Development of an Enzyme-Linked Immunosorbent Assay for the Identification of Smoked Salmon (Salmo salar), Trout (Oncorhynchus mykiss) and Bream (Brama raii)

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ABSTRACT

An indirect enzyme-linked immunosorbent assay (ELISA) has been developed for the identification of smoked meat from salmon (Salmo salar), trout (Oncorhynchus mykiss), and bream (Brama raii). The assay uses polyclonal antibodies raised in rabbits against soluble proteins of muscle from salmon (anti-SSP), trout (anti-TSP), and bream (anti-BSP) which are rendered species-specific by blocking them with the heterologous soluble muscle proteins. The blocked antibodies were used to detect the samples from smoked fish bound to the wells of a microtiter plate. Immunorecognition of polyclonal antibodies adsorbed to fish samples was made with goat anti-rabbit immunoglobulins conjugated to the enzyme horseradish peroxidase. Subsequent enzymic conversion of the substrate allowed clear species identification of smoked meat of salmon, trout, and bream.

Key words: fish species identification, smoked fish, ELISA, Salmo salar, Oncorhynchus mykiss, Brama raii

The marketing of processed fishery products instead of intact fish is increasing. Processing removes the external features which provide a ready means of species identification and, consequently, there is a risk of willful or unintentional adulteration by substituting lower-quality and/or lower-priced seafood products for higher-priced products.

Identification of fish species and seafood products has become relevant because of the labeling regulations imposed by various countries (18, 25). One of the prerequisites for complying with labeling regulations is the existence of a number of techniques that allow the characterization of different species and that could be employed for different food products. Several electrophoretic methods such as starch-gel zone electrophoresis, acrylamide-disc electrophoresis, thin-layer polyacrylamide-gel isoelectric focusing and cellulose acetate strip have been accepted as official methods by the Association of Official Analytical Chemists (3) to differentiate seafood species or seafood products. Methods such as capillary zone electrophoresis (8, 15), liquid chromatography (19) and high-performance liquid chromatography (HPLC) (4) also have been reported. However, these methods are laborious and time consuming and require substantial equipment. Moreover, a large data bank of various seafood species is needed for effective protein profile comparison (1).

The use of immunoassays by the food industry is increasing steadily. The specificity and sensitivity of an antibody for an antigen have allowed the quantification of a variety of food components without the extraction and separation steps that are necessary in more conventional methods of analysis (24). Applications of immunological assays for the detection and quantification of hormones, microorganisms, pesticides, antibiotics, mycotoxins, and proteins in meat and milk products have been well documented (2, 7, 10, 12, 17, 22). In contrast, work relating to fish species identification is scarce (1, 27, 28), partly due to the variety of fish species that are commercialized. Enzyme-linked immunosorbent assays (ELISAs) have been prepared as commercial test kits for rapid screening of a variety of compounds in food products (7, 22). These assays can be performed rapidly in the field or processing plant on a large number of samples by minimally trained personnel, with small quantities of solvents and with no major scientific equipment.

We report in this communication the production of polyclonal antibodies against soluble muscle proteins of smoked meat from three species of fish and the development of an indirect ELISA to identify smoked meat from salmon (Salmo salar), trout (Oncorhynchus mykiss), and bream (Brama raii).

MATERIALS AND METHODS

Preparation of the antigenic extracts

Smoked salmon (Salmo salar), trout (Oncorhynchus mykiss), and bream (Brama raii) samples were provided by Ahumados Dominguez, Alcorcón, Madrid.

Antigenic extracts from salmon, trout, and bream samples
were prepared from 2-kg batches of trimmed, well-mixed, lean smoked muscle. Representative 200-g samples were thoroughly homogenized in 1 liter of a saline solution (8.5 g of NaCl per liter) at 20°C using a mechanical blender (Polytron® SEV, Kinematica, Lucerne, Switzerland). The proteins were extracted by gentle agitation of these homogenates for 2 h at 20°C. The sarcoplasmic extracts were centrifuged at 3,500 × g for 30 min at 10°C and the supernatants were filtered though a Whatman no. 1 filter paper and lyophilized. The dried proteins were placed in an airtight container and stored at −20°C until use.

The protein content of the antigenic extracts was calculated according to the method of Markwell et al. (16).

**Production of polyclonal antisera**

Antibodies against muscle soluble proteins from smoked salmon (SSP), trout (TSP), and bream (BSP) were raised in New Zealand male white rabbits. Immunization commenced by intradermic injection at multiple dorsal sites of the selected lyophilized protein extract (5 mg) in 0.5 ml of deionized and distilled water emulsified in 0.5 ml of Freund's Complete Adjuvant (Difco, Detroit, MI, USA). Nine booster doses made in Freund's Incomplete Adjuvant were applied alternatively by intradermic, subcutaneous, or intramuscular injection every 14 days. After 120 days the rabbits were bled and the blood was allowed to clot for 1 h at room temperature. Serum was collected by centrifugation at 2,000 × g for 10 min.

Immunoglobulins from the crude antisera were recovered by ammonium sulfate precipitation to minimize further nonspecific interactions (11). Samples of precipitated antibodies (1 ml) were stored frozen at −20°C.

**Blocking of polyclonal antibodies**

Polyclonal antibodies against soluble muscle proteins from smoked salmon (anti-SSP), trout (anti-TSP), and bream (anti-BSP), diluted 1:2,000 in phosphate-buffered saline (PBS: 0.14 M NaCl, 0.0015 M KH₂PO₄, 0.081 M Na₂HPO₄ · 12 H₂O, 0.0027 M KCL, pH 7.2) containing 1% Tween 20 and 1% skimmed milk powder, were made species-specific by mixing with an appropriate amount of antigenic extracts from the heterologous species of smoked fish. The anti-SSP was blocked with 0.5 mg of TSP and 0.5 mg of BSP per ml of diluted antiserum. The anti-TSP was blocked with 1.5 mg each of SSP and BSP antigenic extracts and the anti-BSP was blocked with 0.1 mg each of SSP and BSP 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 indirect ELISA.

**Preparation of smoked fish samples for analysis**

Samples (40 g) of smoked salmon, trout, and bream were homogenized in 200 ml of saline solution (8.5 g of NaCl/liter) and maintained for 2 h at 20°C with shaking before centrifugation at 3,500 × g for 30 min at 10°C. Supernatants were filtered through a Whatman no. 1 filter paper and stored frozen at −20°C until used for analysis.

To be used in the indirect ELISA developed in this work, salmon samples were diluted 1:300 in PBS. trout samples were diluted 1:45, and bream samples were diluted 1:800 in the same buffer.

**Indirect ELISA**

Flat-bottomed micro-ELISA plates (Costar, Cambridge, MA, USA) were inoculated with 0.1 ml of the sample extracts diluted in PBS, pH 7.2, and incubated for 1 h at 20°C. The wells were washed five times with PBST (PBS containing 1% Tween 20) and coated with 0.2 ml of 0.1% gelatin in PBS for 30 min at 20°C. After five washes with PBST, 0.1-ml aliquots of the blocked antibodies diluted 1:2,000 in PBSTM (PBST containing 1% skimmed milk powder) were added to the wells and the plates incubated on a plate shaker for 1 h at 20°C. After washing five more times with PBSTM to remove heterologous antigens and unattached antibodies, 0.1-ml aliquots of peroxidase-conjugated goat anti-rabbit immunoglobulins (DAKO, DK 2600 Glostrup, Denmark), diluted 1:2,000 in PBSTM were added to the wells, and the plates were incubated with shaking for 1 h at 20°C. Wells were washed five more times with distilled water before addition of 0.15 ml of a ready-to-use substrate solution of 3,3',5,5'-tetramethylbenzidine (Boehringer Mannheim GmbH, 6800 Mannheim 31, Germany). After a 10-min incubation, the reaction was stopped by addition to each well of 0.05 ml of 1 M H₂SO₄. The yellow color developed by conversion of the substrate was measured at 450 nm with a Titertek Multiskan Plus Spectrophotometer (Flow Laboratories, McLean, VA, USA).

**RESULTS AND DISCUSSION**

Smoked salmon (Salmo salar), trout (Oncorhynchus mykiss), and bream (Brachyrahi Brama raiI) are three popular fish products with similar market presentations, which are frequently offered sliced and used to prepare snack food. Smoked salmon and trout are usually very difficult to distinguish by eye and taste even for trained people, and there is a risk of fraudulent substitution for the more expensive smoked salmon by the cheaper smoked trout. Smoked bream exhibits a color and morphological characteristics different from those of salmon and trout. However, smoked bream slices are generally stained and offered to the consumer as a substitute for smoked salmon or trout.

The cold smoking process for salmon, trout, and bream yields products with an extended shelf life due to their reduced water activity, which results from a large loss of moisture, the action of salt in rather high concentration and the contribution of smoke components (23). In contrast to hot smoking, where the temperature may be as high as 80 to 90°C, cold smoking is performed at temperatures lower than 30°C (20) and this treatment should not denature sarcoplasmic proteins. Dincer et al. (6) studied the effects of curing and cooking on the detection of species origin of meat products; they did not find a significant loss of antigenic determinants of sarcoplasmic proteins due to curing agents. However, in order to obtain the best sensitivity of the immunoassay developed in this work, polyclonal antibodies have been raised in rabbits against soluble muscle proteins from smoked salmon (anti-SSP), trout (anti-TSP) and bream (anti-BSP).

Immunoglobulins from each polyclonal antiserum were recovered by ammonium sulfate precipitation. However, when those antibodies were tested in an indirect ELISA format, strong cross-reactivities were found against the antigenic extracts from the heterologous species (Figure 1). This cross-reactivity was expected since the antigenic extracts used for immunization contained all the soluble muscle proteins from the smoked samples and there might be many shared epitopes in the same proteins from closely related species (14). Therefore, if the immunosera are to be used for the specific detection of individual fish species, the antibodies producing cross-reactivities must be removed.

To be used in the indirect ELISA developed in this
work, the anti-SSP, anti-TSP, and anti-BSP polyclonal antibodies were rendered species-specific by mixing them with lyophilized antigenic extracts from the heterologous species in a process known as "blocking." The blocking treatment is an effective procedure to remove cross-reactions (9, 13) without the need for affinity purification through columns containing the heterologous antigens in a bound form. This procedure has also been successfully used to reduce cross-reactivities from an anti-sardine (Sardina pilchardus) antiserum, but could not improve the specificity of an immunoassay to detect adulteration of tuna (Thunnus spp. or Euthynnus spp.) with bonito (Sarda spp.) (27).

The effectiveness of the blocking procedure for the anti-SSP, anti-TSP, and anti-BSP antibodies is shown in Figure 2. Once the cross-reacting antibodies were blocked by the heterologous proteins, each antiserum recognized only the antigenic extract against which it was produced. However, it should be noted that the sensitivity of the anti-TSP antibody after blocking was lower than that of anti-SSP and anti-BSP antibodies. Probably the lower sensitivity of the anti-TSP antiserum after blocking could be explained because this immunoserum contains more cross-reacting antibodies than the anti-SSP and anti-BSP antisera. In fact, the amount of heterologous antigenic extracts needed to reduce cross-reactivity of this immunoserum is higher than the amount needed for the blocking of the anti-SSP and anti-BSP antisera. Therefore, when the cross-reacting antibodies of the anti-TSP were blocked, the amount of available species-specific antibodies in the antisera was substantially reduced.

The blocked antibodies produced were further used in an indirect ELISA for the specific identification of the commercial smoked fish samples. In the indirect ELISA developed in this work, the soluble proteins from smoked salmon, trout, and bream were bound to the wells of a microtiter plate and detected with the blocked anti-SSP, anti-TSP, and anti-BSP antibodies. After the desired antigen-antibody reactions had occurred in the wells, the washing steps removed the remaining heterologous antigens and the products of the blocking interaction. Further recognition of the polyclonal antibodies bound to the fish proteins was made with the goat anti-rabbit immunoglobulins conjugated to horseradish peroxidase. A tetramethylbenzidine substrate solution was used as peroxidase chromogen. The substrate turns blue in wells containing the homologous antigen; color development was measured spectrophotometrically at 450 nm after stopping the reaction with sulphuric acid to yield a stable yellow end-product. However, since the tetramethylbenzidine forms a dark blue color in the presence of
peroxidase, visual identification of the samples was very clear before the reaction was stopped.

Five samples of different brands of smoked salmon, trout, and bream were tested by the indirect ELISA against the three species-specific polyclonal antibodies obtained in this work (Table 1). It should be stressed that the absorbance values of negative samples for each immunoserum are similar to those of the background, while positive samples reach values higher than 1.2, 1.5, or 2.5 depending on the antisera. According to these results, the indirect ELISA developed in this work allows clear identification of smoked samples of *Salmo salar*, *Oncorhynchus mykiss*, and *Brama raii*. As far as we know, this is the first reported immunosay for the species identification of smoked fish.

Immunological methods like the ELISA described herein allow unequivocal identification of fish samples. The assay is easy to perform, sensitive, and specific and does not require the use of sophisticated apparatus. Therefore, it offers several advantages compared to other chromatographic and electrophoretic techniques where the interpretation of protein band patterns is time-consuming and requires comparison to standard proteins or reference samples that could lead to misidentification of unknown samples (26).

However, for immunoassay techniques to succeed on a large scale they must meet certain standards such as reproducibility, ease of use, and good shelf life (27). The only way to meet these criteria is through commercialization, with the involvement of strict quality-control measures established by industry and tested by government agencies (5). We are interested in cooperative and collaborative trials in order to develop a commercial version of this test.

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**REFERENCES**


**TABLE 1. ELISA results for the analysis of smoked fish samples**

<table>
<thead>
<tr>
<th>Smoked fish sample</th>
<th>Absorbance (450 nm)</th>
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<tbody>
<tr>
<td><em>Salmo salar</em></td>
<td>1.861 ± 0.29</td>
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<tr>
<td><em>Oncorhynchus mykiss</em></td>
<td>0.465 ± 0.21</td>
</tr>
<tr>
<td><em>Brama raii</em></td>
<td>0.253 ± 0.34</td>
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</tbody>
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<table>
<thead>
<tr>
<th></th>
<th>anti-SSP</th>
<th>anti-TSP</th>
<th>anti-BSP</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmo salar</em></td>
<td>0.314 ± 0.02</td>
<td>0.293 ± 0.07</td>
<td></td>
</tr>
<tr>
<td><em>Oncorhynchus mykiss</em></td>
<td>1.256 ± 0.35</td>
<td>0.302 ± 0.16</td>
<td></td>
</tr>
<tr>
<td><em>Brama raii</em></td>
<td>0.302 ± 0.04</td>
<td>2.532 ± 0.12</td>
<td></td>
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