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Detection of chicken meat in unheated meat mixtures by an indirect Enzyme-linked Inmunosorbent Assay

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INTRODUCTION

The possibility that unheated ground 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. In this context, it would be desirable to have at hand a simple and rapid test to determine the addition of undeclared meat in a product and give the consumers greater protection.

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 (Kang'ethe et al., 1982; Whittaker et al ., 1983; Patterson et al., 1984; Johnston et al., 1985). Most of the authors have employed in the test antisera to serum albumins, blood serum or species specific IgG's. However, we think and this has also been stated by others (Griffiths et al ., 1984), that this particular antisera may create problems to detect meat species in undeclared meat mixtures,due to differences of the amount of blood in the various joints of meat or to non- specific interactions between extracted meat components and the antisera. For this reason, and based on previous work carried out in our laboratory (Casas et al., 1984a, 1984b, 1985a, 1985b) about the existence of species specific muscle soluble proteins on each animal species tested, we propose to use antisera to species specific meat soluble proteins to detect the presence of undeclared meat in meat mixtures. In this work,we report the use of antisera to specific chicken meat proteins to detect the presence of chicken meat in unheated meat mixtures.

MATERIALS AND METHODS

Preparation of the antigenic extracts

Skeletal muscle tissue from chicken (Ms. pectoralis and Ms. supracoracoideus) , cow (Ms. rectus femoris, Ms. vastus medialis and Ms. vastus lateralis), pork (Ms. intercostalis externi and Ms. trapezius) and horse (Ms. glutaeous superficialis and Ms. biceps femoris) in a total weight of 100 g were finely triturated, minced and homogenized in 300 ml of 0.85% saline solution. The soluble proteins were extracted by constant agitation of these homogenates for 1 h at 2ºC. The protein extracts were filtered through a Whatman Nº 1 filter paper, lyophilised, and the dried extracts placed in a airtight container and stored at -20ºC until use. Extracts of minced chicken meat beef mixed in weighed proportions were also prepared. To achieve an effective concentration, 50 g amount were homogeneized in 150 ml saline, centrifuged, filtered and stored frozen in 1 ml aliquots until use.

Production of antisera

Serum containing suitable chicken soluble protein (CHSP) antibodies was obtained by injecting, subcutaneously, New Zealand male rabbits with single doses of lyophilised chicken protein extracts (50 mg) in 2 ml of deionised and distilled water emulsified in 0,5 ml of Freund complete adyuvant (Difco). Ten booster doses were applied subcutaneously every 5 days. After 50 days, the rabbits were bled, the blood allowed to clot for 1 h at 20 ºC, the serum collected by centrifugation at 1000 g for 10 min and finally it was stored frozen at -20 ºC.

The isolation of the monoespecific fraction of the anti-CHSP antibodies was carried out passing the serum CHSP antibodies thought four CNBr-activated Sepharose-4B columns (Pharmacia Fine Chemicals) coupled to 52.5 mg of suitable muscle soluble protein antigens from either cow, horse, pork and chicken. The antibodies monospecific to CHSP were realeased from the last column by elution with glycine-HCl buffer (0.1 M, pH 2.5). The fractions eluted were pooled, adjusted to pH 7.2 with solid Tris and dyalised overnight against PBS (phosphate buffered saline, pH 7,2). Aliquots of 1 ml each were stored frozen at -20 ºC.

Double immunodifusion tests

Test plates were made on a supporting glass (7.5 x 5.0 cm) covered with 0.1% agarose in a 0.85% saline solution with sodium azide (0.1%). Hexagonal "Ouchterlony" pattern of wells were used to test the monospecific anti-CHSP antibody (1mg/ml) against the muscle soluble proteins from cow, horse, pork and chicken (14mg/ml). 50 µl of antigen was placed on the outer wells and 150 µl of antibody in the central one. The plates were incubated 24 h at 37 ºC until clear precipitin lines developed after wich they were soaked in saline (0.85%) for 48h to remove free protein and then stained.

Indirect ELISA procedure

Flat bottomed micro-ELISA plates (Costar-3590, 96 wells) were filled with aliquots (0.1ml) of meat extracts (14 mg/ml) diluted in carbonate coating buffer (sodium carbonate-bicarbonate buffer, pH 9.6) and incubated for 16 h at 4ºC. Wells were washed five times with PBST (PBS-containing Tween 20 at 0.5 ml/l) and tapized with PBST-BSA (BSA at 0.1% w/v) for 30 min at ambient temperature (19-21ºC). After another 5 washes with PBST wells were incubated with the monospecific CHSP antisera diluted 1/50 for 1 h at 37ºC. Following a new washing with

PBST, the appropiate immunoconjugate GAR/IgG (H+L)/PO (Nordic Laboratories) diluted 1/25000 was added and incubated 1 h at ambient temperature. After washing 5 more times to remove unattached enzyme conjugate , 0.1 ml aliquots of enzyme substrate solution were added to each well and the reaction allowed to proceed for 30 min, before termination with 0.1 ml of 3N sulphuric acid. The substrate used was 0-phenylendiamine (Sigma Co.,) in citric-citrate buffer (0.1M, pH 5.0) made up immediately before use (1mg/ml) with hydrogen peroxide (30 vol, 10 µl/25 ml). The yellow/brown colour developed by conversion of the substrate was measured in each well as absorbance at 492 nm by micro-ELISA plate reader (Titertek Multiskan PLUS).

RESULTS AND DISCUSSION

As it has been previously stated in the introduction section, previous work carried out in our laboratory through the use of immunoelectrophoresis techniques, revealed the existence of species specific muscle soluble proteins on each animal species tested. We hypothesized then, that those proteins could act as protein markers or diagnosis agents for testing unprocessed meat for contamination by extraneous animal species. Furthermore, those proteins could be better specific markers in the detection of meat adulterations than the serum albumins, whole blood serum or species specific IgG's, since their distribution in the meat mixture should be uniformer and their cuantitation from the saline protein extracts easier and much less variable.

Based on the above mentioned facts, we report here the development of an indirect ELISA assay to detect the presence of chicken meat in unheated meat mixtures. Serum containing suitable chicken soluble protein (CHSP) antibodies, was obtained through the immunization of New Zealand male rabbits. To make this serum monospecific and to eliminate all the antibodies able to crossreact with muscle soluble proteins from cow, horse and pork, we resorted to the use of an affinity chromatography system. Serum containing anti-CHSP antibodies was passed through four columns containing a matrix of CNBr-activated Sepharose-4B coupled to suitable muscle soluble protein antigens from either cow, horse, pork and chicken. As it may be seen in Figure 1, at the end of the purification pattern, the monospecific fraction of the anti-CHSP antibodies was eluted from the last column with the use of an eluting buffer. Immunodiffusion experiments (results not shown), were made to unequivocally demonstrate that the monospecific anti-CHSP antibodies were unreactive against protein extracts from cow, horse and pork. The results demonstrated that the purified antibodies only gave protein precipitin lines against chicken antigens.

Once the monospecific anti-CHSP antibodies were obtained, we hypothesized that these antibodies could be used to detect chicken proteins in meat mixtures, through the use of ELISA methodologies. With the use of an indirect ELISA procedure (Fig. 2), we observed that effectively,

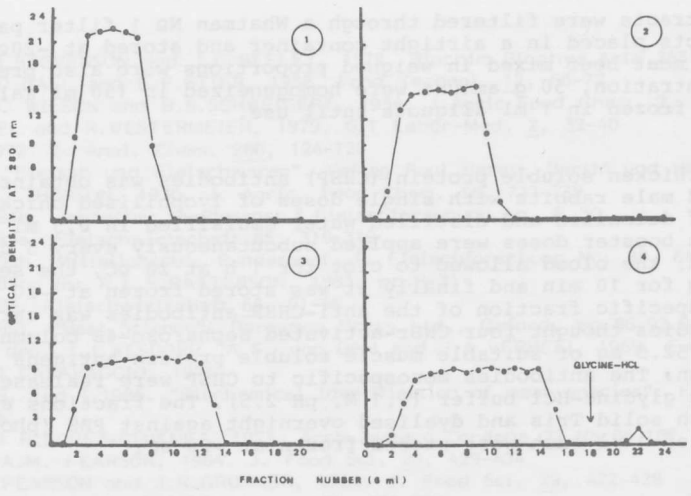

Figure 1. Isolation of the monospecific fraction of the anti-CHSP antibodies on CNBr-activated Sepharose-4B columns coupled to suitable muscle soluble protein antigens from(1) horse, (2) pork,(3) cow and (4) chicken. The eluting buffer was glycine-HCl (0.1 M, pH 2.5).

the monoespecific anti-CHSP antibodies could discriminate between muscle soluble protein extracts from cow, horse, pork and chicken.

Finally, we checked the effectiveness of the monospecific anti-CHSP antibodies to detect the

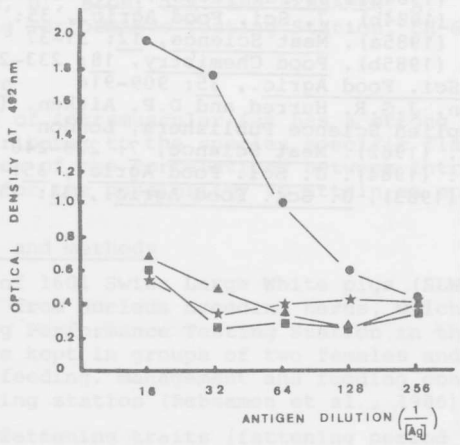

Figure 2. ELISA of the monospecificic anti-CHSP antibodies against muscle soluble protein from (●) chicken, (▲) cow, (■) pork and (★) horse.

presence of chicken proteins in preweighed meat mixtures. Results are shown in figure 3. When the ELISA indirect assay was employed to detect the presence of chicken meat in an artificially made beef/chicken meat mixture, the technique discriminated between a 10% to 30% contamination of chicken in the mixture. From 30% to 100% contamination the optical density of the micro-ELISA plate reached a plateau, meaning that at that optical density the contamination of chicken meat was equal or higher than 30%. From 1% to 10% contamination, the discrimination is difficult to made perhaps due to problems of inespecifc absorption of the immunoconjugate to the antigens or to the wall of the well. We think that the lower and the higher limits of detection may be improved by developing a sandwich ELISA assay. Efforts in that direction are in progress.

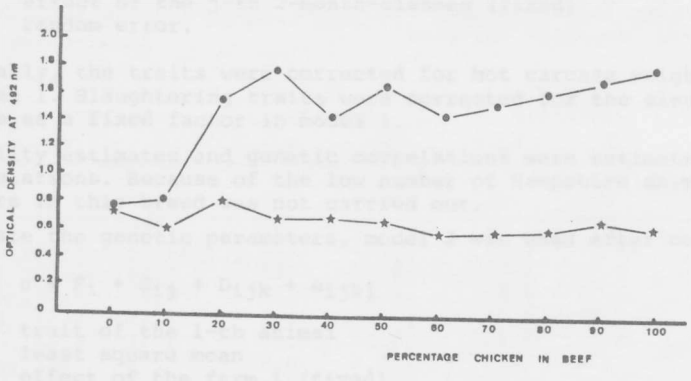

Figure 3. Effectiveness of the monospecific anti-CHSP antibodies to detect the presence of chicken proteins in a preweighed beef/chicken meat mixture. (●) chicken in beef, (★) beef alone.

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