Fish species identification by isoelectric focusing

The use of schematic patterns

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In this paper, flat bed agarose gel isoelectric focusing (AGIF) is tested as a method of identification of fish species. In spite of the large number of species tested, the specificity of the IF-pattern remains evident for each. Intra-specific variations in the IF-pattern are of minor importance, so that one schematic pattern can be constructed for each species. With the aid of these standard patterns unknown fish samples can be identified without any problem. To build up a "library" of standard patterns, a large number of fishes and fish species must be examined in order to assimilate maximum information into the patterns.

Determination of fish species by means of electrophoresis of fish-muscle protein extracts has been investigated by a number of workers. In 1953, CONNELL (4, 5) first tried out moving boundary electrophoresis in order to differentiate twenty fish species. This author described his results as "fingerprints" of the species. During the following years, several experiments with zone electrophoresis have been carried out by other investigators. The media used for this purpose included starch gel (20, 21), polyacrylamide gel (6, 9, 18, 22), agar gel (8) and cellulose acetate (1, 2, 10, 11). The specificity of the electrophoretic pattern for each species was confirmed in these studies, although reproducibility was not satisfactory (1, 10, 11, 22). Three collaborative studies using polyacrylamide (22) or cellulose acetate (10, 11) as the medium, showed lack of reproducibility to be the main cause of incorrect determination of unknown fish samples. Moreover, when using photographic standards (10), only 39% of the samples examined were correctly identified. Analysis of authentic flesh standards simultaneously with unknown samples appeared to be necessary (1, 11, 16).

Isoelectric focusing (IF) is a technique by which a mixture of ampholytes with different isoelectric points (Ip) can be separated by means of an electrically induced linear pH-gradient. As shown in previous papers (9, 13, 14, 15, 16, 19), flat bed polyacrylamide gel IF (PAGIF), applied to sarcoplasmic protein extracts of non heated fish-meat, gives excellent results as a method of differentiation of fish species. According to TINBERGEN et al. (19), closely related species, rather difficult to distinguish from each other by anatomical features, show clearly distinct patterns. Applying enzymatic colouring to be protein bands, CHUA et al. (3) were also able to differentiate interrelated species. DJUPSUND (7), however, had difficulties in distinguishing several fishes of the Coregonidae family from one another.

In a collaborative study with photographic standards, LUNDSTROM (15) and collaborators found PAGIF to be very reproducible. Only one of eight species, which proved later to have sarcoplasmic protein polymorphism (16), could not be identified by 5 out of 7 collaborators.

LUNDSTROM (17) compared PAGIF with flat bed agarose gel IF (AGIF). He demonstrated AGIF to be as specific and reproducible as PAGIF, though substantially less time-consuming, easier to apply and less expensive.

In this work a great number of fish and fish species are examined by AGIF in order to construct schematic IF-patterns which can be applied as standards for determination of unknown fish samples. Some minor variations in the reproducibility of the AGIF-pattern within a few species will be discussed.

Materials and Methods

Solutions

1. Gel solution: 0.18 g "Isogel" Agarose-EF (LKB); 16.6 ml distilled water; 1.4 ml "Ampholine" pH 4.0—6.5 (LKB)

2. Anode solution: Acetic acid 0.5 M

3. Cathode solution: NaOH 0.5 M

4. Fixing solution: Trichloroacetic acid 100 g; sulphosalicylic acid. $2H_2O$ 11.65 g; aq. dest. ad 1000 ml

5. Washing solution: methanol 100%

6. Staining solution: Coomassie Brilliant Blue R 250 2.5 g; Destaining solution ad 1000 ml

7. Destaining solution: ethanol 95% 350 ml; Acetic acid 100% 100 ml; aq. dest. ad 1000 ml

Fish samples examined in this study were obtained at the fishing port of Zeebrugge, at the fish auction at Rungis near Paris, or were kindly supplied by veterinarians employed as inspectors in the fish trade.

Apparatus used included the Multiphor (LKB, Sweden) focusing unit, the Consort 2000 (Belgium) high voltage constant-power supply and the Ultra-turrax (Germany) homogenizer.

Extracts were made by filtration as described by LUNDSTROM (17). Some homogenates were difficult to filter: these were centrifuged (3000 rpm) before filtration of the supernatant. The extracts were deep-frozen until IF was performed.

The AGIF procedure was carried out according to the LKB instruction sheet for high-performance analytical electrofocusing in 0.5 mm thin-layer agarose gel (12). We used a narrow pH-gradient because most of the protein bands of interest were localized in the pH-gradient 4.0—6.5. Gels with wide gradients, as shown by LUNDSTROM (13, 14, 15, 16, 17), seemed overloaded in this particular pH-zone. Moreover, a narrow gradient results in better separation of proteins with neighbouring Ip: this leads to a clearer distinction between closely related fish species.

A few modifications were introduced in the AGIF-procedure. After preparation of the agarose gel, the surface of the gel is dried with a Whatman 1 MM filter paper for max. 1 min immediately before use. The gel is then placed on top of the template. Electrode solutions are poured out in large watch-glasses; electrode strips are pulled through, blotted between Whatman paper, cut to size and placed on the gel. Twenty µl extract is applied to the sample application strip, which is placed on the gel surface ± 3 cm from the cathode. Up to 22 samples may be examined on one gel without having distorted protein-band patterns at the gel ends. The gel is prerun for 5 min using low voltage conditions (approx. 200 V) before setting at 8.5 Watt. After 15 min focusing, sample application strips are removed and focusing continued up to 60 min. Power is then increased by 50% (12.6 Watt) and IF is terminated 10 min later. After IF, the gel is fixed for 10 min, and is then washed for 10 min. Before drying, the bottom of the GelBond film is thoroughly cleaned of remaining kerosene drops with a wad of dry cottonwool in order to avoid white spots on the film after drying. The dried gel is stained for 7 min. Decoloration generally took place overnight and was judged visually.

Results and Discussion

Species specificity of the IF-pattern

A total of 53 different species (tab.) was examined. All 53 species show a specific IF sarcoplasmic protein pattern, characterized by the (approximate) number, relative position and relative color intensity of the protein bands. This result is in agreement with those of other investigators (9, 13, 14, 15, 16, 17, 19). Patterns obtained by IF clearly show more protein bands than those produced by

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Tab.: List of fish species, examined in this work. The first number in brackets is the number of fish examined per species; the second refers to the schematic pattern of the species in fig. 5

I. OSTEICHTHYES

A. Fam. Anarichadidae
Atlantic catfish (3, 36), Anarhichas lupus

B. Fam. Anguillidae
European eel (2, 35), Anguilla anguilla

C. Fam. Ariidae
sea catfish (1, /), Galeichthys felis

D. Fam. Atherinidae
atherine (3, 47), Atherina presbyter

E. Fam. Balistidae
gray triggerfish (1, /), Balistes capriscus

F. Fam. Berycidae
red bream (1, 44), Beryx decadactylus

G. Fam. Carangidae
horse mackerel (8, 26), Trachurus trachurus

H. Fam. Clupeidae
herring (29, 31), Clupea harengus
sardine (14, 49), Sardina pilchardus
sprat (2, 32), Clupea sprattus

I. Fam. Congridae
conger (1, 34), Conger conger

J. Fam. Cyprinidae
carp (1, 42), Cyprinus carpio

K. Fam. Engraulidae
anchovy (4, 46), Engraulis encrasicolus

L. Fam. Gadidae
cod (10, 6), Gadus morhua
forkbeard (1, /), Phycis
haddock (6, 4), Melanogrammus aeglefinus
hake (3, 3), Merluccius merluccius
ling (4, 1), Molva molva
pollack (1, 8), Pollachius pollachius
pout (12, 2), Gadus fusca
saithe (2, 7), Pollachius virens
whiting (5, 5), Merlangus merlangus

M. Fam. Lophiidae
anglerfish (4, 20), Lophius piscatorius

N. Fam. Mugilidae
grey mullet (5, 22), Mugil auratus Risso

O. Fam. Mullidae
surmullet (5, 22), Mullus surmeletus

P. Fam. Pleuronectidae
Atlantic halibut (1, 18), Hippoglossus hippoglossus
brill (3, 14), Rhombus laevis Rondelet
common dab (8, 16), Limanda limanda
common sole (7, 12), Solea solea
flounder (2, 9), Pleuronectes flessus
Greenland halibut (3, 48), Reinhardtius hippoglossoides
lemon sole (4, 15), Microstomos kitt Walbaum
megrim (2, 11), Lepidorhombus whiffiagonis Walbaum
plaice (7, 10), Pleuronectes platessa
Senegalese sole (6, 50), Glyptocephalus glyptocephalus
turbot (3, 13), Rhombus maximus
witch (3, 17), Glyptocephalus cynoglossus

Q. Fam. Salmonidae
Atlantic salmon (2, 37), Salmo salar
sea trout (2, 38), Salmo trutta

R. Fam. Scombresocidae
garfish (1, 33), Belone belone

S. Fam. Scombridae
mackerel (4, 30), Scomber scombrus

T. Fam. Scorpaenidae
redfish (2, 23), Sebastes marinus

U. Fam. Serranidae
bass (2, 25), Morone labrax

V. Fam. Sparidae
bogue (1, 45), Boops boops
black seabream (3, 24), Cantharus lineatus Montagu

W. Fam. Trachinidae
greater weever (2, 19), Trachinus draco

X. Fam. Triglidae
grey gurnard (3, 29), Trigla gurnardus
red gurnard (1, 27), Trigla cuculus
yellow gurnard (4, 28), Trigla lucerna

Y. Fam. Zeidae
John Dory (5, 21), Zeus faber

II. CHONDRICHTHYES

A. Fam. Lamnidae
porbeagle (2, 39), Lamna nasus

B. Fam. Scyliorhinidae
lesser spotted dogfish (2, 41), Scy. canicula

C. Fam. Squalidae
picked dogfish (2, 40), Squalus acanthias

cellogel (1) or PAG (9, 16) electrophoresis with extracts of the same species. This high degree of resolution, together with the sharpness of the protein bands is characteristic of IF.

Morphologically interrelated species generally show visually similar patterns (fig. 1). This suggests that additional phylogenetic information may be acquired by means of IF sarcoplasmic protein patterns.

There are, however, exceptions to this rule: the yellow gurnard pattern (No. 28 in fig. 5) appears to be quite different from the patterns of red and grey gurnard (No. 7 to 10, No. 27 and 29 in fig. 5).

LUNDSTROM (14) observed the absence of strong protein bands in the IF-pattern of mackerel in a narrow pH-gradient, practically all the proteins being accumulated close to the cathode with Ip values higher than 6.5. Our experiments confirm this result (No. 11 and 12 in fig. 1). One shark species (porbeagle) also shows this type of pattern. (This fish species is often marketed as "tuna" in a number of fish products.) Should further experiments reveal more fish species with such IF-patterns in a narrow pH-gradient, the use of a wide gradient (e.g. 3.5–9.5) would become necessary to obtain distinct patterns for these species.

Reproducibility of the IF-pattern of each extract

LUNDSTROM (14) concluded that PAGIF is quite reproducible. Essentially AGIF does not differ from PAGIF, so the reproducibility of the former was not investigated systematically during this work. However, the same extracts of some fish species were submitted regularly to the IF-procedure. The reproducibility of their IF-pattern was quite good, except for cod, haddock and pout. IF-patterns of these last three fishes gradually lost certain protein bands during storage. Two possible explanations have to be considered here: alteration of the extract or a decrease in solubility due to deep-freezing and thawing between the IF-experiments.

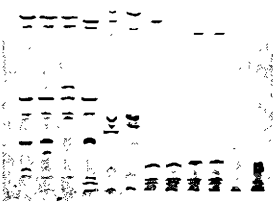

Fig. 1: Resemblance between IF-patterns of interrelated fish species.
fam. Gadidae: cod (tracks 1, 2 and 3) and haddock (track 4)
fam. Pleuronectidae: brill (track 5) and turbot (track 6)
fam. Triglidae: red gurnard (track 7 and 8) and grey gurnard (track 9 and 10)
Note: tracks 11 and 12 are two extracts from mackerel. No strong protein bands are seen in this pH- range (4.0–6.5)

An extract of 2 fresh pouts was quickly deep-frozen (−24 °C) and thawed (+20 °C) and this cycle was repeated five times in succession. This extract was compared with a fresh, non-frozen extract of the same fishes. The two pouts were then quickly deep-frozen and thawed five times in succession, after which a third extract was prepared. The results of this experiment are shown in fig. 2 (tracks 1 to 7). No fading of the protein bands is evident after deep-freezing. On the contrary, certain protein bands are considerably more pronounced in the patterns of deep-frozen and thawed fish, indicating better solubility of these proteins after the deep freeze-thawing cycles.

In fig. 2 the effect of storage of pout extracts at room temperature (20 °C) on the IF-pattern is also demonstrated. One extract of a fresh pout was divided into ten equal portions. One portion was immediately deep-frozen until examination, the remaining nine portions being stored at room temperature.

Each consecutive day one portion was deep-frozen. Fig. 2 (tracks 7 to 14) shows IF-patterns of the portions obtained after storage for 0 (immediately deep-frozen), 1, 3, 5, 6, 7, 8 and 9 days. It can be seen that some protein bands disappear after 3 days storage. These are the same bands that faded away when repeatedly using the pout extract mentioned above. Alteration of the extract is thus most probably the cause of this phenomenon. After 5 days of storage, it becomes difficult to interpret the IF-pattern correctly, unless the upper (acidic) part, which is obviously more resistant to alteration, has enough specificity to determine the species.

It should be noted that the IF-patterns of the same extract on different agarose gels may differ from one another since the pH-gradient is never perfectly linear. This leads to an "accordion" effect: some parts of an IF-pattern will be slightly more stretched and other parts more contracted in comparison with the same pattern on a second gel.

Neither the fading of certain protein bands, nor the "accordion" effect caused any difficulty in interpretation of the IF-patterns of fish species at any time.

Variation of the IF-pattern within a fish species

The total number of fish examined for each species is given in the table. As explained in the previous chapter, the only gel-to-gel varia-

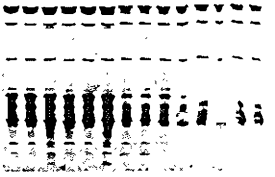

Fig. 2: Effect of freeze-thaw cycles and aging on the IF-pattern of pout
tracks 1 to 3 incl. and 4 to 6 incl. derive from the same fish
tracks 1 and 4: fresh fish extract put five times through a deep-freeze/thaw cycle
tracks 2 and 5: extracts from fresh fish (the extracts have never been deep-frozen)
tracks 3 and 6: extracts obtained after freezing and thawing the fish five times in succession
tracks 7 to 14 incl. are portions of the same extract of one fresh pout stored at room temperature (+20 °C) for 0, 1, 3, 5, 6, 7, 8 and 9 days respectively)

tion of fresh extracts consisted in the "accordion" effect. Any other variation between IF-patterns of extracts from different fish of the same species is due to properties inherent in these fish.

IF-patterns of fish extracts of the same species generally exhibit slight variations. Some faint protein bands may be slightly more pronounced or may even disappear completely. Only within a few species (e.g. cod, pout, herring) do certain protein bands show a continuous gradation between strong and absent.

Fig. 3 shows the IF-patterns of four cod and six pout. These patterns may be compared, for cod with those in fig. 1 (tracks 1 to 3 inclusive) and for pout with those in fig. 2. The variation of some bands is evident. For pout it is also noticeable that the same bands, when present in the fresh extract (fig. 2 No. 7), disappear with alteration of the extract (fig. 2, No. 8 to 14 inclusive).

Fishing ground, age, sex, season and sampling point were investigated in order to determine their influence on intra-specific variations. No causality whatsoever could be found. Nevertheless, physiological conditions are likely to be the major reason for this variability.

Two species showed a somewhat different type of variation in their IF-pattern: surmullet and Senegalese sole. Here no continuous gradation of the variable protein

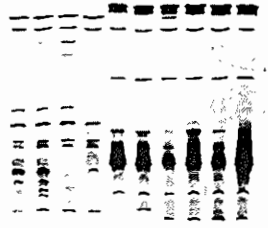

Fig. 3: Variation of the IF-pattern within a fish species
tracks 1 to 4 incl.: four cod
tracks 5 to 10 incl.: six pout

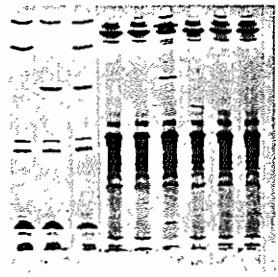

Fig. 4: Biological variation in the species of surmullet (tracks 1 to 3 incl.) and Senegalese sole (tracks 4 to 9 incl.)

bands was detected: as demonstrated in fig. 4, these bands were either clearly present or absent. A possible explanation could be a genetic protein polymorphism, as was previously found in American monkfish by LUNDSTROM (16). All intra-specific variations here described, when present, concern only a minor part of the IF-pattern. The remaining bands are constant for the species.

The construction and use of schematic protein band patterns

In the course of the present work, a limited IF-experiment was carried out to see whether it was possible to determine unknown fish samples from IF-agarose gel patterns obtained in previous experiments. A total of 53 fish, to 25 species, was examined on 3 agarose gels. IF-patterns in agarose gels, obtained in previous IF-procedures, were already available for 20 of these 25 species. With the aid of these IF-patterns, an untrained worker was asked to identify all 53 patterns of this experiment. The position of the extracts on the 3 agarose gels was unknown to him. All fish belonging to the group of 20 were correctly identified, while all the others were classified as unknown.

Because of this good result, and because variations within a species were only of minor importance, one schematic, full pattern was drawn up for each species, indicating the protein bands which are always present (the invariable part of the species pattern) as well as the variable protein bands. These patterns, of course, have to be considered as preliminary since the number of fish examined is still limited and certain variations may remain undiscovered. Fig. 5 shows such patterns for 50 species. A two-colour system (red and black) was chosen in order to simplify the scheme. Each point represents the location of a protein band. All red points are variable bands: they may be present or absent in certain extracts of a particular fish species. On the contrary, black points are always present in the IF-pattern of a fish species. Broad bands are represented by vertical lines proportional to the thickness of the protein band. Points or lines from bands that have a dense colour are extended to the left with a horizontal line (or a dotted line if this strong density is not always present). The directions for use are simple. The IF-agarose gel pattern to be determined is placed immediately to the right of the first schematic pattern. Strong bands are now compared with the horizontal lines. If they do not match with one another, the next pattern is taken. It should be remembered that red points or bands can be absent. Also a slight up and down movement of the agarose gel is needed, because the "accordion" effect may put the protein bands a little further apart or a little closer together. If the pattern correlates with that on the gel, the latter is placed on the standard pattern and faint bands are compared for confirmation. Again, a slight up and down movement of the gel is necessary. The interpretation of the 53 extracts was repeated with the aid of the schematic patterns instead of known IF-agarose gel patterns. All fish species were correctly identified by untrained observers.

Conclusion

In this work, flat bed agarose gel IF is shown to be a reliable and practical method of identification of the species of raw fish meat. It is also demonstrated that the patterns for each species examined here may be represented by a simple schematic pattern. With the aid of these standard patterns, fish species identification does not give rise to any problem. These standard patterns are simple to set up and easy to handle. IF-examination of a great number of fishes per species however is necessary in order to assimilate maximum information into each schematic pattern.

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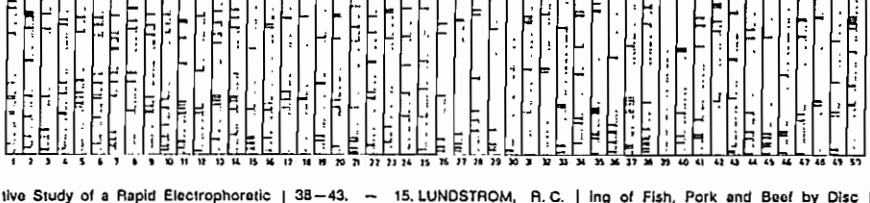

Fig. 5: Schematic Patterns of fifty fish species. The second number after each name in the table refers to the corresponding pattern in this fig.

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