

Analytical, Nutritional and Clinical Methods Section

# Specific enzyme detection following isoelectric focusing as a complimentary tool for the differentiation of related Gadoid fish species

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Received 8 August 1999; received in revised form 1 December 1999; accepted 1 December 1999

## Abstract

The presence of specific enzymes in the sarcoplasmic protein fraction of eight gadoid fish species has been investigated following isoelectric focusing (IEF) in the 3.5–9.5 pH range. Lactate dehydrogenase (LDH) and glycerol-3-phosphate-dehydrogenase (G-3-PD) allowed the differentiation of most of the gadoids tested, even in the case of closely related species belonging to the genus *Merluccius*. Investigation of arginine kinase and creatine kinase afforded non-specific enzyme patterns in the species tested. Adenylate kinase and malate dehydrogenase did not give specific or reproducible enzyme patterns. Analysis of LDH and G-3-PD activities in commercial IEF gels was shown to be a reliable and reproducible technique for distinguishing closely related *Merluccius* spp. and other gadoid fish species. © 2000 Elsevier Science Ltd. All rights reserved.

**Keywords:** Gadoid fish species identification; Enzyme detection; Isoelectric focusing

## 1. Introduction

Several biochemical techniques have been applied to study and identify fish species of commercial interest in raw products. Among these, protein analysis by isoelectric focusing (IEF) (An, Wei, Zhao, Marshall & Lee, 1989; Mackie, 1980; Rehbein, 1990), polyacrylamide gel electrophoresis (PAGE) (Mackie, 1980; Mackie & Jones, 1978; Piñeiro, Barros-Velázquez, Pérez-Martín et al., 1999; Scobbie & Mackie, 1988), starch gel electrophoresis (Gabriel & Gersten, 1992; Keenan & Shaklee, 1985), two-dimensional (2-D) gel electrophoresis (Martínez, Solberg, Lauritzen & Ofstad, 1992; Piñeiro, Barros-Velázquez, Sotelo, Pérez-Martín & Gallardo, 1998; Piñeiro, Barros-Velázquez, Sotelo & Gallardo, 1999), HPLC (Armstrong, Leach & Wyllie, 1992; Osman, Ashoor & Marsh, 1987; Piñeiro, Sotelo, Medina,

Gallardo & Pérez-Martín, 1997) and DNA-based techniques (Barlett & Davidson, 1991) have been considered, although some of them are time-consuming and require specialized personnel.

Specific protein staining methods for certain seafood products using starch gel electrophoresis or PAGE have been reported (Redfield & Salini, 1980; Shaklee & Keenan, 1986) for inspection and control purposes. However, most reports on enzyme detection in raw fish have been aimed at studying the population genetics of selected taxa, both for phylogenetic and systematic purposes (Bischoff, Shi & Kennelly, 1998; Grant, Becker & Leslie, 1988; Shaklee, Allendorf, Morizot & Whitt, 1990). Thus, little attention has been paid to the application of specific enzyme staining as a tool for the identification of related fish species of commercial value, from the point of view of seafood inspection and analysis. The present study was undertaken to investigate the presence of specific enzymes in the sarcoplasmic protein fraction of selected gadoid fish species separated by IEF, and to evaluate the accuracy, reproducibility and reliability of such experimental protocols to achieve species-specific patterns and thereby facilitate identification of species in seafood products.

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## 2. Materials and methods

### 2.1. Fish material

Eight gadoid fish species (four specimens from each): European hake (*Merluccius merluccius*), Southern hake (*Merluccius australis*), Argentinian hake (*Merluccius hubbsi*), Chilean hake (*Merluccius gayi*) and Cape hake (*Merluccius capensis*), cod (*Gadus morhua*), pollack (*Pollachius pollachius*) and blue whiting (*Micromesistius poutassou*) were investigated. Specimens of *M. merluccius*, *P. pollachius*, *M. poutassou* (all of them caught near the Galician coast, Northwestern Spain) and *G. morhua* (from Gran Sol fishing bank) were purchased as fresh fish at a local market, frozen in the laboratory and stored at  $-30^{\circ}\text{C}$  until processing. *M. australis*, *M. gayi* (caught off the coast of Chile), *M. hubbsi* (caught off the coast of Argentina) and *M. capensis* (caught off the coast of South Africa), were shipped on ice by overnight delivery, frozen at the laboratory and stored at  $-30^{\circ}\text{C}$  until processed. The weight of the specimens were in the 3–6 kg range. The whole specimens were classified before freezing, by means of anatomical and morphological analyses.

### 2.2. Preparation of protein extracts

Samples of raw white muscle were dissected from each specimen, and portions of 5 g minced and homogenized with 10 ml of twice-distilled water for 3 min at 9000 rpm with an Ultraturrax homogenizer. The mixtures were spun at 12,500g for 15 min at  $4^{\circ}\text{C}$ , and the supernatants recovered by gentle pipetting, filtered and stored at  $-80^{\circ}\text{C}$  until required for processing.

### 2.3. Reagents

Lactate dehydrogenase (LDH), glycerol-3-phosphate-dehydrogenase (G-3-PD), adenylate kinase (AK), malate dehydrogenase (MDH), arginine kinase (ARGK) and creatine kinase (CK) protein standards were purchased from Sigma-Aldrich (St. Louis, MO). Coomassie Blue R-250, reagents used for specific enzyme staining and both LDH and CK specific staining kits were also purchased from Sigma-Aldrich.

### 2.4. Isoelectric focusing and general protein staining

A Multiphor II electrophoresis unit provided with a MultiTemp III refrigerated bath circulator (Amersham Pharmacia Biotech, Uppsala, Sweden) was employed in the IEF studies. Precast polyacrylamide gels in the 3.5–9.5 pH range (Ampholine PAGplate, Amersham Pharmacia Biotech) for analytical IEF were used. The anode solution was 0.1 M phosphoric acid and the cathode solution was 0.1 M sodium hydroxide. Sample

application papers were loaded with 10–15  $\mu\text{g}$  protein. Each gel was loaded with two equal sets of samples: one set was destined for general protein staining with Coomassie Blue while the other set was subjected to specific enzyme staining. Technical conditions were as follows: 1500 V/50 mA/30 W until 4000 V·h were reached. IEF gels that were not destined for specific enzyme staining were stained with 0.1% Coomassie Blue R-250 according to the Amersham Pharmacia Biotech procedure. A protein standard in the 3–10 pH range (Broad pI Standard, Amersham Pharmacia Biotech) was included in the IEF gels, and consisted of: amyloglucosidase (pI: 3.50), methyl red (pI: 3.75), soybean trypsin inhibitor (pI: 4.55),  $\beta$ -lactoglobulin A (pI: 5.20), bovine carbonic anhydrase B (pI: 5.85), human carbonic anhydrase B (pI: 6.55), horse myoglobin-acidic band (pI: 6.85), horse myoglobin-basic band (pI: 7.35), lentil lectin-acidic band (pI: 8.15), lentil lectin-middle band (pI: 8.45), lentil lectin-basic band (pI: 8.65) and trypsinogen (pI: 9.30).

### 2.5. Specific enzyme staining

IEF gels destined for enzyme staining were processed immediately after IEF was completed. All the enzyme staining experiments were done in triplicate. Six enzymes were investigated, with a view to evaluating their potential usefulness for species identification. Thus, LDH, AK, G-3-PD, MDH, ARGK and CK were evaluated in five species in a preliminary phase of this research.

The detection of LDH activity was conducted by means of a commercial kit from Sigma-Aldrich, following the manufacturer's instructions. This protocol involved a three-step reaction based on the specific oxidation of L(+) lactate to pyruvate and subsequent stoichiometric reduction of trinitrobluetetrazolium (TNBT) to its highly-coloured and insoluble reduced formazan, which was localized in the electrophoretic zones of LDH activity.

G-3-PD was detected according to Manchenko (1994). This involved a three-step reaction based on the specific oxidation of glycerol-3-P to glycerone-P and stoichiometric reduction of TNBT to its formazan derivative. The detection of AK and MDH was carried out according to Manchenko, while the investigation of ARGK and CK was carried out according to Shaklee and Keenan (1986), and by using a commercial kit (Sigma-Aldrich), respectively. LDH, G-3-PD, AK, MDH, ARGK and CK enzyme standards were included in the IEF gels as positive controls. The mixture of proteins used as pI markers was used as a negative control in the specific staining protocols.

The pI values of the enzymes detected were determined by comparison with the IEF protein standards using the Whole Band Analyzer software (BioImage

Systems Corp., MI) in a Sun SPARC station 5 (Sun Microsystems Inc) equipped with a Scanmaster 3+ device (Howtek Inc., NH).

### 3. Results and discussion

#### 3.1. IEF of sarcoplasmic proteins and selection of specific enzymes

The major water-soluble proteins of the fish species tested were successfully separated by IEF analysis in the 3.5–9.5 pH range. Fig. 1 shows the protein profiles obtained for all the eight gadoids analyzed, after a general protein staining step with Coomassie Blue. The homogeneity and reproducibility of these IEF profiles has been checked in our laboratory by conducting multiple analyses with different specimens belonging to each species. These results allowed us to obtain reproducible and characteristic protein profiles in the eight gadoid fish species tested. In this sense — and from the point of view of seafood inspection and control — the profiles obtained by IEF are expected to be less affected — and, in consequence, more reproducible — by intra-specific phenotypic variability that might result from a mutation in the DNA encoding for certain proteins. Thus, single nucleotide substitution, deletion or addition mutations would not result in a detectable change in the pI of the proteins, but, by contrast, would rather imply subtle charge changes that might affect the protein profiles obtained by native gel electrophoresis (Keenan & Shaklee, 1985; Ramshaw, Coyne & Lewontin, 1979). Thus, from the point of view of the seafood inspection and control, IEF profiles — either obtained by general

protein staining or by specific enzyme staining — might afford a higher reproducibility, not being so affected by possible protein polymorphisms.

Duplicates of the IEF gels, run in parallel to those destined for Coomassie Blue-staining, were subjected to specific enzyme detection. As stated above, six different enzymes — LDH, AK, G-3-PD, MDH, ARGK and CK — were investigated in five fish species: *M. merluccius*, *M. australis*, *M. gayi*, *P. pollachius* and *G. morhua* as a preliminary study. ARGK (Fig. 2a) and CK (Fig. 2b) staining protocols proved to be non-specific, since each of the fish species tested showed identical enzyme patterns under the technical conditions used in this work. Thus, both staining protocols did not seem to be specific for the substrate (creatine or arginine) and they rather afforded a non-specific kinase pattern in the fish species tested.

Specific staining of AK activity also yielded non species-specific profiles in the species tested (data not shown). The detection of AK was tedious because of the need to pour an agar overlay containing the substrate and co-factors of the enzymatic reaction, followed by the visualization of AK bands under UV light. Although King (1984) had reported the recovery of adenylate kinase and creatine kinase activities from cooked meat products by guanidine hydrochloride extraction and that the investigation of these enzymes following IEF separation allowed the differentiation of certain cooked meats, we did not find a similar result in the raw gadoid fish species examined in this work.

The investigation of MDH in the IEF gels resulted in a significant background, which complicated the detection of this enzyme in the fish species tested. In an effort to improve the results obtained with this MDH staining protocol, a post-coupling technique consisting of delaying the addition of Fast Garnet GBC, a reagent used for

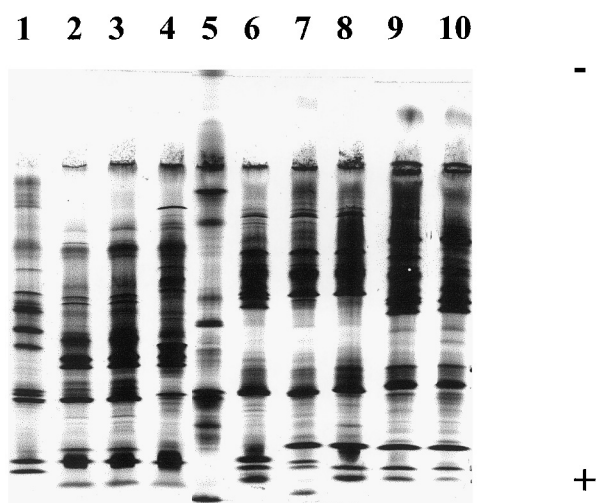


Fig. 1. Sarcoplasmic protein patterns obtained by IEF after Coomassie Blue staining. Lane 1: cod; lane 2: pollack; lane 3: pollack; lane 4: blue whiting; lane 5: pI protein standards; lane 6: Southern hake; lane 7: Chilean hake; lane 8: Argentinian hake; lane 9: Cape hake; lane 10: European hake.

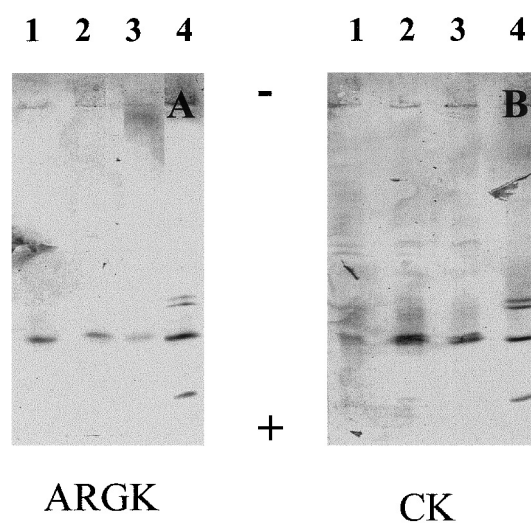


Fig. 2. Enzyme staining for ARGK (A) and CK (B). Lane 1: European hake; lane 2: Southern hake; lane 3: Chilean hake; lane 4: pollack.

the detection of MDH, was considered, as suggested by Manchenko (1994). This modification did not give good results as it did not prevent the apparent precipitation of the dye. As a result a strong brown background which complicated the detection of MDH activity was obtained (data not shown). By contrast, LDH and G-3-PD staining protocols proved to be specific and reproducible. Accordingly, the latter two enzymes were selected for further investigation in all the eight gadoid fish species.

### 3.2. Investigation of LDH and G-3-PD activities

Fig. 3 displays the specific detection of LDH activities in the eight gadoid fish species tested. All the species yielded reproducible and characteristic LDH patterns. The five hakes analyzed presented LDH patterns that were more similar among themselves than with respect to pollack, cod and blue whiting. The pH region in the 5–6 range proved to be specific in all the species tested (Fig. 3). LDH staining also allowed the classification of the five hakes tested in two groups: (i) *M. merluccius* and *M. capensis* (Fig. 3, group 1); and (ii) *M. hubbsi*, *M. gayi* and *M. australis* (Fig. 3, group 2). Thus, this staining technique allowed us to distinguish both the European hake (*M. merluccius*) and Cape hake (*M. capensis*) from those belonging to the South American fishing banks (*M. hubbsi*, *M. gayi* and *M. australis*), a result that agrees with those reported by Quinteiro, Vidal and Rey-Méndez (in press) and Piñeiro et al. (1998) using DNA-based techniques and 2-D electrophoresis, respectively. In this sense, certain bands (indicated with arrows in Fig. 3) proved to afford valuable information with a view to distinguish among the hakes belonging to each of the above-cited groups. Mackie &

Jones (1978) had investigated LDH activity for identification purposes in closely related species of hake, two of which – *M. merluccius* and *M. australis* – were also considered in this work. However, these authors, when using polyacrylamide disc gel electrophoresis previous to specific LDH detection, did not find significant differences between both species which did not allow the differentiation of both hakes.

Investigation of G-3-PD in the eight species tested also proved to afford valuable information concerning species identification (Fig. 4). Thus — and as it was also observed for LDH — this specific enzyme staining yielded reproducible and well-resolved patterns. The five hakes were easily distinguished from cod, blue whiting and pollack according to their G-3-PD enzyme profiles. *M. merluccius* and *M. capensis* showed almost identical G-3-PD patterns, which complicated the differentiation of these closely-related hakes only based on their G-3-PD profiles (Fig. 4). By contrast, *M. australis* showed species-specific G-3-PD patterns that allowed the differentiation of this species with respect to the closely related *M. hubbsi* and *M. gayi*. The characteristic G-3-PD bands obtained for the two groups of hakes (on one hand, European and Cape hake, and, on the other hand, South American hakes) are highlighted with brackets (Fig. 4).

Keenan and Shaklee (1985) investigated — by enzyme staining following horizontal starch electrophoresis and PAGE — the electrophoretic mobilities of seven enzymes — among them, AK, CK-A<sub>2</sub> and LDH-A<sub>4</sub> — in 23 species of fishes. These authors reported that the combination of a general protein staining of polyacrylamide gels together with specific enzyme staining following starch gel electrophoresis — when used together — resulted in an extremely powerful tool for the differentiation of certain fish species. However, this study was aimed at the identification of fish fillets

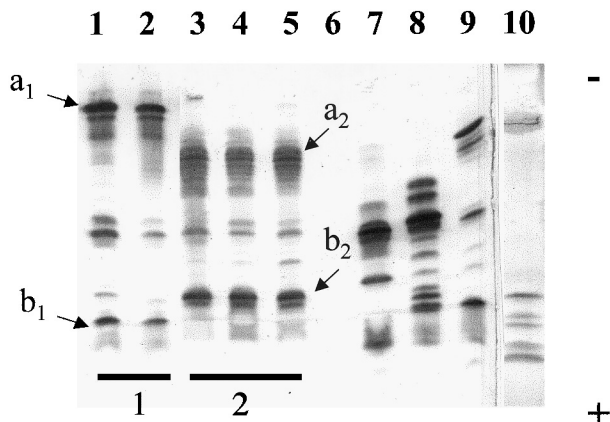


Fig. 3. Specific enzyme staining for LDH. Lane 1: European hake; lane 2: Cape hake; lane 3: Argentinian hake; lane 4: Chilean hake; lane 5: Southern hake; lane 6: pI protein standards; lane 7: pollack; lane 8: cod; lane 9: blue whiting; lane 10: LDH standard. Groups 1 (European hake and Cape hake) and 2 (South American hakes) are indicated, and specific bands of each group highlighted.

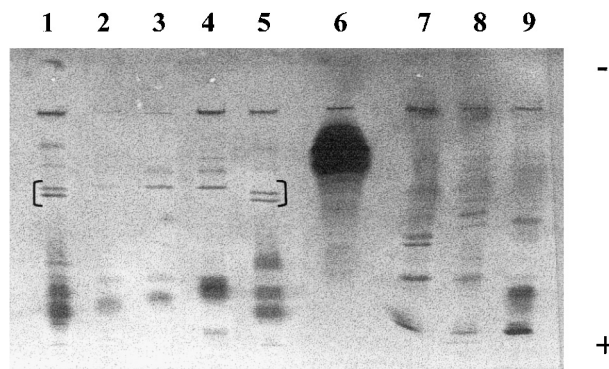


Fig. 4. Specific enzyme staining for G-3-PD. Lane 1: European hake; lane 2: Southern hake; lane 3: Chilean hake; lane 4: Argentinian hake; lane 5: Cape hake; lane 6: G-3-PD standard; lane 7: pollack; lane 8: cod; lane 9: blue whiting. Characteristic bands for the differentiation of European and Cape hakes (lanes 1 and 5) from South American hakes (lanes 2–4) are highlighted between brackets.

obtained from fish belonging to taxa not related to the species considered in the earlier study.

#### 4. Conclusion

The results show that specific staining of LDH and G-3-PD activities — following IEF in commercial gels in the 3.5–9.5 pH range — of the water-soluble protein extracts, constituted an auxiliary tool for identification purposes in the eight gadoid fish species tested, even allowing the differentiation among closely related species of the genus *Merluccius*. Thus, when applied to the raw gadoid fish species tested, specific staining for selected enzymes is revealed as a fast, simple and sensitive technique which proved to be a reproducible method for identification purposes.

#### Acknowledgements

The authors thank the financial support of CICYT (Project ALI95-0053). The authors also thank Helena Pazó for her excellent technical assistance.

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