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PCR-ELISA for the Semiquantitative Detection of Nile Perch (*Lates niloticus*) in Sterilized Fish Muscle Mixtures

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A PCR-ELISA technique was developed for the semiquantitative detection of Nile perch (*Lates niloticus*) in experimentally sterilized fish muscle mixtures. Specific oligonucleotides derived from the 5S rDNA gene of Nile perch were selected. A forward primer, together with a reverse digoxigenin-labeled primer, permitted the amplification of specific 185 bp DNA fragments showing DNA intensities proportional to the contents of Nile perch muscle tissue in the fish mixtures. A biotinylated probe immobilized onto streptavidin-coated microplates was used to capture the digoxigenin-labeled fragments that were detected with peroxidase antidigoxigenin conjugate. Subsequent enzymatic conversion of substrate gave distinct absorbance differences when assaying fish binary mixtures containing different percentages of Nile perch muscle.

KEYWORDS: ***Lates niloticus*; fish mixtures; PCR-ELISA; 5S rDNA gene**

INTRODUCTION

In recent years, there has been a tremendous growth in seafood consumption due to changes in consumer attitudes toward health and nutrition. Some of the world's fish catch is sold unprocessed, but the market for processed fishery products is steadily increasing. A considerable number of these products may contain muscle or other tissue from one or more fish species. Examples are cooked or sterilized fish commodities such as cakes, pies, pastries, soups, pâtés, and also industrial products such as fish meal. Detection and identification of the fish species comprising highly processed products is a very difficult task, since biological characteristics are lost and proteins may be denatured on processing and heating, leading to subsequent loss of analytical specificity.

One form of adulteration for economic gain may be the addition of undeclared cheaper fish such as Nile perch (*Lates niloticus*) in heat-treated products that are labeled using the names of higher price and quality fish species. Consequently, to ensure compliance with labeling regulations, there is a need for suitable methods for qualitative and also quantitative monitoring of fish species in the market of heat-processed fishery products (*1*, *2*).

Numerous analytical techniques that rely on protein analysis have been developed for fish species identification: electrophoretic techniques such as isoelectric focusing or SDS−PAGE (*3*, *4*); chromatographic techniques (*5*, *6*) and immunological techniques such as immunodiffusion and ELISA (*7*, *8*). However, proteins lose their biological activity after animal death, and their presence and characteristics depend on the cell types. Furthermore, most of them are heat-labile. Thus, for fish species identification in heat-processed matrices, a DNA method rather than protein analysis is preferable.

Among DNA-based approaches, the Polymerase Chain Reaction (PCR) has shown to be the most reliable technique for fish species identification (*9*, *10*). Moreover, PCR offers the possibility to also identify fish species in sterilized products, by its ability to amplify short DNA fragments from degraded samples (*10*, *11*). The potential of PCR to amplify a small gene copy number of target cells has prompted the development of protocols for food analysis that are adapted to the quantification of amplified PCR sequences (*12*). For example, realtime PCR, quantitative competitive PCR, and PCR-ELISA have been developed to detect and quantify genetically modified organisms (GMOs) (*13*), pathogenic and spoilage microorganisms (*14*−*16*), nut residues (*17*), and species of meat samples from different origins (*18*−*20*). However, as far as we know, quantitative PCR assays have not yet been reported for fishery products.

In the present work, we describe the development of a combined PCR-ELISA method targeting the 5S ADNr gene for the rapid semiquantitative detection of Nile perch (*Lates niloticus*) in experimentally sterilized fish mixtures.

MATERIALS AND METHODS

Preparation of the Samples and DNA Extraction. Nile perch and other fish species such as grouper (*Epinephelus marginatus*), hake (*Merluccius merluccius)*, bluefin (*Thunnus thynnus*), and bonito (*Sarda sarda*) were obtained from MercaMadrid (Madrid, Spain) and other local markets and were identified by Veterinary Public Health Inspectors. Binary muscle mixtures of Nile perch/grouper, Nile perch/hake, Nile perch/bluefin, and Nile perch/bonito were prepared containing 15, 10, 5, 2, 1, and 0% (w/w) of Nile perch in the other fish species in a final weight of 5 g. Samples were autoclaved at 120 °C for 20

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min and placed into a falcon tube. Five milliliters of TSM extraction buffer (Tris 0,2 M; EDTA 0,1 M; SDS 1%) and 60 μL of proteinase K (20 mg/mL) were added and samples were incubated at 55 °C overnight with shaking. Finally, 750 μL of each homogenate were taken and DNA was extracted according to a previously described procedure (*21*).

PCR Amplification of a Specific Fragment from Nile Perch DNA. The set of primers used for PCR amplification were those described by Asensio et al. (*9*) for the specific amplification of a 185 bp DNA fragment from the 5S rDNA gene of Nile perch: 5S1 (5′-TACGCCCGATCTCGTCCGATC-3′) and 5SP-dig (5′-TACGCTGACGTGCAGATGCA-3′, digoxigenin-labeled). A third sequence (5S3-bio, 5′-CATCTTTCACCCGTAGAGGTC-3′, biotin-labeled) was used as an internal capture probe in the PCR-ELISA experiments.

Double-stranded amplifications were carried out in a final volume of 50 μL containing 2 mM $MgCl_2$, 10 pmol of each primer (5S1 and 5SP-dig), 200 ng of template DNA, and 2 U of DNA polymerase (Biotools, Madrid, Spain) in a reaction buffer containing 75 mM Tris-HCl, pH 9.0, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, and 0.001% bovine serum albumin.

The polymerase chain reaction (PCR) was carried out in a Progene Thermal Cycler (Techne Ltd., Cambridge, U.K.) programmed to perform a denaturation step of 94 °C for 3 min, followed by 35 cycles consisting of 45 s at 94 °C, 45 s at 67 °C, and 45 s at 72 °C. The last extension step was 5 min longer. The amplification products were tested in a 1.5% D1 (Hispanlab S.A., Alcobendas, Spain) agarose gel, containing ethidium bromide (1 μg/mL) in Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0). DNA fragments were visualized by UV transillumination and analyzed using a Geldoc 1000 UV Fluorescent Gel Documentation System-PC (Bio-Rad Laboratories, Hercules, CA).

Hybridization Conditions. Amplified PCR products were denatured at 100 °C for 10 min and cooled on ice. For hybridization, 2 μL PCR products were added to 98 μL hybridization solution [6 × standard saline citrate (SSC), 0.05% skim milk powder, and 100 μg/mL of denatured herring sperm DNA] in a Eppendorf tube prior to being used in the ELISA procedure described below.

PCR-ELISA Procedure. Flat-bottom micro-ELISA plates (Nunc, Roskilde, Denmark) were filled with 0.1 mL streptavidin (10 μg/mL, Sigma) diluted in phosphate-buffered saline (PBS), pH 7.2, and incubated for 1 h at 37 °C. The wells were washed five times with PBST (PBS containing 1% Tween-20) and coated with 0.2 mL denatured herring sperm DNA (1 μg/mL) (Roche) in PBS for 30 min at 37 °C. After five washes with PBST, 0.1 mL 5S3-bio biotinylated probe diluted in PBST (0.5 pmol per well) was added to the wells and the plates were incubated on a plate shaker (LKB; Pharmacia, Uppsala, Sweden) for 1 h at room temperature. Following another washing with PBST, 0.1 mL of the denatured PCR amplified products in the hybridization solution was added to the wells and the plate incubated for 2 h at 53 °C by shaking. The plates were subsequently washed once with 2 × SSC, 1% Tween for 10 min. The wells were then filled with 0.1 mL peroxidase antidigoxigenin conjugate (Roche) diluted 1:1500 in PBST and incubated for 1 h at room temperature. The plates were washed five times with distilled water before addition of 0.1 mL of the substrate solution consisting of ready-to-use 3,3′,5,5′-tetramethylbenzidine (Roche). After a 10 min incubation, the reaction was stopped by addition of 0.05 mL 1 mol/mL H_2SO_4. The yellow color developed by conversion of the substrate was measured at 450 nm with an iEMS Reader MF Spectrophotometer (Labsystems, Oy, Helsinki, Finland).

RESULTS AND DISCUSSION

The purpose of this study was the development and evaluation of a PCR-ELISA technique for the detection and quantification of Nile perch in sterilized fish mixtures. Nile perch (*L. niloticus*) is a low-quality fish species frequently sold at about $5/kg in the marketplace. Due to its low cost, when Nile perch loses its morphological characteristics it is highly susceptible to being used in fishery products for the substitution of more expensive fish species such as grouper (*E. marginatus*), hake (*M. merluccius*), bluefin (*T. thynnus*), and bonito (*S. sarda*).

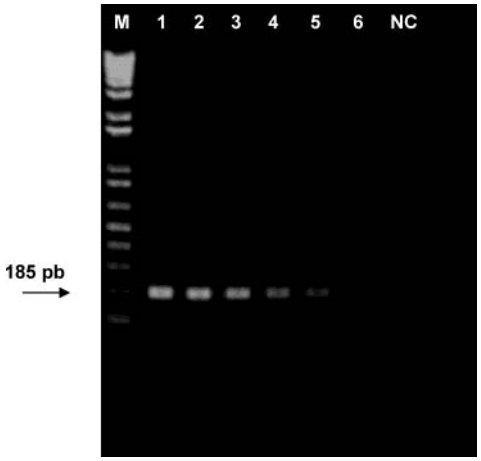

Figure 1. Agarose gel electrophoresis of PCR products obtained from DNA extracted from sterilized Nile perch/grouper mixtures. Line 1, 15% Nile perch; line 2, 10% Nile perch; line 3, 5% Nile perch; line 4, 2% Nile perch; line 5, 1% Nile perch; line 6, 100% grouper. NC = negative control. M = 1 Kb BRL marker (Gibco).

Since mitochondrial DNA is relatively more abundant than nuclear DNA in cells, evolves much faster, and thus contains more sequence diversity compared to nuclear DNA, qualitative DNA-based detection of fish species preferably relies on mitochondrial genes. However, quantitative assays should be performed using nuclear genes to avoid a large variation in target cell numbers (*22*). Thus, the PCR-ELISA developed herein uses two primers (5S1 and 5SP-dig) and an internal oligonucleotide probe (5S3-bio), complementary to DNA sequences of the nuclear 5S rDNA gene from Nile perch.

In agreement with the results obtained in a previously reported work (*9*), the reverse oligonucleotide 5SP-dig, together with the direct 5S1 primer, amplified a specific 185 bp DNA fragment in all the sterilized fish mixtures containing 15%, 10%, 5%, 2%, and 1% Nile perch. As can be seen in **Figure 1**, the intensity of the amplified DNA bands increased proportionally to the amount of Nile perch muscle tissue present in the Nile perch/grouper mixture. Similar results were obtained when DNA extracted from the binary mixtures Nile perch/hake, Nile perch/bluefin, and Nile perch/bonito was analyzed (data not shown). Optimal PCR specificity and sensitivity was achieved when primers 5S1 and 5SP-dig were assayed under the conditions described.

Optimum PCR-ELISA detection conditions were determined by adjusting the streptavidin, probe, PCR product, and conjugate concentrations. The biotinylated probe was immobilized onto streptavidin-coated microplates and was used to capture the digoxygenin-labeled fragments that were detected with peroxidase antidigoxigenin conjugate. Subsequent enzymatic conversion of the substrate gave distinct absorbance differences when assaying fish binary mixtures containing different percentages of Nile perch muscle.

Figure 2 shows the absorbance values obtained in the PCR-ELISA performed with the PCR amplification products obtained with primers 5S1 and 5SP-dig. It was observed that over a limited range of Nile perch (1−15%) in the different binary mixtures analyzed in this work, the absorbance values increased with the concentration of the PCR products obtained. In these fish mixtures, absorbance was related to the percentage of Nile perch muscle present in the samples in the range 1−15% by the equation:

$$y = 0.6135x + 0.5049$$

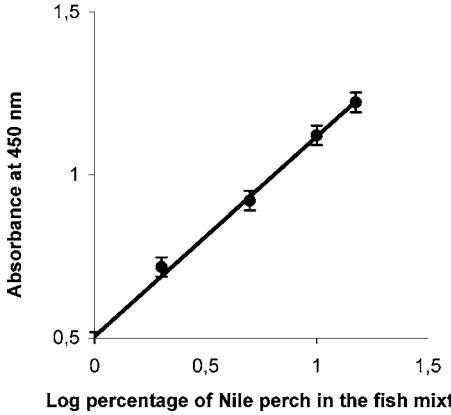

Figure 2. Relationship between absorbance values obtained in the PCR-ELISA and the logarithm of the percentage of Nile perch in the fish mixtures. Each point represents the mean of the experimental values obtained by analyzing three different mixtures in triplicate assays of each binary mixture analyzed. The bars represent the standard deviation.

with a correlation coefficient of $r = 0.9965$.

The high specificity that is crucial to avoid false positives, especially at the limit of detection (1%), was achieved in this work by verification of the DNA sequence from the PCR products using a sequence-specific hybridization probe (5S3-bio) in combination with the specific primer set. In contrast, detection of PCR products by simple agarose gel electrophoresis without further sequence verification would not be specific enough, as it is too imprecise for accurate size determination of amplicons and because artifacts similar in size to the specific product could lead to false positive results (*17*). The positive signals detected with the ELISA technique were exclusively derived from Nile perch material. No crossreactions with the other fish species of commercial significance were observed, thus reflecting a very specific detection and quantification system.

The PCR-ELISA described herein allows for the processing of numerous samples, is relatively inexpensive, and eliminates the need for electrophoretic and photographic equipment as well as the use of potential carcinogens such as ethidium bromide, which is utilized in the gel electrophoretic detection of PCR products. Based on common ELISA equipment, the PCR-ELISA can be performed with a thermocycler as the only additional apparatus needed when ELISA techniques are already set up. The time required for sample preparation and analysis is comparable to that of ELISA methods, and results can be obtained in less than one working day (*17*, *23*).

In conclusion, the potential of the PCR-ELISA technique describe herein for quantification of Nile perch in admixtures has been confirmed to a detection limit of 1%. This methodology should prove useful for enforcing labeling regulations in the authentication of sterilized or otherwise-processed fish products and as a routine analysis in food control laboratories.

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