# Use of an Indirect-ELISA to asses the meat proteins denaturation by heat

MARIN, M.L. ; CASAS, C. and J.A. ORDOÑEZ
Departamento de Higiene y Tecnología de los Alimentos. Facultad de Veterinaria. Universidad Complutense. 28040 Madrid ( Spain ).

## INTRODUCTION

Heat treatment is a very used and, sometimes, essential process to produce many comminuted meat products. However, this treatment induces proteins denaturation and modifications of their functional properties (Li-Chan *et al*, 1985).

Studies on the antigenic structures of globular proteins have shown that antibodies against to the native proteins are directed mainly against conformational rather than sequential sites (Iwabuchi & Shibasaki,1981). Consecuently, only poor cross-reaction is observed between denatured proteins and antibodies to the same proteins in their natural forms. In this context, immunological techniques provide a sensitive tool for the detection of structural changes produced in a given antigen by the application of various treatments.

The aim of the present work was to study the effects of heat treatments on the denaturation of meat proteins. For that, the residual antigen (unaltered meat proteins after the heat treatment) was measured by an enzyme-linked immunosorbent assay (Indirect-ELISA). A potential application of this method is the estimation of the degree of heating that meat products have received.

## MATERIALS AND METHODS

### Preparation of meat proteins extracts

Salt-soluble proteins extracts of beef were prepared in 0.01 M sodium phosphate buffer (pH 7.0), containing 0.6 M NaCl and 1mM $MgCl_2$, as described by Li-Chan *et al*, (1984). Extracts were analized for protein concentration using the Lowry method (Lowry *et al*, 1951).

### Heat treatments

Salt-soluble protein extracts in the buffer above mentioned were subjected to the heat treatment in a water bath with constant stirring to reach internal temperatures from 40°C to 100°C for 30 min. After achieving the desired temperatures, the solutions were rapidly cooled in an ice water bath, then homogeneized in a Sorvall Omni-mixer blender at approximately 2,000 rpm for 15 sec.

### Preparation of the antiserum

Antibodies against salt-soluble meat proteins (anti-SSMP) were produced in New Zealand male white rabbits. Immunization started by subcutaneous injection at multiple sites along the back of 2 ml of beef protein extracts emulsified in 0.5 ml of Freund's Complete Adyuvant (Difco Laboratories, Detroit, MI, USA). Ten booster doses were applied subcutaneously every 5 days. After 50 days, the rabbits were bled, the blood was allowed to clot for 1 h at room temperature and the serum was

collected by centrifugation at 2,000 g for 10 min. Samples of the serum (1 ml) were stored by freezing at -20°C.

**Indirect-ELISA procedure**

Flat bottomed micro-ELISA plates (Costar, Cambrige, MA, USA), were filled with samples (0.1 ml) of salt-soluble protein extracts (50 µg/ml of protein) diluted in carbonate coating buffer (sodium carbonate-bicarbonate buffer, pH 9.6) and incubated for 1 h at 37°C. The wells were washed five times with PBST (PBS containing 0.5 ml of Tween 20) and blocked with PBS-gelatine (1% gelatine w/v) for 1 h at 37°C. After 5 additional washes with PBST, 0.1 ml of anti-SSMP antiserum diluted 1/20 in PBS were added to the wells and the plates were incubated for 1 h at room temperature (19-21°C). After a new washing with PBST, 0.1 ml of horseradish-peroxidase protein A conjugate (Sigma Chemical Co., St. Louis, MO, USA) at 1/5,000 were added to the wells and the plate incubated 1 h at room temperature. After washing five times more with PBST to remove the unattached enzyme conjugate, 0.1 ml portions of the enzyme substrate solution (2,2´-Azino-bis at 15% in citric acid, buffer pH 3.9) were added to each well and the absorbance at 405 nm ($A_{405}$) measured after exactly 10 min.

For each heated and unheated meat extracts, the absorbance (duplicate assays) were plotted against $\log_{10}$ concentration of sample solution. The percentage of protein (residual antigen) undenatured after heat treatment was obtained according to the equation:

$$UP = 100 \times A_{405}\ 1.0\ HS / A_{405}\ 1.0\ UHS$$

where, **UP** is the undenaturated protein (residual antigen), $A_{405}$ **1.0 HS** is the concentration of the heated sample giving the absorbance of 1.0 and $A_{405}$ **1.0 UHS** is the concentration of the unheated sample giving the absorbance of 1.0.

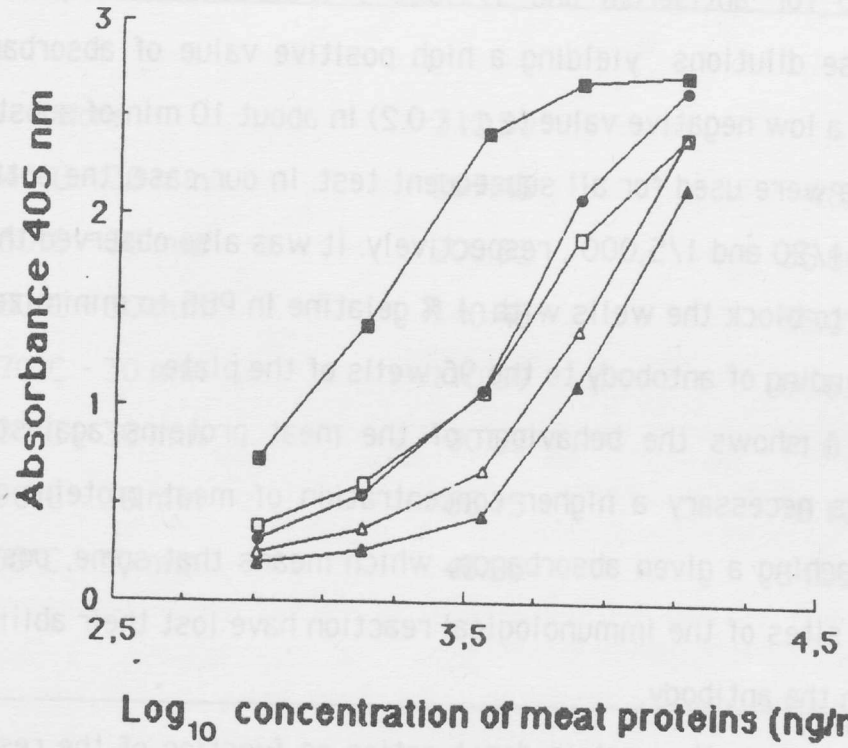


**Fig.1.** Assay of meat proteins receiving several heat treatments by the indirect-Elisa method. Unheated meat proteins ( ■ ), meat proteins heated at 50°C ( □ ), 60°C ( ● ), 80°C ( △ ) and 100°C ( ▲ ) for 30 min.

## RESULTS AND DISCUSSION

We have used an enzyme-linked immunosorbent assay (Indirect-ELISA) to study the effects of heat treatment in the range 40-100ºC on meat proteins denaturation measuring the undenaturated residual antigen.

To optimize the indirect-ELISA method, an exhaustive checkerboard titration of antibodies (anti-SSMP) and conjugate (HRPO-Protein A) were carried out. Three dilutions of antiserum and conjugate were tested : 1/10, 1/20 and 1/40 for antiserum and 1/2,500 , 1/5,000 and 1/7,500 for conjugate. Those dilutions yielding a high positive value of absorbances (e.g., > 1.5) and a low negative value (e.g., < 0.2) in about 10 min of substrate incubation time were used for all subsequent test. In our case, the optimun dilutions were 1/20 and 1/5,000 , respectively. It was also observed that it was necessary to block the wells with 1 % gelatine in PBS to minimize the non-specific binding of antobody to the 96 wells of the plate.

Figure 1 shows the behaviour of the meat proteins against the heating. It was necessary a higher concentration of meat proteins after heating for reaching a given absorbance, which means that some, possibly many, original sites of the immunological reaction have lost their ability to conjugate with the antibody.

Table 1 shows the protein denaturation as function of the residual antigenic meat proteins. When a heat treatment of 40°C was applied, a low but measurable denaturation was observed. However, at higher temperatures (50 and 60°C) a clear protein denaturation was already detected . From 312 ng/ml of the original antigen only reacted 200 ng/ml and 140 ng/ml respectively, which means that 33% and 55% of original meat proteins have been denatured by the treatment. Heat treatment at 80°C and 100°C for 30

min caused a more severe denaturation (71% and 84%).

**TABLE 1.**- Percentage of the denatured protein of meat subjected to several heat treatments.

| *Heat treatment* | *Residual antigenic meat protein (ng/ml)* | *% of denaturated protein* |
|---|---|---|
| None | 312.50 | - |
| 40°C - 30 min | 297.43 | 4.82 (±1.15) |
| 50°C - 30 min | 200.62 | 35.80 (± 0.6) |
| 60°C - 30 min | 140.46 | 55.05 (± 0.17) |
| 70°C - 30 min | 110.59 | 64.61 (± 0.53) |
| 80°C - 30 min | 90.53 | 71.03 (± 0.56) |
| 90°C - 30 min | 68.15 | 78.19 (± 1.42) |
| 100°C - 30 min | 48.28 | 84.55 (± 1.53) |

By plotting the $\log_{10}$ of the residual antigenic meat protein *versus* the temperature, a straight line was obtained (Fig. 2). This is obviously related to the denaturation of proteins is highly dependent of temperature. Therefore, a simple equation may be used for assessing the temperature at which the meat was subjected. The equation derived from Fig. 2 was

$$y = 2.937 - 0.0125x$$

where, x = temperature

y = $\log_{10}$ of the concentration of residual antigenic meat proteins.

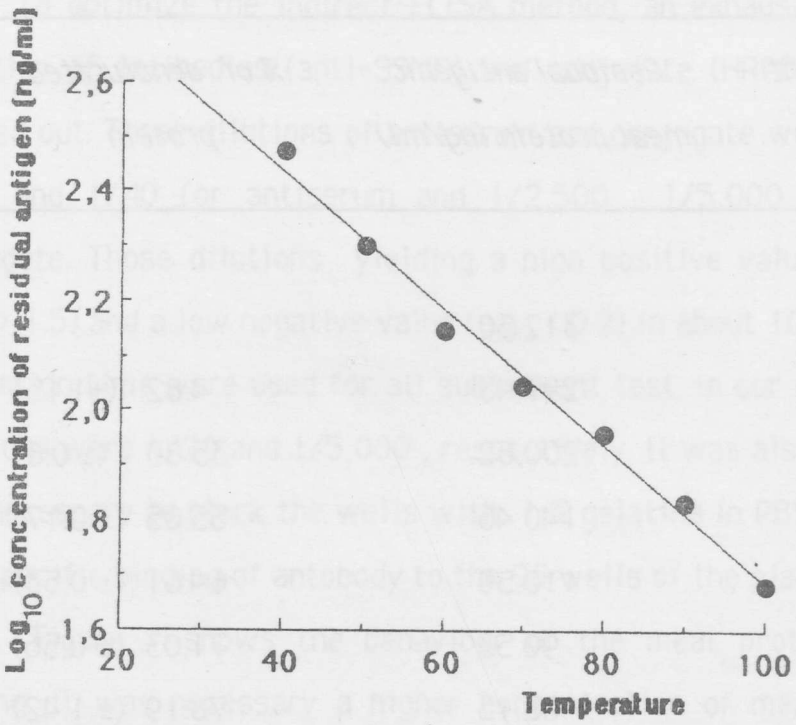


**Fig.2.** Relationship between the concentration of residual antigen (on logarithmic scale) and the temperarure of heating

Variations can occur between duplicates on the same assay and between results determined in different days. The variation coefficient (% VC) of absorbances for a sample of unheated meat proteins was less than 3% for duplicates on the same plate and near to the 4% for plates of different days.

It has been also proved that the assay may detect levels of undenatured antigens lower than 10-12 ng/ml, as measured at the mid-point

of the assay curve, i.e. at an absorbance of 1.0.

The results allow to deduce that the indirect-ELISA assay may clearly discriminate between samples of meat proteins receiving heat treatments with 10°C intervals. The assay is simple, reproducible and it may detect small levels of unaltered proteins on the heated meat proteins.

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