JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Identification of Grouper (*Epinephelus guaza*), Wreck Fish (*Polyprion americanus*), and Nile Perch (*Lates niloticus*) Fillets by Polyclonal Antibody-Based Enzyme-Linked Immunosorbent Assay

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An indirect enzyme-linked immunosorbent assay (ELISA) has been developed for the species identification of grouper (*Epinephelus guaza*), wreck fish (*Polyprion americanus*), and Nile perch (*Lates niloticus*) fillets. The assay was performed in two different formats, microtiter plates and immunostick tubes, and uses polyclonal antibodies raised in rabbits against muscle-soluble proteins of grouper (anti-GSP), wreck fish (anti-WSP), and Nile perch (anti-PSP). The antibodies were made species-specific by blocking them with the heterologous soluble muscle proteins. Immunorecognition of polyclonal antibodies adsorbed to their specific fish samples was made with swine antirabbit immunoglobulins conjugated to the enzyme horseradish peroxidase. Subsequent enzymatic conversion of the substrate allowed unequivocal identification of the species studied.

KEYWORDS: Fish species identification; ELISA; *Epinephelus guaza*; *Polyprion americanus*; *Lates niloticus*

INTRODUCTION

The ability to identify fish species following the removal of external characteristics by processing such as canning, smoking, and filleting, although problematic, is of great commercial importance. Once the morphological characteristicss have been removed during processing, the identification becomes difficult and there is a risk of fraudulent substitution of lower-valued fish species for high-valued fish in seafood products (*1*).

Grouper (*Epinephelus guaza*), wreck fish (*Polyprion americanus*), and Nile perch (*Lates niloticus*) represent commercially important fish species that are frequently subjected to adulteration when they are sold as fillets in the marketplace. Nile perch fillets are many times labeled and marketed either as grouper or as wreck fish because, with the three fish species being similar in texture, the quality attributes and price are higher for the last two ($20−30/kg versus $5−10/kg). Additionally, grouper and wreck fish are closely related species that may be misidentified in the marketplace and are commonly sold as grouper, which is more demanded by consumers. To prevent fraudulent fish substitutions, food laboratories need to have available techniques to ascertain the fish species used in the manufacture of fish products (*2*).

Chromatographic and electrophoretic techniques have proved to be useful in fish species identification (*3*−*5*). However, although they are of considerable value in certain instances, these methods are not convenient for routine sample analyses because they are relatively costly, time-consuming, and complex to perform. Consequently, fish species identification has been mostly performed in the past few years by genetic (*6*−*9*) and immunological techniques (*10*−*12*) that are suitable for routine analysis of a large number of samples.

Genetic techniques are the most specific and sensitive methods for species identification. However, they require some expensive laboratory equipment and a certain degree of expertise. Immunological techniques are not only inexpensive but also simpler to develop. Applications of immunological assays for the detection and quantification of microorganisms, pesticides, hormones, antibiotics, mycotoxins, and proteins in meat and milk products have been well documented (*13*−*19*). In contrast, work relating to fish species identification is scarce partly because of the variety of fish species that are commercialized (*11*, *12*). The use of immunoassays, especially the enzyme-linked immunosorbent assay (ELISA), is therefore an interesting approach to the development of rapid methods for assessing the authenticity of fish products.

We report in this work the accurate specific identification of grouper (*Epinephelus guaza*), wreck fish (*Polyprion america-*

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10.1021/jf026020x CCC: $25.00
Published on Web 01/23/2003

nus), and Nile perch (*Lates niloticus*) fillets by using two indirect ELISA formats: microtiter plates and immunostick tubes.

MATERIAL AND METHODS

Fish Samples. Nile perch (*Lates niloticus*), grouper (*Epinephelus guaza*), and wreck fish (*Polyprion americanus*) samples were obtained from Mercamadrid (Madrid, Spain) and other local markets. Every specimen was morphologically identified according to the keys of Bauchot and Pras (*20*) and Corbera et al. (*21*). The samples were obtained fresh and transported to the laboratory under refrigeration conditions. At arrival, they were immediately processed by aseptically cutting small muscle portions (1−2 g) and subsequently storing the samples at −20 °C until use.

Preparation of Antigenic Extracts. Antigenic extracts from 15 to 30 different individuals of grouper, wreck fish, and Nile perch were prepared as follows. Muscle samples (40 g) were thoroughly homogenized at room temperature in 200 mL of saline solution (8.5 g of NaCl/L). Homogenates were maintained overnight at 4 °C with shaking before centrifugation at 3500*g* for 30 min at 10 °C. Supernatants containing muscle-soluble proteins were filtered through borosilicate glass filter (pore diameter of 40−90 μm) using a vacuum aspirator A-3S (Eyela, Tokio, Japan) and then lyophilized. The dried proteins were stored at −20 °C until use.

The protein content of the lyophilized extracts was calculated with the Bio-Rad protein assay (Bio-Rad Laboratories GmbH, Munchen, Germany) using albumin fraction V from bovine serum (BSA) (Merck, Darmstadt, Germany) as the protein standard.

Production of Polyclonal Antisera. Polyclonal antibodies against muscle-soluble proteins from grouper (anti-GSP), wreck fish (anti-WSP), and Nile perch (anti-PSP) were raised in New Zealand male white rabbits. Immunization commenced by intradermic injection at multiple dorsal sites of 5 mg of the selected lyophilized protein extract in 0.5 mL of saline solution (8.5 g of NaCl/L), emulsified in 0.5 mL of Freund's complete adjuvant (Difco Laboratories, Detroit, MI). Six booster doses made in Freund's incomplete adjuvant were applied by intradermic, subcutaneous, or intramuscular injection alternately every 2 weeks. The immunization schedule was completed after 3 months. The blood was collected 10 days later. It was allowed to clot for 1 h at room temperature and then overnight at 4 °C. The serum was recovered by centrifugation at 2000*g* for 10 min at 4 °C.

After crude antisera from rabbits were obtained, immunoglobulins were purified by ammonium sulfate precipitation (*22*). Samples of precipitated antibodies were stored frozen at −20 °C.

Blocking of Polyclonal Antibodies. Polyclonal antibodies against soluble muscle proteins from grouper (anti-GSP), wreck fish (anti-WSP), and Nile perch (anti-PSP) were diluted 1/2000 in phosphate-buffered saline (PBS: 0.14 M NaCl, 0.0015 M $KH_2PO_4 \cdot 12H_2O$, 0.0027 M KCl, pH 7.2) containing 1% Tween 20 and 1% skimmed milk powder. The diluted antibodies were then made species-specific by mixing with an appropriate amount of antigenic extracts from the heterologous species analyzed in this work. The anti-GSP was blocked with 0.5 mg of each WSP and PSP extract per milliliter of antiserum. Similarly, the anti-WSP was blocked with 0.5 mg/mL of GSP and PSP, and the anti-PSP was blocked with 0.5 mg/mL of GSP and WSP antigenic extracts.

The mixtures of each antiserum with the heterologous antigenic extracts were incubated for 1 h at 37 °C and then used in the two indirect ELISA formats, microtiter plates and immunostick tubes.

Preparation of Fish Samples for Analysis. Grouper, wreck fish, and Nile perch muscle samples (1 g) were homogenized in 5 mL of saline solution (8.5 g of NaCl/L) and maintained for 10 min in a stomacher. Extracts were filtered through Whatman no. 1 filter paper and diluted 1/50 in PBS before they were used for ELISA analysis.

Other fish samples of salmon (*Salmo salar*), trout (*Oncorhynchus mykiss*), bream (*Brama raii*), sole (*Solea solea*), European plaice (*Pleuronectes platessa*), flounder (*Platichthys flesus*), Greenland halibut (*Reinhardtius hippoglossoides*), conger eel (*Conger conger*), and cod (*Gadus morhua*) were also prepared for subsequent analysis. These fish species were obtained fresh and were transported, stored, and processed as described for grouper, wreck fish, and Nile perch samples.

Indirect ELISA Using Microtiter Plates. Flat-bottomed micro-ELISA plates (Costar, Corning, NY) were filled with 0.1 mL of the filtered muscle sample extracts diluted in phosphate-buffered saline (PBS) and incubated for 1 h at 37 °C. The wells were washed with PBS and coated with 0.2 mL of albumin bovine serum (BSA) for 30 min at 37 °C. After washing with PBST (PBS containing 1% Tween 20), 0.1 mL aliquots of the blocked polyclonal antibodies were added to the wells and the plates were shaken for 1 h at room temperature. After washing with PBST, 0.1 mL aliquots of peroxidase-conjugated swine antirabbit immunoglobulins (DAKO, Glostrup, Denmark), diluted 1/2000 in PBST, were added to the wells, and the plates were incubated with shaking for 1 h at room temperature. Wells were washed with distilled water before addition of 0.1 mL of a ready-to-use substrate solution of 3,3′,5,5′-tetramethylbenzidine (Boehringer Mannheim GmbH, Mannheim, Germany). After a 10 min incubation, the reaction was stopped by the addition of 0.05 mL of 1 M H_2SO_4 to each well. The yellow color developed by conversion of the substrate was measured at 450 nm with an iEMS Reader MF spectrophotometer (Labsystems, Oy, Helsinki, Finland).

Indirect ELISA Using Immunostick Tubes. The paddles of immunostick tubes (Nunc, Roskilde, Denmark) were coated by adding to the tubes 1 mL of the filtered sample extracts diluted 1/50 in PBS. After 20 min of incubation, the paddles and tubes were washed using cold running tap water. The paddles were blocked by adding 1 mL of gelatin (Difco, Detroit, MI) to the tubes and incubating for 15 min. After another washing step, 1 mL of the species-specific polyclonal antibodies was added, and the tubes were incubated for 15 min. Subsequently, the paddles and tubes were washed and filled with 1 mL of peroxidase-conjugated swine antirabbit immunoglobulins (DAKO) diluted 1/2000 in PBSTM. After 15 min of incubation, the paddles were washed and introduced in clean tubes containing 1 mL of a ready-to-use substrate solution of 3,3′,5,5′-tetramethylbenzidine. A positive reaction was identified by the development of a blue color. All the incubation steps in this ELISA format were performed at room temperature.

RESULTS AND DISCUSSION

The aim of the present work was to develop a simple colorimetric ELISA using microtiter plates and immunostick tubes for the specific identification of grouper (*Epinephelus guaza*), wreck fish (*Polyprion americanus*), and Nile perch (*Lates niloticus*) fillets.

Polyclonal antisera used in this work were raised in rabbits against sarcoplasmic proteins of grouper (GSP), wreck fish (WSP), and Nile perch (PSP) species. The sarcoplasmic proteins were chosen because they may vary both qualitatively and quantitatively among closely related species (*23*).

Immunoglobulins from each polyclonal antiserum were purified by ammonium sulfate precipitation in order to minimize further nonspecific interactions (*22*). However, from an indirect ELISA, the anti-GSP, anti-WSP, and anti-PSP antibodies showed cross-reactivities against all antigenic extracts tested (data not shown). This cross-reactivity was expected because the antigenic extracts used for immunization contained all the soluble muscle proteins from the samples and there might be many shared epitopes in the same proteins from closely related species (*24*). Therefore, if the immunosera are to be used for the specific detection of individual fish species, the antibodies producing cross-reactivities must be removed.

The simple antibody−antigen blocking technique (*24*), in which the proteins with which cross-reactivity is not desired are added to the antiserum to block the antibodies directed at them, has been extensively demonstrated to be an effective method for removing cross-reactions (*12*, *25*). This technique was therefore the approach taken to adjust the specificity of the polyclonal antisera. The effectiveness of the blocking procedure for the anti-GSP, anti-WSP, and anti-PSP antibodies

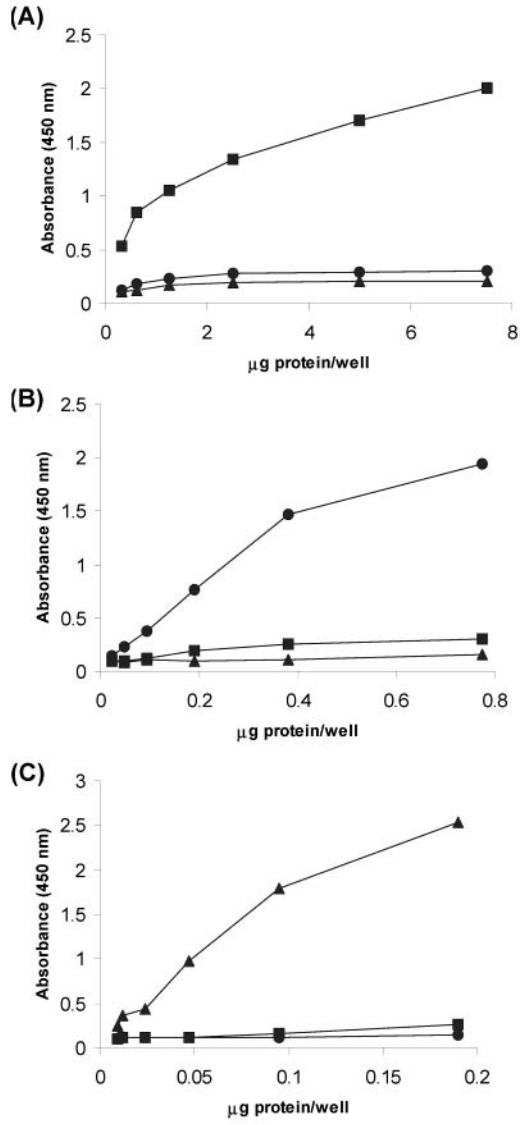

Figure 1. Indirect ELISA results for the analysis of muscle-soluble proteins from grouper (■), wreck fish (●), and Nile perch (▲), using anti-GSP (A), anti-WSP (B) and anti-PSP (C) polyclonal antibodies after blocking with the heterologous antigenic extracts. The blocked antibodies were diluted 1:2000 in PBSTM, and the swine antirabbit immunoglobulins were diluted 1:2000 in PBST.

is shown in **Figure 1**. Optimal conditions for the assay were obtained using the antigenic extracts (0−10 μg/well), the blocked antibodies (1/2000), and the peroxidase-conjugated swine antirabbit immunoglobulins (1/2000). Once the polyclonal antibodies were blocked with the heterologous proteins, cross-reactivity against heterologous species was eliminated and each blocked antiserum recognized only the species against which it was produced.

The blocked antibodies were further used in an indirect ELISA in microtiter plates for the specific identification of a number of grouper, wreck fish, and Nile perch commercial samples obtained from different marketplaces. An total of 30 Nile perch samples and 15 samples of each grouper and wreck fish were tested against the three species-specific polyclonal antibodies obtained in this work (**Table 1**). The absorbance values of negative samples for each immunoserum were similar to those of the background readings (0.092−0.2), while positive samples reached values higher than 2.0 in the different antiserum. Moreover, we verified the specificity of these antibodies

Table 1. Indirect ELISA Results for the Analysis of Commercial Grouper, Wreck Fish, and Nile Perch Samples[a]

		absorbance[b] (450 nm)		
fish samples	*n*[c]	anti-GSP	anti-WSP	anti-PSP
grouper	15	1.923 ± 0.25	0.272 ± 0.05	0.226 ± 0.02
wreck fish	15	0.321 ± 0.02	1.978 ± 0.35	0.154 ± 0.06
Nile perch	30	0.242 ± 0.05	0.141 ± 0.35	2.214 ± 0.12

[a] The blocked antibodies were diluted 1:2000 in PBSTM, and the swine antirabbit immunoglobulins were diluted 1:2000 in PBST. [b] Data are the mean ± standard deviation. Background readings were 0.092–0.200. [c] Number of individual samples tested.

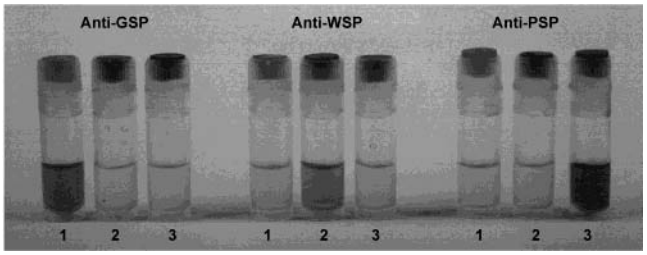

Figure 2. Immunostick ELISA results for the identification of grouper (1), wreck fish (2), and Nile perch (3), using the anti-GSP, anti-WSP, and anti-PSP species-specific antibodies. The blocked antibodies were diluted 1:2000 in PBSTM, and the swine antirabbit immunoglobulins were diluted 1:2000 in PBST.

by testing them against other fish species: salmon (*Salmo salar*), trout (*Oncorhynchus mykiss*), bream (*Brama raii*), sole (*Solea solea*), European plaice (*Pleuronectes platessa*), flounder (*Platichthys flesus*), Greenland halibut (*Reinhardtius hippoglossoides*), conger eel (*Conger conger*), and cod (*Gadus morhua*). The results of the indirect ELISA performed (data not shown) proved that each blocked antiserum recognized only the species against which it was produced. According to these results, the indirect ELISA developed in this work allows clear identification of fish fillets from grouper, wreck fish, and Nile perch.

An immunostick colorimetric ELISA was also developed for faster identification of the fish species studied. A total of 15 individual samples each of grouper and wreck fish and 30 samples of Nile perch were tested against the three species-specific polyclonal antibodies obtained in this work. The blue color developed by enzymatic conversion of the substrate solution allowed the accurate visual identification of grouper, wreck fish, and Nile perch fillets (**Figure 2**). This ELISA format, which can be performed in less than 1 h, could be useful for the commercial production of stable kits that contain all the necessary components and supplies. Moreover, the simplicity of the procedure and the short time required for the analysis make it suitable for screening purposes in inspection programs without the need for laboratory equipment.

In conclusion, the two indirect ELISA formats described in this work are powerful approaches for the specific identification of grouper, wreck fish, and Nile perch. Since ELISA techniques are specific, sensitive, cheap, and very simple to develop, they should prove to be useful as routine analysis tools in food control laboratories for enforcing labeling regulations in the authentication of these fish species.

ACKNOWLEDGMENT

The authors are grateful to Ángel Mendizábal (S.V.O., Mercamadrid) for kindly supplying fish samples and to Pilar Bringas (Centro de Asistencia a la Investigación "Animalario de la

Universidad Complutense", Madrid, Spain) for her help in immunizing and bleeding the rabbits used to produce the antisera.

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Received for review October 9, 2002. Revised manuscript received December 16, 2002. Accepted December 17, 2002. This work was supported by the Comunidad Autónoma de Madrid (Project 07G/0027/2000). Miguel A. Rodríguez and Belén Mayoral are recipients of fellowships from the Comunidad Autónoma de Madrid. Inés López-Calleja and Luis Asensio are recipients of fellowships from the Universidad Complutense de Madrid and the Ministerio de Educación y Ciencia.

JF026020X