Sandwich ELISA for Detection of Pig Meat in Raw Beef Using Antisera to Muscle Soluble Proteins

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ABSTRACT

A double-antibody sandwich ELISA (enzyme-linked immunosorbent assay) has been successfully developed for the detection of defined amounts of pig meat (1-50%) in raw beef. Antibodies against pig sarcoplasmic extracts were produced in rabbits. Pig-specific antibodies were affinity purified by removing antibodies which crossreacted with horse, chicken or beef extracts followed by immunoadsorption and elution from a pig-extract column. The ELISA involved capturing antigens in sarcoplasmic extracts with pig specific antibodies immobilized on 96-well plates, detecting bound antigen with pig specific horseradish peroxidase-labeling antibody, and measuring peroxidase activity by the conversion of a clear substrate to a colored product.

The possibility that unheated meat products may contain the flesh of species not indicated by the product description, whether by accident or intention, is certainly not a new problem. While improvements of technology and processing have led to more economical transportation and utilization of deboned carcass meat, they have also facilitated the use of undeclared meats, because unequivocal identification of species becomes very difficult once meat has been taken off the carcass and the anatomical features have been destroyed. Meats of different animal species are virtually impossible to distinguish by eye once they have been frozen in large blocks, or flaked and incorporated into comminuted meat products. A simple, rapid test would make it possible to determine if undeclared meat had been added to a product thereby giving the manufacturers and consumers greater protection.

Enzyme-linked immunosorbent assay (ELISA) has emerged recently as a rapid, convenient method of assaying antigens and antibodies and it may have an immense range of applications for measuring components of foods which are capable of acting as antigens. The method has been applied to the species identification of fresh meat (11,12,15,23,24,34). These investigators have employed in the assay antisera against whole blood serum or species-specific serum albumins.

However, we think and this has also been stated by others (9), that even when the antisera against serum proteins may constitute a fairly good qualitative reagent to identify meat species in undeclared meat mixtures, it may have problems to quantify the extent of meat substitution since the concentration of those proteins in meat is affected by the residual blood left in the muscle tissue after slaughter (33). Thus it would be expedient to raise species-specific antibodies to a less variable component of meat (e.g. muscle or specific muscle proteins), making it possible to determine more accurately the percentage of different types of meat in a meat product.

The proteins of the muscle can be roughly categorized into contractile, soluble and insoluble fractions. The soluble fraction can be extracted from muscle with water or dilute salt solutions and comprises mostly enzymes of the intermediate metabolism, the pigment myoglobin and the serum albumins. The muscle soluble protein fraction is also known as the sarcoplasmic extract.

Whereas the contractile proteins of vertebrates skeletal muscle show a universal similarity, and are often indistinguishable regardless of source, the sarcoplasmic proteins of skeletal muscle vary both qualitatively and quantitatively even between closely related species (10,17,27,28). For this reason and based on previous work carried out in our laboratory (3,4,5,6) on the detection by immunoelectrophoresis in agarose gels of species-specific muscle soluble proteins on each animal species tested, we propose to use species-specific polyclonal antibodies against sarcoplasmic extracts to detect the presence of undeclared meat in unheated meat mixtures.

We describe in this report the use of affinity chromatography purified pig-specific antibodies and a double-antibody sandwich ELISA to detect and quantify the presence of pig muscle soluble proteins in raw beef.

MATERIALS AND METHODS

Preparation of the antigenic extracts

Antigenic extracts from pig, horse, chicken and beef meat were prepared from ca. 1 kg batches of trimmed, well mixed, lean meat. Representative 100 g samples were thoroughly homogenized in 300 ml of a saline (0.85% NaCl) solution and the proteins were extracted by gentle agitation of these homogenates for 24 h at 4°C. The sarcoplasmic extracts were centrifuged at 1500
g for 5 min and the supernatants were filtered through a Whatman No. 1 filter paper and lyophilized. The dried proteins were placed in an airtight container and stored at -20°C until use.

**Pig-specific antibodies**

Antibodies against pig sarcoplasmic extracts were raised in New Zealand male white rabbits. Immunization commenced by subcutaneous injection at multiple sites along the back of lyophilized pig protein extracts (50 mg) in 2 ml of deionized and distilled water emulsified in 0.5 ml of Freund’s Complete Adjuvant (Difco Laboratories, Detroit, MI, USA). Ten booster doses were applied subcutaneously every 5 d. After 50 d, the rabbits were bled, the blood allowed to clot for 1 h at room temperature and the serum was collected by centrifugation at 2000 g for 10 min. Samples of the serum (1 ml) were stored frozen at -20°C.

Antibodies in the crude antisera were rendered species-specific by immunoadsorption chromatography. The isolation of the pig-specific fraction was carried out passing 8-ml samples of the crude anti-pig serum, diluted 1:2 with 2x concentrated phosphate buffered saline (PBS), pH 7.2 containing Tween 80 (0.05%), through four affinity chromatography columns. Each column contained 3 g of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) coupled, according to the manufacturers instructions, to 52.5 mg of sarcoplasmic extracts from either horse, chicken, beef or pig. The crude anti-pig serum was deposited in the first column containing the horse sarcoplasmic extracts and elution was performed with PBS buffer, pH 7.2. The optical density of the eluted fractions (6 ml) was read at 280 nm and the content of the fractions showing an OD higher than 0.1 was mixed and deposited in a second column containing the chicken sarcoplasmic extracts. The content of this column was eluted as described above and the fractions showing an OD higher than 0.1 were mixed and deposited in a third column containing the beef sarcoplasmic extracts. The eluted fractions from this column showing an OD higher than 0.1 were mixed and deposited in a fourth column containing the pig sarcoplasmic extracts. When the absorbance values of the eluted fractions reached zero, the absorbed pig-specific antibodies (anti-PSP) were released from the pig column by elution with 0.1 M glycine-HCl buffer, pH 2.5. The eluted fractions showing an absorbance at 280 nm higher than 0.1 were pooled, adjusted to pH 7.2 with solid TRIS (Merck, Darmstadt, FRG) and dialysed overnight against PBS, pH 7.2. Samples of 1.0 ml each were stored frozen at -20°C. Affinity chromatography was performed at 4°C in a refrigerated walk-in room. This immunopurification step was performed several times to obtain enough concentration of anti-PSP to be utilized in the ELISA experiments.

**Agarose gel immunoelectrophoresis**

The basic technique was that of Grabar and Williams (8), modified by Scheidegger (25). Consequently, only the details relevant to this application are given here. One percent agarose gel in veronal buffer, pH 8.6, was used. The gels were punched with a well cutter, giving a hole of 1 mm diameter and a slot of 0.2 x 5.0 cm. One μl (24 μg) of the antigenic extracts was deposited in the hole. The plate was then electrophoresis ed for 4 h at 130 V. When the electrophoresis was concluded, the slot was filled with 0.3 ml of the antisera. Immunodiffusion was for 18-24 h at 37°C. The protein precipitin bands were visualized with Amido Black.

**Conjugation of horseradish peroxidase**

The anti-PSP antibodies were conjugated to horseradish peroxidase (HRPO, Nordic Laboratories, Tilburg, The Netherlands) by the periodate oxidation method described by Nakane and Kawoi (20) omitting the NaBH₄ treatment since sodium borohydride is difficult to handle; besides it may significantly reduce the antibody activity of the conjugate while the peroxidase activity remains high (22). The anti-PSP antibodies conjugated to HRPO were isolated from polymeric antibodies and free enzyme by gel filtration on a column (1.5 x 90 cm) of Sephadex G-200 (Pharmacia) equilibrated in PBS, pH 7.2.

**Extraction of meat samples**

Sarcoplasmic extracts from pure pig, horse, chicken and beef meat to be tested by ELISA, were prepared following the methodology used in obtaining antigenic extracts but omitting the lyophilization step. Samples of the extracts (3 ml) were stored frozen until use. Sarcoplasmic extracts of raw beef containing weighed portions of pig meat were also obtained as described. The extracts from two independently made beef-pig mixtures were tested by ELISA.

**Sandwich ELISA procedure**

Flat bottomed micro-ELISA plates (Costar, Cambridge, MA, USA) were filled with samples (0.1 ml) of captured anti-PSP antibodies (0.6 mg/ml) diluted 1/50 in carbonate coating buffer (sodium carbonate-bicarbonate buffer, pH 9.6) and incubated for 16 h at 4°C. The wells were washed five times with PBST (PBS containing Tween 20 at 0.5 ml/l) and blocked with PBST-BSA (BSA at 0.1% w/v) for 30 min at ambient temperature (19-21°C). After another 5 washes with PBST, 0.1 ml of the meat extract dilutions in PBST (15 mg/ml, initial concentration) were added on each of 2 wells and the plates were incubated for 1 h at 37°C. Following a new washing with PBST, 0.1 ml of the anti-PSP antibodies conjugated to HRPO (1 mg/ml) and diluted 1/50 in PBST were added to the wells and the plate incubated 1 h at ambient temperature. After washing five more times with PBST to remove unattached enzyme conjugate, 0.1 ml portions of enzyme substrate solution were added to each well and the reaction allowed to proceed for 30 min, before termination with 3 N sulphuric acid. The substrate used (1 mg/ml) was o-phenylenediamine (Sigma Chemical Co., St. Louis, MO, USA) made up 1 h before use in 0.1 M citric-citrate buffer, pH 5.0 with hydrogen peroxide of 110 volumes (10 μl H₂O₂/25 ml buffer). The yellow/brown color developed by conversion of the substrate was measured at 492 nm with a Titertek Multiskan spectrophotometer (Flow Laboratories, McLean, VA, USA).

**RESULTS AND DISCUSSION**

A problem currently concerning meat product manufacturers is the detection of small amounts of substituted meat in meat supplies intended for use in product manufacture (12). Enzyme-linked immunosorbent assay (ELISA) has been developed in several independent versions for the identification of meat species, thus demonstrating the effectiveness of this approach to the analysis of meat mixtures.

Most of the ELISAs recently developed for the identification of meat species in meat mixtures (11,12,15,23, 24,34), use antibodies against serum proteins. We have used species-specific polyclonal antibodies against pig muscle soluble proteins, to "capture" and "detect" from complex meat mixtures those pig proteins which should be evenly distributed through the muscular system, as they constitute an intrinsic part of it. These proteins may not be...
activated Sepharose 4B coupled to sarcoplasmic extracts from the change in the eluting buffer to 0.1 M glycine-HCl, pH 2.5. through four affinity chromatography columns containing CNBr-

Figure 1. Purification of an 8 ml-sample crude anti-pig serum through four affinity chromatography columns containing CNBr-activated Sepharose 4B coupled to sarcoplasmic extracts from horse (1), chicken (2), beef (3) and pig (4). The arrow indicates the change in the eluting buffer to 0.1 M glycine-HCl, pH 2.5.

subjected to the fluctuations encountered with the serum albumins whose presence in the meat is related to the muscle blood content.

Initial experiments using immunoelectrophoresis in agarose gels demonstrated that the crude pig antisera gave reactions of identity with the antigenic extracts against which it was raised and with other heterologous meat extracts. Effectively, the unpurified antisera showed four protein precipitin bands against pig sarcoplasmic extracts, two against beef, and none against the horse and chicken antigenic extracts.

To make the crude pig antisera specific, eliminating those antibodies capable of crossreacting with sarcoplasmic extracts from horse, chicken and beef, we utilized an affinity chromatography purification step. As it may be observed in Fig. 1, samples of the crude anti-pig serum were circulated once on columns containing sarcoplasmic extracts from the horse, chicken, beef or pig, followed by elution of the pig-specific antibodies (anti-PSP) off the pig column, thus allowing antibodies to bind to the column to which they have the highest affinity regardless of true specificity. However, it should be noted that if the antiserum was passed over the horse, chicken and beef columns repeatedly and then over the pig column followed by elution of the pig specific antibodies off the pig column, this procedure will tend to remove a much greater percentage of the crossreacting antibodies. Immunelectrophoresis in agarose gels of the purified anti-PSP antibodies showed two protein precipitin bands against pig muscle soluble proteins but not against sarcoplasmic extracts from horse, chicken and beef.

A portion of the purified anti-PSP antibodies were conjugated to horseradish peroxidase by the periodate oxidation method (20) omitting the NaBH₄ treatment. However, it should be noted that since sodium borohydride is necessary to stabilize the Schiff's base formed in linking the carbohydrate side chains of peroxidase to the lysine groups of the antibody, use of NaBH₄ to reduce the Schiffs base should result in a more stable conjugate. In the ELISA procedure the substrate for horseradish peroxidase was o-phenylenediamine. This compound being very sensitive to peroxidase is mutagenic (1,32) and since the use of mutagenic substances may be hazardous it should be convenient to utilize as peroxidase substrates other sensitive non-mutagenic compounds such as 3,3',5,5'-tetramethylbenzidine (2,29).

The double-antibody sandwich ELISA developed uses purified anti-PSP antibodies adsorbed to a plastic support to sequester pig proteins. Further immunorecognition was made with the same antibodies conjugated to horseradish peroxidase. Using this assay, it is observed (Fig. 2) that the anti-PSP antibodies effectively discriminate pig from horse, chicken and beef in a wide range of meat extract dilutions from 1:320 to 1:20480. Correspondingly low values were obtained (OD<0.200) for the controls. From these results it is clear that the immunoadsorption step removes most of the crossreacting antibodies and it enables confident identification of pig meat using the ELISA procedure. This is essential to obtain results free of interference from co-extracted components of product mixtures and to avoid the possibility of "false-positive" identifications (9).

However, it should be noted that at the lower dilutions of the meat extract dilutions the antigens from horse, chicken and beef show a certain degree of binding to the anti-PSP antibodies. This binding may be due to non-specific interactions of the sarcoplasmic extracts with the antibodies or to the presence in the purified antibodies of immunoglobulins (not detected by immunoelectrophoresis) that interact with those proteins. Since detection of crossreacting antibodies by immunoelectrophoresis is insensitive, the estimated percentage crossreactivity of horse, chicken and beef sarcoplasmic extracts in the final ELISA...
SANDWICH ELISA FOR DETECTING PIG MEAT IN BEEF

Figure 3. Sandwich ELISA response to pig meat substitution (■) in beef (□). Controls of the conjugate without antigen (○) and conjugate without antigen nor antibody (●). Bars represent standard deviation of triplicate assays from two independent meat mixtures.

The logarithm of the pig meat content in raw beef (Ln %P) may be calculated from data in Fig. 2, where it looks like it will take about a 1/32,000 fold dilution of pig extracts to produce the same OD as a 1/320 dilution of beef or horse or chicken extracts in which case the crossreactivity would be about 1%.

The sandwich ELISA was further used to detect the presence of pig muscle soluble proteins in raw beef. An exhaustive checkerboard titration of all components of the assay showed that optimum conditions for the test were obtained using the capture antibodies at a final concentration of 12 μg/ml and the conjugate at 20 μg/ml, whereas the sarcoplasmic extracts were diluted 1/320. Other quality control procedures were also performed. The coefficient of variation (CV) of optical densities for a standard sample of the pig extract allocated over 2 entire 96-well plates was less than 2% for intra assay results and near 3% for the inter assay ones. We have not observed the existence of defined edge effects, as reported by other investigators (7,21). However, it should be stressed that these results have been obtained by highly trained personnel and when this is not the case higher CV values for the above situations may be observed. Nevertheless, if it is found that binding varies significantly across the plate it may be convenient to use alternative recommended methods for allocation of the samples in the plates (30).

Figure 3 shows the absorbance values from two independent batches of beef meats weighted to include 0, 1, 2.5, 5, 10, 15, 20, 25, 30, 40, 50 and 100% of pig in 100 g. The absorbance values increased until a 50% substitution of pig meat in beef. In these meat batches absorbance was related to pig meat content (%P) in the range 1 to 50% by the equation: Absorbance = 0.268 + 0.114 Ln (%P), with a correlation coefficient of r = 0.983. This expression constitutes a logarithmic regression analysis of the experimental data, according to the formula y = A + B Ln x. Input data items are the logarithm of the pig meat content in raw beef (Ln %P) and y corresponds to the optical density reading at 492 nm of the raw beef extracts containing weighed amounts of pig meat. This relationship may be used as a reference curve when trying to evaluate the addition of pig meat to raw beef. However, since the reference curve is likely to differ slightly under the conditions given at different days of work and laboratories, it is advisable to run a reference curve on each plate.

The assay used facilitates the detection of pig meat in raw beef differentiating 1-50% of pig substitution in prepared lean mixtures. The assay is more sensitive than previous indirect ELISAs (13,15,19,34) being in the range of those using a sandwich methodology. It appears that an additional advantage of this sensitivity over a wide range of substitution. A disadvantage of the sandwich ELISA is that it uses more purified antiserum than competitive or indirect ELISA procedures.

This test also offers the advantage of potential automation over other procedures developed to determine the species of origin of raw meat. These include Ouchterlony immunodiffusion tests (16,26,31), haemagglutination inhibition (14) and isoelectric focusing of enzymes (10,17,18, 28).

The sandwich ELISA developed demonstrates that the same polyclonal pig-specific antibodies may be used as "capture" and immuno-recognition antibodies to detect pig meat in raw beef. The limiting factor for application of this technique in the meat industry is the costly and time-consuming isolation of the anti-PSP antibodies since the immunopurification process has to be repeated many times to obtain reasonable amounts of purified antibodies. Instead, monoclonal antibodies could be raised against pig-specific antigens to detect pig meat in meat mixtures. This would also permit the use of a reactive to be universally utilized. Using immobilized anti-PSP antibodies onto a Protein A-Sepharose CL-4B column, we are isolating pig-specific antigens to immunize mice as a first step in the obtention of those monoclonals of interest.

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