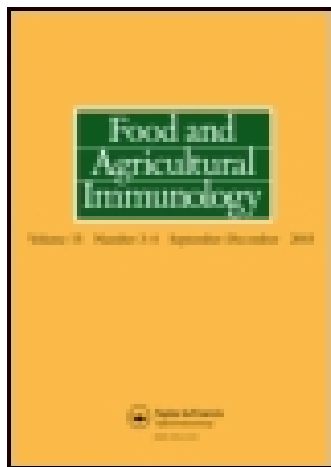




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Detection of Chicken Meat in Raw Meat Mixtures by the Double Method of an Enzyme Immunoassay and an Immunoblotting Technique

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Polyclonal antibodies were raised in male New Zealand White rabbits against a 0.5% (v/v) Lubrol PX protein extract of crude chicken red bone marrow. Following serum protein removal by ammonium sulphate precipitation, the serum was affinity-purified against muscle protein from a selection of different species. The antiserum, when assayed by enzyme-linked immunosorbent assay, was shown not to recognize any proteins from specimens other than chicken hand-deboned (HDM) and mechanically separated (MSM) meats, with the reaction towards MSM chicken being significantly stronger than that against HDM chicken. When the same samples were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis, transferred to nitrocellulose by Western blotting and immunochemically stained with the affinity-purified antiserum, they showed specific staining of protein bands within chicken HDM and MSM, with no reaction towards any proteins of any other species. A band common to both chicken HDM and MSM at about 45 kDa, and a band specific for MSM at 70 kDa were the major immunoreactive proteins.

Keywords: Immunoassay, chicken meat, HDM, MSM

INTRODUCTION

The fraudulent substitution of meat with a less expensive meat from a different species, or the addition of a less expensive form of meat from the same species (e.g. mechanically separated meat, MSM), in products such as sausages, burgers, pies and spreads, are practices known to occur in the processed meat manufacturing industry. On some occasions, such practices are not only unethical, but may also cause concern for health, religious and

economic reasons. For example, the consumption of products containing undeclared meat species has been reported to cause allergies in sensitized individuals.

Poultry tissue represents a major source of protein which, in general, is less expensive than red meat. In addition, the most abundant type of MSM comes from poultry. Such factors highlight the potential for the adulteration of red meat with poultry and, as a consequence, the need to be able to detect the presence of poultry tissue in other meat products.

Several methods have been reported for the detection of adulteration of meat and meat products with meat from different species. Certain methods involve the use of high-performance liquid chromatography (HPLC) (Ashoor & Osman, 1987) for the detection of species-specific proteins within a suspected adulterated sample. This method, although being useful in that it detects muscle proteins, suffers from the drawback of requiring expensive dedicated HPLC, data acquisition and data processing equipment.

Enzyme-linked immunosorbent assay (ELISA) is undoubtedly the most widely used immunological assay in food analysis due to its simplicity, enabling specific and reliable analysis of food components without necessitating expensive apparatus or skilled operators. Most of the methods currently available for meat species analysis use polyclonal antisera raised against serum proteins (Kang'ethe *et al.*, 1982; Griffiths & Billington, 1984; Patterson *et al.*, 1984). However, these antisera raised against serum proteins are unable to confirm the presence of actual muscle tissue proteins in a sample because of the possible contamination of meat proteins by blood from other species during meat processing. Consequently, such assays have drawbacks when used to provide evidence for use in a court of law.

The following report describes the use of a polyclonal antibody which recognizes a chicken muscle soluble protein and which, when used in a ELISA or an immunoblotting system, is able to detect the presence of chicken meat in raw meat mixtures. Further, when using immunoblotting, this antibody also has the capacity to detect the presence of chicken MSM in hand-deboned meat (HDM) mixtures.

MATERIALS AND METHODS

Reagents

Chemicals used for electrophoresis were of Electran grade. Those used for buffers and immunochemical determinations were of AnalaR grade. All other chemicals were general-purpose reagent grade.

Acrylamide, *N,N*-methylenebisacrylamide, glycine, urea, sodium dodecyl sulphate (SDS), *N,N,N',N'*-tetramethylethylenediamine, ammonium persulphate, glycerol, Page blue, acetic acid, methanol, ethanolamine, dimethyl sulphoxide (DMSO), sodium chloride, potassium chloride, sodium phosphate, dibasic sodium phosphate and dibasic potassium phosphate were obtained from The British Drugs Houses, Poole, Dorset, UK.

Lubrol PX, hydrogen peroxide, protein molecular weight standards, Freund's complete and incomplete adjuvants, sodium acetate, sodium azide, polyoxyethylene sorbitan monolaurate, ponceau S red, 3,3',5,5'-tetramethyl benzidine, cyanogen bromide (CNBr)-activated Sepharose 4B, and antibodies (anti-rabbit IgG horseradish peroxidase (HRP) conjugate), were obtained from the Sigma Chemical Company, Poole, Dorset, UK.

Marvel dried, skimmed milk powder was obtained from Cadbury Premier Brand (UK), Bournville, Birmingham, UK.

MSM samples were supplied by the Laboratory of the Government Chemist (LGC), Queens Road, Teddington, UK. HDM samples were supplied by the Laboratory of the Government Chemist, and also by several reputable meat retailers.

Extraction of Meat Samples

Soluble muscle proteins from beef, lamb, pork, chicken and chicken adulterated meat mixtures were prepared from samples of trimmed, well-mixed lean meat. Representative 50 g samples were homogenized in 150 ml of 7 M-urea and the proteins were extracted by gentle stirring of the homogenates for 3 h at 4°C. Homogenates were centrifuged at $1500 \times g$ for 10 min at 4°C to remove insoluble material, then filtered through Whatman No. 1 filter paper.

Antigen Preparation

Antigenic extracts of whole chicken red bone marrow were prepared by collecting bone marrow (5 ml) from freshly killed chicken leg and rib bones. This was achieved by aseptically cutting open the bones using a pair of sharp scissors and scraping out the red bone marrow. This was then resuspended in 0.5% (v/v) Lubrol PX (50 ml), and the proteins extracted by gentle agitation for 24 h at 4°C. The extract was clarified by centrifugation ($2000 \times g$ for 15 min), then filtered through Whatman No. 1 filter paper into sterile universal bottles. The protein extracts were then stored at -20°C until required.

Polyclonal Antibody Production

Antibodies against chicken red bone marrow extracts were raised in male New Zealand White rabbits, as outlined by the method of Hudson and Hay (1980).

Polyclonal Antibody Purification

Following ammonium sulphate precipitation to remove the serum proteins (Johnstone & Thorpe, 1987), the antibodies in the crude serum were rendered species-specific by immunoadsorption chromatography against 7 M-urea muscle protein extracts from beef, lamb and pork, coupled to CNBr-activated Sepharose 4B columns (3 g dry weight column material coupled to 40–50 mg protein extract).

Heterologous Non-competitive Solid-phase ELISA

Meat samples were assayed using a simple, single-site, solid-phase ELISA, using microtitre plates (96-well microtest, Becton Dickinson Labware, Oxnard, CA, USA).

A total of 50 μl of 7 M-urea extracts of meat proteins (1:1000 dilution in 7 M-urea) was applied to wells in the microtitre plate, and allowed to bind for 30 min at 37°C. Following three washes with phosphate-buffered saline (PBS), the remaining protein binding sites in the wells were blocked by applying 200 μl of 2% (w/v) Marvel in PBS to each well and incubating for 30 min at 37°C, after which the wells were aspirated three times with PBS plus 0.1% (v/v) Tween 80. Then, 50 μl of the primary antibody (anti-chicken bone marrow), appropriately diluted in PBS plus 2% (w/v) Marvel, was added, and allowed to react for 30 min at 37°C. The wells were then washed three times in PBS plus 0.1% (v/v) Tween 80 and twice in distilled water, after which 50 μl of a 1:5000 dilution (in PBS plus 2% (w/v) Marvel) of goat anti-rabbit IgG conjugated to HRP was added to each well and allowed to incubate for 30 min at 37°C. After the wells had been washed twice in PBS plus 0.1% (v/v) Tween 80 and twice in distilled water, 100 μl of substrate (20 ml of 0.1 M-sodium acetate, pH 6.0, 25 μl of 3% (w/v) hydrogen peroxide and 150 μl of a stock solution of tetramethyl benzidine (10 mg ml^{-1} in DMSO)) were added to each well and left for 5–10 min or until full colour development, after which the reaction was stopped by the addition of 50 μl of 2.5 M-sulphuric acid to each well. The absorbance of the plate was then read at 450 nm using a microplate reader (Titertek from ICN Flow, Thame, Oxon, UK).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The method employed was a modification of that described by Laemmli (1970). Polyacrylamide gels of 10% (w/v) were found to be optimal for resolution of the meat protein. The Protean 11 or mini Protean (Bio-Rad, Richmond, CA, USA) gel mould apparatus for vertical slab gel electrophoresis was used and assembled according to the manufacturer's instructions.

Protein standard molecular weight markers (myosin, 205 kDa; β -galactosidase, 116 kDa; phosphorylase b, 94 kDa; bovine serum albumin 67 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 21.5 kDa; and lysozyme, 13.4 kDa) (Little Chalfont, Amersham, Bucks, UK) were run alongside samples for comparison.

Western Blotting

Western blotting of proteins onto nitrocellulose (Gelman, Northampton, UK), previously electrophoretically separated on 10% (w/v) SDS-polyacrylamide gels, was performed using a modified version of the method of Towbin *et al.* (1979).

Immunoprobng of Western Blots

Immunoprobng of Western blots was performed using rabbit anti-chicken bone marrow (affinity-purified IgG) diluted 1:300 in buffer A (100 mM-Tris-HCl; 150 mM-sodium chloride; 0.05% (v/v) Tween 80; 2% (w/v) Marvel, pH 9.0) and incubated overnight at 4°C with gentle agitation.

Proteins were revealed using anti-rabbit IgG conjugated to HRP, diluted 1:2500 in buffer B (50 mM-Tris-HCl; 200 mM-sodium chloride; 0.1% (v/v) Tween 80, pH 7.4) plus 2% (w/v) Marvel, followed by incubation with 4-chloronaphthol reagent.

RESULTS

Comparison of Crude and Purified Antisera

By using a non-competitive solid phase ELISA, it was shown that the crude antiserum raised to 0.5% (v/v) Lubrol PX extracts of crude chicken bone marrow reacted strongly with 7 M-urea extracts of chicken meat (HDM and MSM), while showing only 5–10% of the reaction against chicken HDM and MSM with similarly extracted proteins of beef, lamb and pork meats. Protein extraction using 0.5% (w/v) Lubrol PX was found to interfere slightly with the ELISA, whereas extracting in 7 M-urea did not, and for this reason 7 M-urea was used in all further investigations.

In order to reduce cross-reactivity with other animal species, the crude antiserum was first purified by ammonium sulphate precipitation to remove serum proteins and leave an enriched immunoglobulin fraction. This fraction was then further purified by affinity chromatography using 7 M-urea muscle extracts of HDM from beef, lamb and pork conjugated to CNBr-activated Sepharose 4B. After sequential incubation with the three immunoadsorbants, the resultant supernatant was reassayed against the same extracts as before, where it was found that while the strong reactivity against chicken meat remained, the slight reaction against the other meat species had disappeared.

The affinity-purified anti-bone marrow antibodies effectively discriminated whole chicken (HDM or MSM) from whole beef, lamb and pork meat in a large range of dilutions of meat extracts between 1:10 and 1:10 000 (Figure 1).

Correspondingly low values (absorbance at 450 nm [A_{450}] < 0.2) were obtained for the control blanks. However, at the lower antigen dilutions (1:10–1:100), slightly elevated A_{450} values were obtained for beef, lamb and pork, indicating some binding of the antigens from beef, lamb and pork to the anti-chicken bone marrow antibodies, which is likely to be caused by non-specific binding.

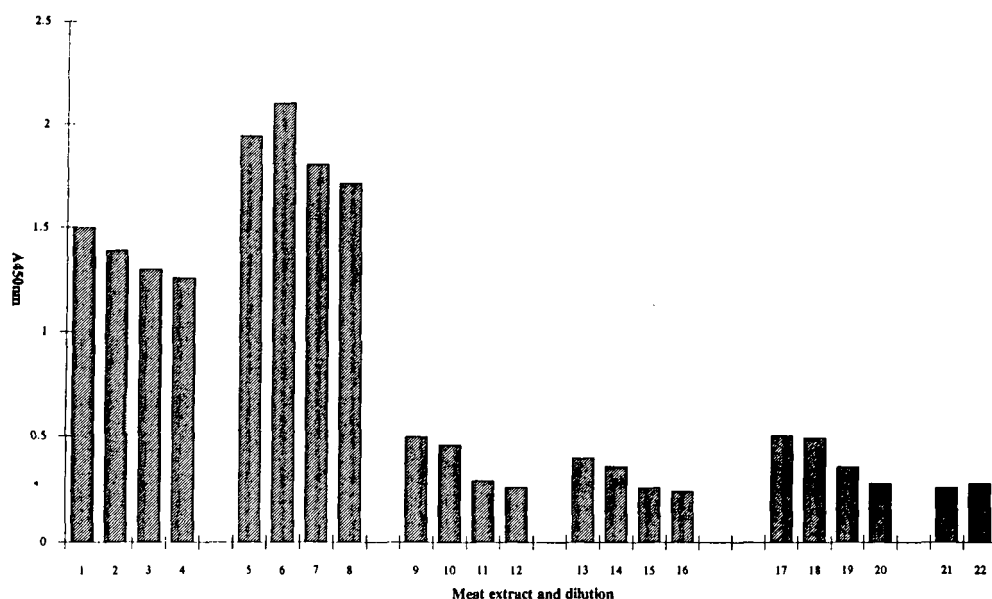


FIG. 1. ELISA investigating the effect of antigen dilution on the specificity of affinity-purified anti-chicken bone marrow. Samples: 1–4, chicken HDM; 5–8, chicken MSM; 9–12, bovine HDM; 13–16, ovine HDM; and 17–20, porcine HDM. Meat extract dilutions were as follows: (1,5,9,13,17) 1:10; (2,6,10,14,18) 1:100; (3,7,11,15, 19) 1:1000; and (4,8,12,16,20) 1:10 000. Control blanks: (21) minus antigen/plus antibody; (22) plus antigen/minus antibody.

The assay was found to give approximately 30% greater A_{450} values for the chicken MSM samples tested compared with the chicken HDM samples.

Characterization of the Immunogenic Components

Urea (7 M) protein extracts of chicken meat and chicken blood, separated by SDS-PAGE, were transferred on to nitrocellulose and immunoprobed with affinity-purified anti-chicken bone marrow in order to determine whether the antigenicity was due to a muscle soluble or a blood soluble protein.

The immunoblot shown in Figure 2, indicates a major band in chicken HDM (Figure 2, lane 1) which was found to react with the anti-bone marrow antisera at approximately 45 kDa; a very faint band at 70 kDa was also visible. Within chicken MSM (Figure 2, lanes 2 and 3), there was a strong reaction to a 45 kDa band; however the reaction to the 70 kDa band was much stronger than in HDM. An immunoreactive band just below 70 kDa and a lesser reactive band at about 14 kDa, specific for MSM, was also shown to be present.

In chicken blood, it was found that two immunoreactive bands were present, a major protein of about 70 kDa and a lesser one at about 16 kDa. Preliminary studies involving the immunoprobings of Western blots using affinity-purified antiserum as above but then further affinity purified against chicken serum albumin have failed to produce a positive band at about 70 kDa. This indicates that the 70 kDa immunoreactive protein (Figure 2, lanes 2 and 3), abundant in chicken MSM, present in trace amounts in chicken HDM and abundant within chicken blood, is most probably serum albumin.

The immunoreactive protein at about 45 kDa, found in chicken meat (HDM/MSM), was not detectable in chicken blood (data not shown) suggesting that it was likely to be a muscle soluble protein.

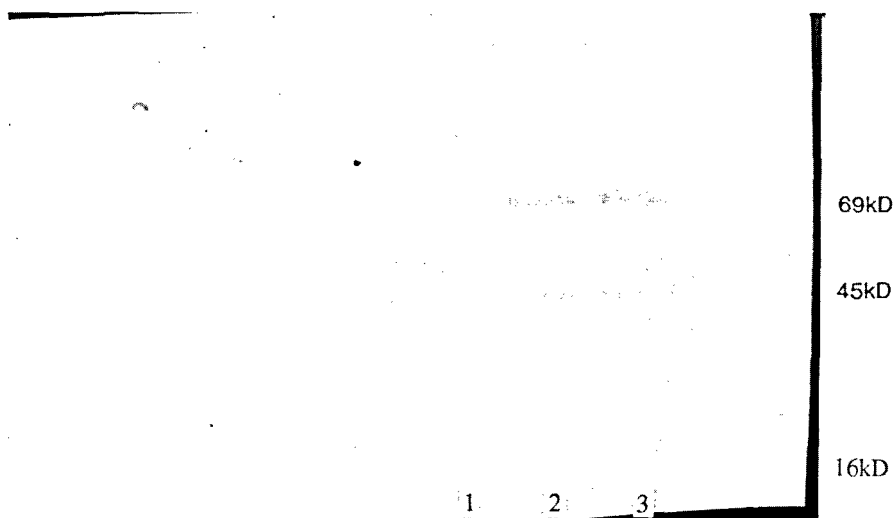


FIG. 2. Western blot of 10% (w/v) SDS-PAGE-separated proteins extracted from lane 1 (HDM chicken) and lanes 2 and 3 (MSM chicken) with 7 M urea. The blot was immunochemically developed with affinity-purified anti-chicken bone marrow.

It was found that the detection of chicken meat (HDM/MSM) by immunoprob-
ing of Western blots with the least amount of background staining necessitated critical
optimization of antibody dilution, and that a dilution of 1:300 in PBS plus 2% (w/v) Marvel
gave the best results.

Detection of Chicken Meat (MSM) in Beef (HDM) by ELISA

The data in Figure 3, shows the ELISA absorbance values (A_{450}) from batches of
beef HDM containing 0, 1, 5, 10, 15, 25, 50 and 100% (w/w) chicken MSM. Results
are shown from an assay performed using a dilution of primary antibody, anti-chicken
red bone marrow, of 1:1000. The results indicate that the presence of increasing con-
centrations of chicken MSM in beef HDM results in an increase in A_{450} . A comparable
result to using chicken MSM was shown when using chicken HDM as the adulterant
(data not shown).

DISCUSSION

The majority of the antisera currently available for the identification of meat species in meat
mixtures are raised against whole serum or specific serum proteins.

Part of the immunological response of the anti-chicken serum discussed here has
been shown to be directed against the blood soluble protein, albumin. This was proven
by the incubation of the antiserum with an affinity matrix complexed to purified chicken
serum albumin, which was then shown to have lost its recognition of the 70 kDa protein
band. However, a significant percentage of the antibodies within the serum are directed
towards a 45 kDa muscle soluble protein, the characterization of which is still to be
undertaken.

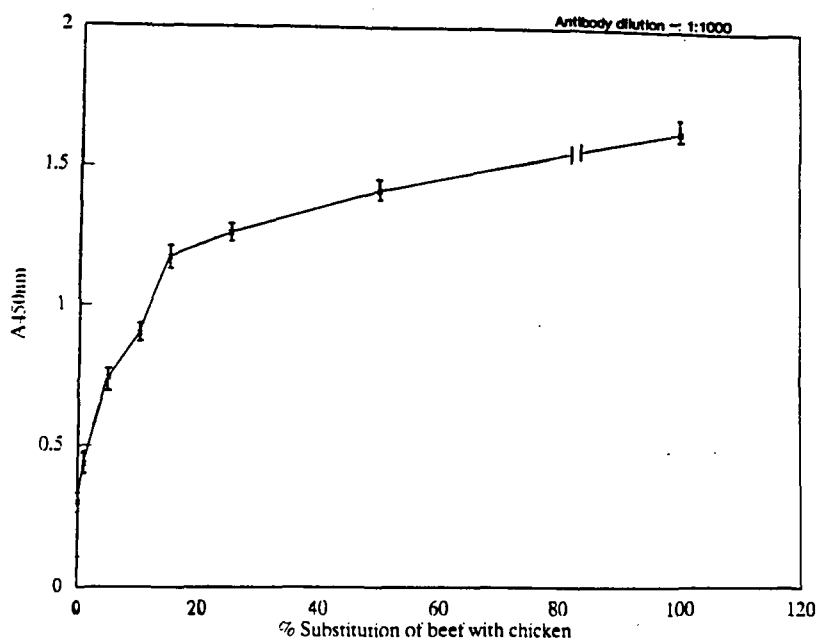


FIG. 3. ELISA detecting the adulteration of bovine HDM with chicken MSM from 0–100%. Samples were extracted and diluted (1:1000) in 7 M-urea for use in the ELISA as described in the text. Affinity purified anti-chicken bone marrow antiserum was used at a dilution of 1:500 in PBS/2% (w/v) Marvel, pH 7.4.

The response to soluble muscle proteins is of significant importance in the identification of meat species, since these proteins are more evenly distributed throughout the muscular system and are not subject to the fluctuations encountered with the serum albumins, whose presence in the meat is related to the muscle blood content. Further, meat adulteration assays based on blood soluble proteins are very difficult to substantiate in a court of law, because blood serum proteins could be transferred from one species to another by drip of the serum when many different types of meats are being transported together.

The ELISA developed in this study should facilitate the detection of chicken MSM in beef (Figure 3), lamb and pork HDMs at low levels of adulteration, (1%, w/w) over a wide range of adulteration levels. Similar results should also be achievable with chicken HDM as the adulterant within other species HDMs.

However, although being able to discriminate between 100% chicken HDM and chicken MSM, the ELISA has proven not to be very sensitive when determining low levels (< 10%) of chicken HDM adulterated with chicken MSM (data not shown). This is probably due to the fact that one of the major immunoreactive proteins (45 kDa) is present in both HDM and MSM. Thus, at low levels of MSM adulteration, the increased A_{450} value confirmed by the MSM abundant 70 kDa and MSM-specific 14 kDa proteins will only be slightly higher than that of HDM.

In contrast, the immunoblot assay has the advantage over the ELISA of usually being able to demonstrate the presence of the chicken muscle protein by the immunostaining of the 45 kDa band.

A further advantage of the immunoblot assay over the ELISA is that chicken MSM can be distinguished from chicken HDM by the size distribution pattern of the protein bands recognized by the anti-chicken bone marrow antiserum.

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