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Identification of freshwater fish commercially labelled "perch" by isoelectric focusing and two-dimensional electrophoresis

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Abstract

Isoelectric focusing (IEF) and two-dimensional electrophoresis (2-DE) were used to distinguish four freshwater fish species which are sold under the generic label of "perch": *Perca fluviatilis* (European perch), *Lates niloticus* (Nile perch), *Stizostedion lucioperca* (European pikeperch) and *Morone chrysops x saxatilis* (sunshine bass). These species have different commercial values but are easily "interchangeable" because they are sold already filleted, in view of the numerous bones of the whole fish. IEF of the water-soluble proteins extracted from fish muscle resolved in species-specific patterns. Intra-species polymorphism was low, and did not concern the bands identified as characteristic of the species. As well, 2-DE maps showed numerous species-specific protein spots. Interestingly, while none of the IEF bands was common to all four species, several major 2-DE spots were similar. Therefore, IEF of water soluble sarcoplasmic proteins is sufficient to unambiguously discriminate among the four species considered. Analysis by 2-DE, which has a higher resolution power but it is more expensive and time consuming, may be applied to obtain further knowledge of the proteome of poorly characterized species.

Keywords: IEF; 2-DE; Fish species; Perch; Identification

1. Introduction

Numerous freshwater fish species are sold daily under the generic label of "perch". These are: (1) the European perch (*Perca fluviatilis*), which lives in European freshwaters, with the exception of southern Italy, Spain and Portugal, and was introduced to Australia and New Zealand from Europe around 1860; (2) the Nile perch (*Lates niloticus*), which is one of the world's largest freshwater fish; it is native to the River Nile but now also lives in Lake Victoria; (3) the European pikeperch (*Stizostedion lucioperca*), which lives in the North-East of Europe; (4) the sunshine bass (*Morone chrysops x saxatilis*), which is a hybrid between the Striped bass (*Morone saxatilis*) and White bass (*Morone chrysops*) and is bred in USA and now also in Europe. These fish have significantly different commercial values, the European perch being the most appreciated by consumers and commanding the highest prices.

All these species are mostly sold as fillets, because the whole fish contain numerous bones. Filleted fish does not retain the anatomical features of the whole fish, thus complicating identification.

In an effort to overcome fraudulent practices, new regulations concerning the labelling of seafood products have been recently introduced in Europe. There is therefore a compelling need to identify these species unambiguously. The generic label "perch" could make for confusion among consumers and increase the possibility of commercial fraud.

Several biochemical methods have been applied to identify fish species of commercial interest. Most are

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based on sarcoplasmic protein analysis by electrophoretic methods (Lundstrom, 1979; Carpenè, Hakim, & Cortesi, 1983; Rehbein, 1990; Pineiro et al., 1999), HPLC (Knuutinen & Harjula, 1998), or enzyme immunoassay (Cespedes et al., 1999a; Asensio et al., 2003). Methods based on DNA amplification by the polymerase chain reaction have also been used to identify species (Cespedes et al., 1999b; Rehbein et al., 1999; Martinez, Friis, & Seppola, 2001).

Among the electrophoretic methods, isoelectric focusing (IEF) has been successfully applied on either raw products (Lundstrom, 1979; Weaver, Lundstrom, & Colbert, 1999; Bossier & Cooreman, 2000; Tepedino et al., 2001; Chen, Shiau, Noguchi, Wei, & Wwang, 2003) or heat-processed or smoked fish (Hsieh, Chien, & Nur, 1997; Etienne et al., 1999; Rehbein et al., 1999; Mackie et al., 2000; Etienne et al., 2001). When IEF gives identical patterns and does not differentiate between closely related species, two-dimensional electrophoresis (2-DE) may be helpful to complete the identification (Huang, Marshall, & Wei, 1995; Pineiro, Barros-Velasquez, Sotelo, & Gallardo, 1999). Electrophoretic techniques applied to fish products, in combination with the recently developed proteomic tools such as in-gel digestion and mass spectrometry, have already been used to characterize species-specific proteins (Pineiro, Vasquez, Marina, Barros-Velasquez, & Gallardo, 2001). Moreover, comparison of 2-DE maps recently permitted to identify marker spots of post-mortem changes and the effects of additives during the processing of fish muscle (Martinez & Friis, 2004).

In this paper, we employed native IEF and 2-DE for objective identification of the four freshwater fish species commercially labelled as "perch".

2. Materials and methods

2.1. Fish material

Whole specimens of European perch, European pikeperch, and sunshine bass were purchased as fresh fish at a local market. The Nile perch was obtained from a fishing bank and shipped frozen by overnight delivery. Fish (six of each species) were identified by an expert veterinary inspector according to their external anatomical and morphological features (Manzoni, 1993). All the specimens of European perch, European pikeperch and Nile perch were wild, while the specimens of sunshine bass were cultivated and belonged from an Italian breeding (Agroittica Lombarda, Viadana di Calvisano, BS, Italy). Samples of white muscle, free from blood, red muscle or other tissues (Rehbein, 1990), of each fish were frozen and stored at −80 °C until used. Frozen muscle samples can be stored indefinitely.

2.2. Extraction of sarcoplasmic proteins

(1) *Native IEF*. Water-soluble proteins were extracted from white muscle as previously described (Tepedino et al., 2001; Rehbein et al., 1995). Supernatants were recovered, assayed for protein content by the method of Lowry, Rosebrough, Farr, and Randall (1951) and used immediately for IEF or stored at −80 °C (not longer than one week).

(2) *2-DE*. Sarcoplasmic proteins were extracted by suspending 0.1 g of tissue in lysis solution (8 M urea, 4% CHAPS, and 0.2% Ampholine pH 3.5–10.0). Tissue was minced using a spatula and the suspension was vigorously vortexed. After 15 min incubation, the samples were sonicated in a bath for 1 min (three times at 5 min intervals), then centrifuged in an Eppendorf bench centrifuge for 5 min at 14,000*g*. The supernatants (2 μL) were assayed for protein content by the method of Lowry et al. (1951), with 2 μL of lysis solution as reference blank, and used immediately for 2-DE or aliquoted and stored at −80 °C.

2.3. Native IEF

Native IEF was done using precast polyacrylamide gels (Ampholine PAGplate, 245 × 110 × 1 mm, pH 3.5–9.5; Amersham Biosciences Europe, Freiburg, Germany), as previously described (Tepedino et al., 2001). Forty micrograms of total proteins were applied near the cathode using pieces of filter paper. Three lanes of protein standards in the 3.5–9.5 pH range (Broad pI calibration Kit, Amersham Biosciences) were included in each gel. The running conditions were: 30 W constant (1500 V maximum) for 1.5 h, with 15 min prefocusing. Gels were stained with Coomassie Brilliant Blue R250, equilibrated in 10% glycerol and 7% acetic acid, and dried in air between sheets of cellophane.

2.4. 2-DE

The first-dimension IEF was carried out on an immobilized pH gradient (Immobiline DryStrip pH 3–10, 13 cm; Amersham Biosciences Europe, Freiburg, Germany) using the Ettan IPGphor system (Amersham Biosciences Europe, Freiburg, Germany) following the procedure described by Berkelman and Stenstedt (2002). Samples (30 μg total proteins) were diluted to 250 μL with DeStreak solution supplemented with 0.5% IPG Buffer pH 3-10 (Amersham Biosciences Europe, Freiburg, Germany), and used for rehydration of the IPG dry strip. After 13 h rehydration at 20 °C, the IEF started with 500 V for a total of 500 V/h (1 h), followed by 1000 V for 1000 V/h (1 h), and 8000 V for 14500 V/h (2.5 h). During the run a maximum of

50 μA/IPG strip was applied and the temperature was maintained at 20 °C.

The second dimension (SDS–PAGE) was carried out on 12.5% polyacrylamide gels (160 × 150 × 1 mm). IPG strips were equilibrated 15 min in urea 6 M, Tris–HCl 50 mM pH 8.8, glycerol 30%, SDS 2%, bromophenol blue 0.002%, applied to the top of SDS gels and sealed with agarose 0.5%. A constant current of 10 mA/gel was applied for 15 min, followed by 15 mA/gel for a further 15 min, and finally 20 mA/gel until the dye front reached the bottom of the gel. Gels were stained with silver nitrate and dried in air between two sheets of cellophane.

2.5. Analysis of patterns

IEF gels were scanned using an Epson densitometer and the images were analysed using the GelComparII software (Applied Maths, Saint-Martens-Latem, Belgium). Bands over a threshold of 10% of the overall intensity of the image were considered characteristic of the species. Different gels were normalized for differences in running conditions by comparing the pattern of pI standard proteins separated in the gel to be analysed with the pI standard pattern chosen as a data base standard. Intra-gel distortions between lines were compensated by aligning the band position of the three lines of standards loaded at different positions on the gel. Calculations were done using the Pearson (product-moment) correlation coefficient (Bossier & Cooreman, 2000).

3. Results and discussion

3.1. Native IEF

The aim was to obtain and compare the IEF profile of the sarcoplasmic proteins extracted from the white muscle of the four species of fish sold as ''perch'', and to analyse the patterns for species identification. Six fish of each species were analysed, in either the same or different gels: the band patterns were scanned by densitometry and the characteristic bands were identified and their pI calculated. A representative pattern for each species is shown in Fig. 1(a). Fig. 1(b) reports a schematic diagram of the patterns and the mean pI (±SD) calculated for the characteristic bands.

Each species analysed has a characteristic band pattern, with differences making for easy discrimination. Interestingly, IEF showed that there are no protein bands common to the four species of fish tested. The band patterns were consistent for the six specimens examined for each species. Only a sample of European pikeperch showed a significant band at pI 4.26 which was not visible in the other five. The presence of this

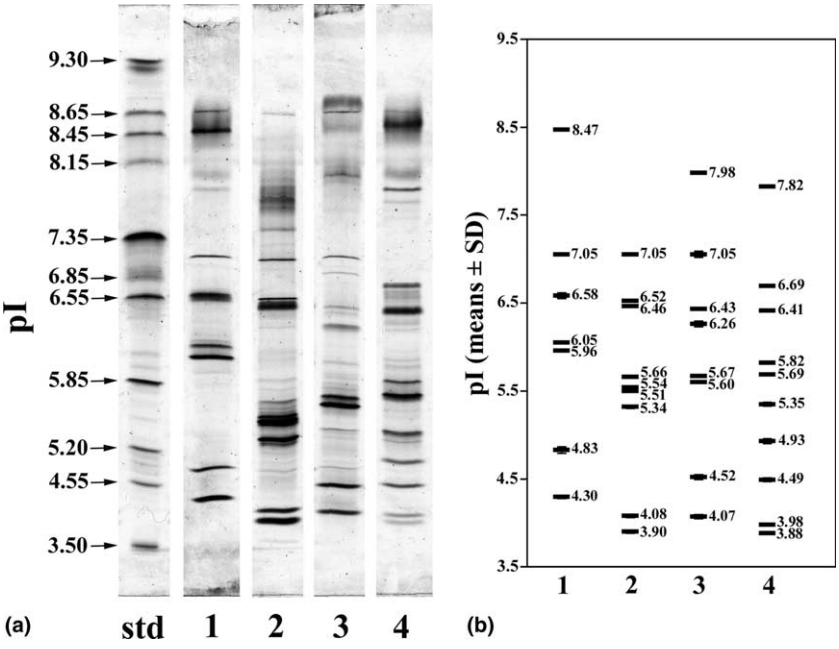

Fig. 1. (a) IEF in polyacrylamide gel (pH range 3.5–9.5) of the water soluble sarcoplasmic proteins extracted from the four freshwater fish species sold under the generic label of ''perch'' (European perch, *Perca fluviatilis*; Nile perch, *Lates niloticus*; European pikeperch, *Stizostedion lucioperca*; sunshine bass, *Morone chrysops x saxatilis*). On the left, the pattern of standard proteins and their pI. (b) Schematic representation of IEF patterns of the four freshwater fish species sold as ''perch''. Bands with threshold over 10% of the overall intensity of the image are shown, considered characteristic of the species. Numbers on the right of each band are the mean pI, calculated from the patterns of the six fish of each species analysed. The SD values varied between 0.01 and 0.03.

band, however, did not prevent correct identification of the species either by visual inspection of the gel or by image analysis with Gel Compar II software. The low intra-species polymorphism found for these species agrees with our previous results (Tepedino et al., 2001). However, it might be necessary to test larger number of specimens from each species to detect intra-specific protein pattern differences. Significant polymorphism among bands was reported for other fish species, when more than 25 individuals were analysed (Weaver et al., 1999). Nevertheless, it is clear that in the analytical conditions used in this work, the inter-specific band variation is greater then the intra-specific variation and good enough for unambiguous species identification.

Most of the bands are in the pH range 4.0–7.0, as for other fish species (Lundstrom, 1979; Weaver et al., 1999; Tepedino et al., 2001; Pineiro, Barros-Velasquez, Sotelo, et al., 1999). On the basis of the colour of their fillet, two types of fish can be distinguished: white-fleshed and red-fleshed. The four species analysed are white-fleshed, and we extracted proteins from their white muscle. White and red muscle differ in physiological function, metabolism and protein composition (Hamoir, Focant, & Disteche, 1972; Rowlerson, Scapolo, Mascarello, Carpenè, & Veggetti, 1985) and both gave species-specific protein pattern. Rehbein (1990) reported that white muscle has an IEF pattern with pronounced anodal bands representing principally parvalbumins. These are small, acidic, heat-stable calcium-binding proteins (Rehbein, Kundiger, Pineiro, & Perez-Martin, 2000), accounting for a major part of the sarcoplasmic proteins of fish muscle. Their pIs in native conditions range between 3.8 and 5.3 and they are species-specific (Rehbein, 1990; Ross, Tilghman, Hartmann, & Mari, 1997; Pineiro, Barros-Velasquez, Sotelo, et al., 1999). Two major bands of the European perch (pI 4.30 and 4.83), of the Nile perch (pI 3.91 and 4.08) and of the European pikeperch (pI 4.07 and 4.52) can be included in this group of proteins, while the sunshine bass shows four bands (pI 3.88, 3.98, 4.49 and 4.93) that can be classified as parvalbumins. Another difference is a band at pI 7.05, present in European perch, Nile perch and European pikeperch but not in the sunshine bass. These differences might be due to the fact that the sunshine bass is a hybrid produced by crossing a female white bass (*Morone chrysops*) with a male striped bass (*Morone saxatilis*) and it is a breeding species only obtained by aquaculture. Differences in the expression of proteins as a result of dietary manipulation have been recently observed in farmed fish (Martin et al., 2003).

3.2. 2-DE

Fig. 2 shows typical silver-stained 2-DE gels of white muscle proteins extracted from European perch and Nile perch (Fig. 2(a)), and European pikeperch and sunshine bass (Fig. 2(b)). The proteins were extracted in the presence of urea 8 M and CHAPS 4% and the suspension was sonicated in order to ensure a complete solubilisation of the proteins. The maps showed numerous spots, distributed mainly in the acidic part of the IPG pH gradient. The $Mr \times 10^3$ varies from 100 to 10. Major spots and gel areas containing spots are numbered from 1 to 21 in the figures.

The 2-DE patterns were species-specific. The sunshine bass is easily recognizable by the presence of five spots (6–10) with Mr about 20,000 and acidic pI. The spots are well defined and are not present in the other species. Spots no. 12–15 are seen only in the European perch, whose pattern also includes lots of little spots distributed in the acidic pH gradient, with Mr between 45,000 and 25,000. Spots 16–18 are characteristic of the Nile perch, and spot no. 19 and those in the area no. 20 are typical of the European pikeperch.

Despite these significant differences, several spots were common to all four species. Spot no. 1 is common, well marked, shows an apparent acidic pI, and a Mr of about 33,000. This protein was tentatively identified as tropomyosin by comparison to the results obtained in previously published works on other fish species (Martinez & Friis, 2004). Amino acid substitutions are probably present in tropomyosin from the four species but the differences do not cause noticeable changes of pI or Mr.

Spot no. 2, common to the four species, can be tentatively identified by comparison to previous published papers on other species (Martinez & Friis, 2004), as actin (pI 5.22; Mr 41700; Swiss-Prot data base at: http://us.expasy.org/sprot/).

All the species show large spots, in the gel area no. 21, with acidic pI (<pH 5.0), migrating just above the dye front in the second dimension (Mr 10,000). These spots are marked in European perch, Nile perch and European pikeperch, weaker and with higher Mr in the sunshine bass, and can be ascribed to the parvalbumin family.

Spot no. 3 is present and marked in European pikeperch and in Nile perch, but is less evident in the European perch and absent in the sunshine bass. Spots present in the circled areas 4 and 5 can be considered common for the four species, with some differences: in the European perch and European pikeperch the spots in area no. 5 are spread from about pI 6.5 to about 7.5, while in the Nile perch they range from about pI 6.5 to 7.0 and in the sunshine bass from about pI 7.0 to 7.5. In addition the number and intensity of spots in the two areas vary, as do the number and intensity of the minor spots visible around the principal ones.

Spots in the gel area no. 11 lie in a similar position in the four maps, but the European pikeperch displays a characteristic pattern with three spots at the same Mr that double at a slightly lower Mr. This pattern was consistent for all five European pikeperch tested.

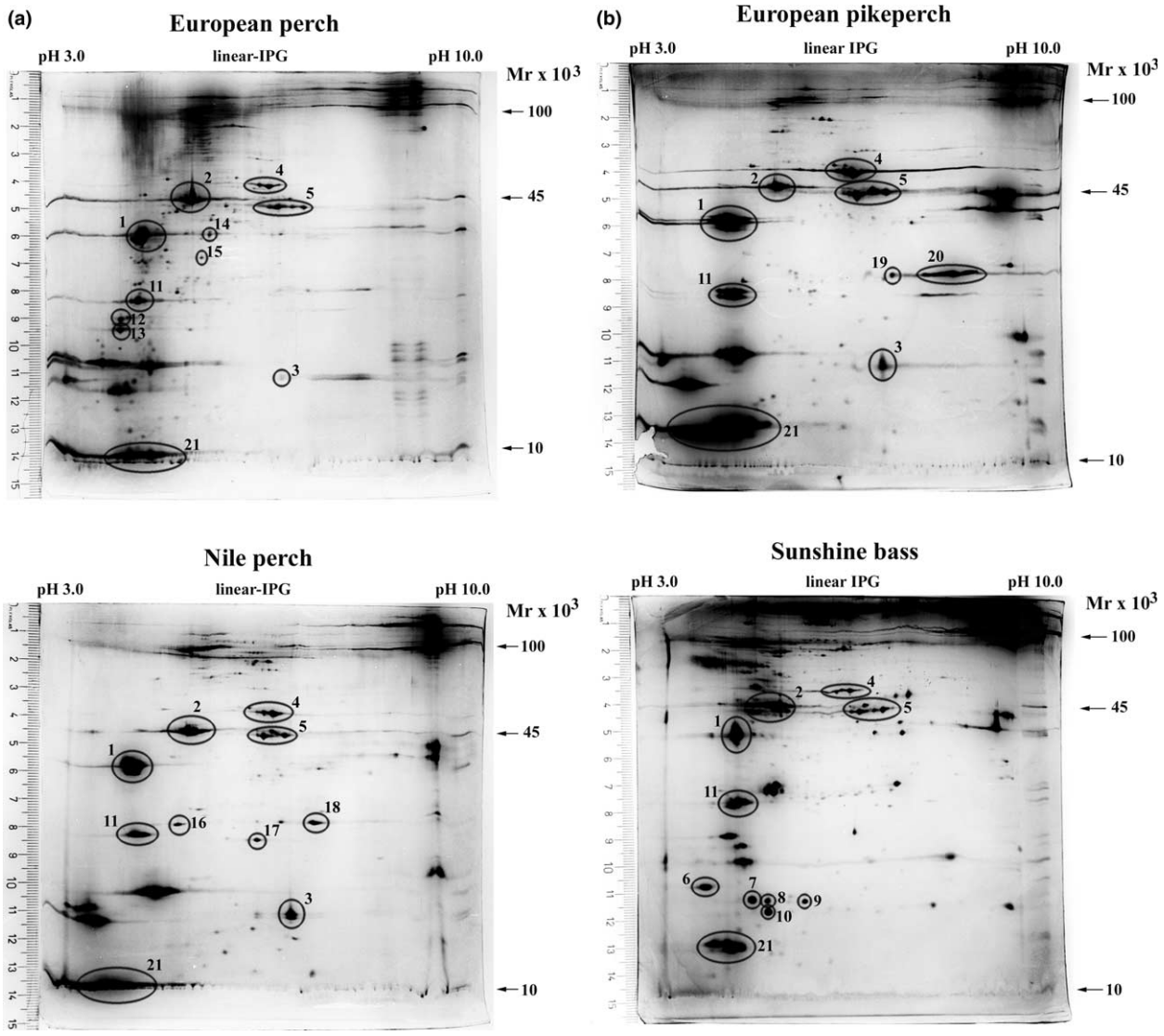

Fig. 2. 2-DE of: (a) European perch (*Perca fluviatilis*) and Nile perch (*Lates niloticus*); (b) European pikeperch (*Stizostedion lucioperca*) and sunshine bass (*Morone chrysops x saxatilis*). Major spots referred to in the text are circled and numbered. Spots similar in the four species are assigned the same number. Gels were stained with silver nitrate.

The fact that 2-DE but not IEF analysis revealed similarities among proteins extracted from muscle of the species of fish sold as "perch", most likely was due to the different resolving power of these two techniques.

4. Conclusions

In conclusion, native IEF of the water-soluble proteins extracted from the white muscle of the four species of fish sold under the generic name of "perch" serves to distinguish the species. The patterns, analysed with suitable software and compared with standard patterns archived in a data-base, permit correct identification of species in less than two days. Thus, this simple and quick technique could help reduce or even avoid mislabelling and fraud.

The more sophisticated, expensive and time-consuming analysis by 2-DE, besides supporting the species identification when IEF fails, as in the case of tuna (Rehbein, 1990), may have major application to give an insight into the proteome of poorly characterized species. Furthermore, proteomics within seafood quality control has been recently shown to be suitable to characterize health status of the organism, contamination levels and post-mortem treatments (Martinez & Friis, 2004).

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