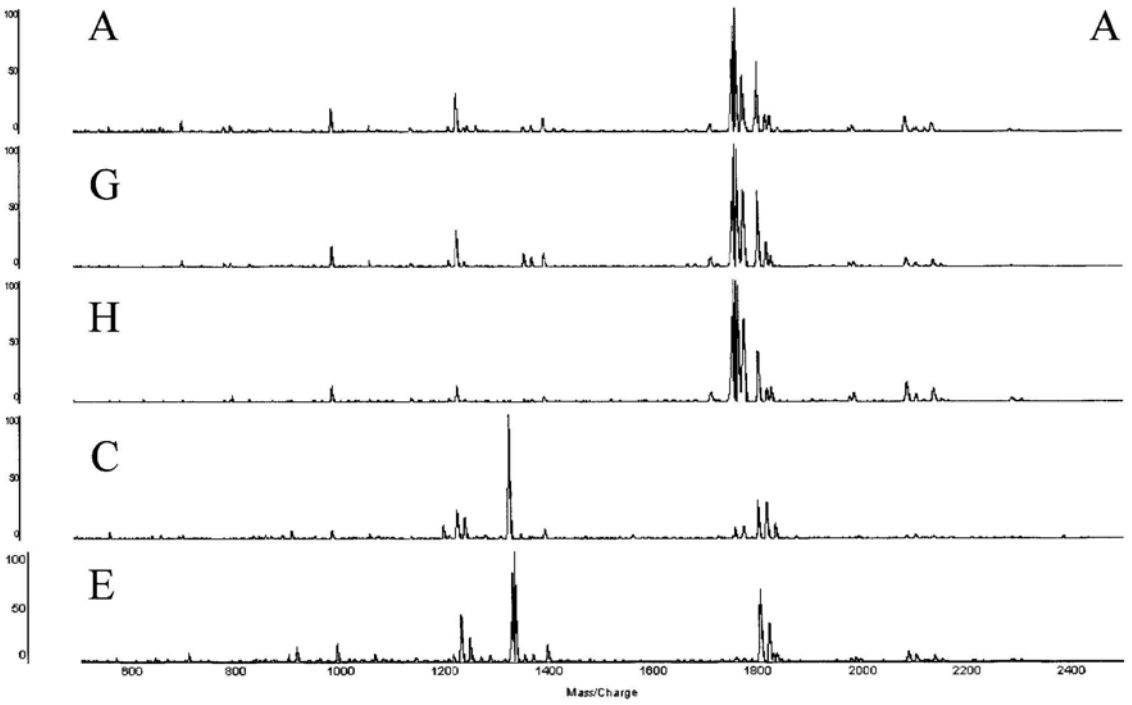


Figure 2. 2-DE patterns of the five hake species tested. (A) *M. australis,* (C) *M. capensis;* (E) *M. merluccius;* (G) *M. gayi; (H) M. hubbsi*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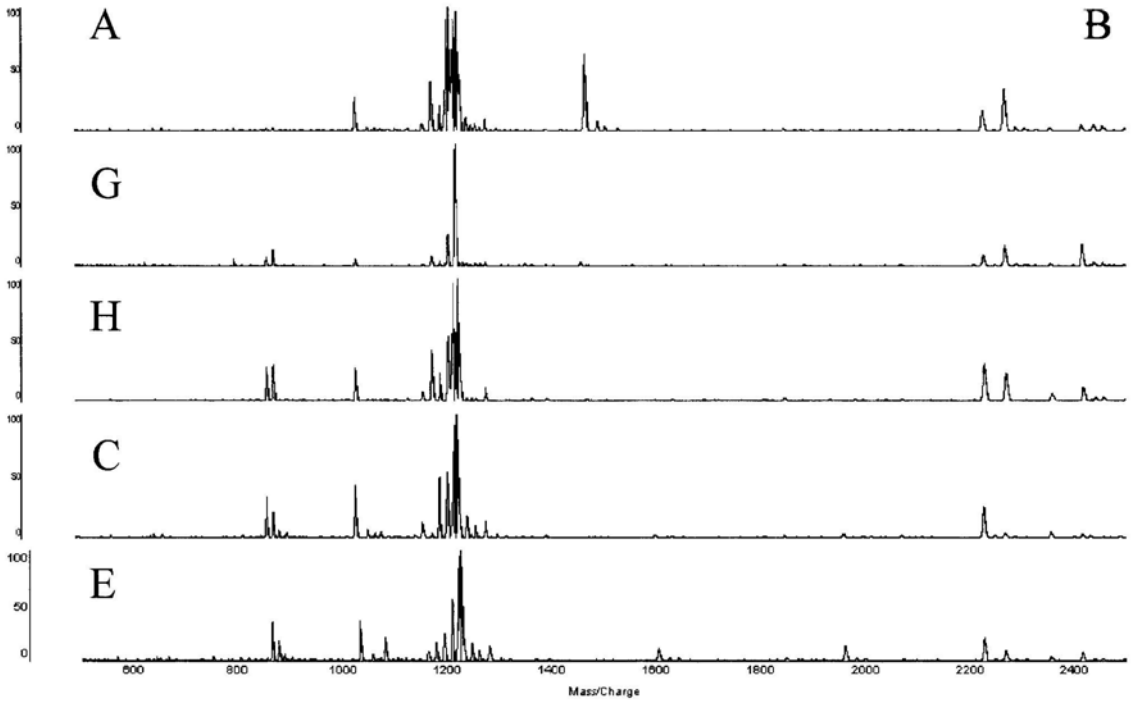
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Figure **3**. Peptide mass maps obtained by MALDI-TOF-MS analysis of(A) group A proteins (pl ~ 5.5, 17 kDa molecular mass); (B) group B polypeptides (parvalbumin fraction). (A) *M. australis*; (G) *M. gayi*; (H) *M. hubbsi*; (C) *M. capensis*; (E) *M. merluccius*.

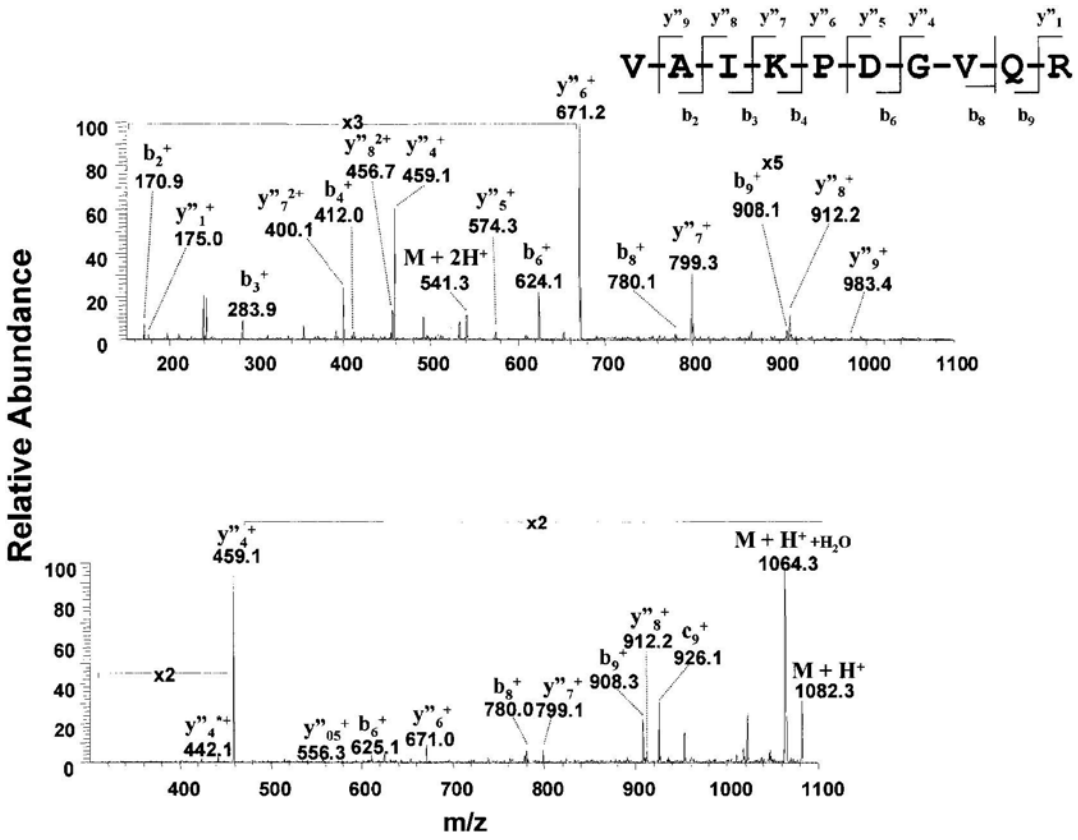

Figure **4**. nESI-IT-MS/MS spectra from the fragmentation of one of the peptides obtained after tryptic digestion of group A polypeptides from European hake (*M. merluccius)*. Upper panel, fragmentation of the doubly-charged ion; lower panel, fragmentation of the singly-charged ion. The manually interpreted sequence, as well as the assignment of peaks belonging to the different fragmentation series is indicated in standard notation. Note that amino acids I and L, and amino acids K and Q cannot be differentiated by this method. K in position 4 and Q in position 9 were tentatively assumed since the other possibility was never found after sequence identity analysis with the homolog peptide from all NDK proteins present in databases. Similarly, I was present in position 3 in the peptide from 32 different NDK sequences, per only 3 cases of L in this position.

Table 1. Sequence of peptides from protein A of European hake (*M. merluccius*) and identity with rat NDKA

Peptide sequence[a)]	VAIKPDGVQR (1083)[b]
	|||||||||
Rat NDKA	..FIAIKPDGVQRG..
Petide sequence[a)]	MMIGETNPADSKPGSIR (1805)[b]
	|:||||||||||:||
Rat NDKA	..RVMLGETNPADSKPGTIRG..
Peptide sequence[a)]	NIIHGSDTVENA K (1398)[b]
	||||||:|| | |
Rat NDKA	..RNIIHGSDSVESAEKE..

a) Determined by nESI-IT-MS. For simplicity, the assumptions stated in legend to Fig. 4 have been followed.

b) b) Average mass ($M + H^+$) determined by MALDI-TOF-MS