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Analytical Methods Section

Study of the effect of heat (treatments) on meat protein denaturation as determined by ELISA

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An enzyme-linked immunosorbent assay (indirect-ELISA) has been used to examine the effects of graded heat treatments on meat proteins denaturation. Approximately 14% of the proteins lost their antigenicity by heating up to 40°C, 50% up to 70°C and 70% at 100°C. Linear regression analysis showed that the remaining per cent antigenicity in the heated meat proteins was significantly correlated with the heating temperature ($r = 0{\cdot}993$, $n = 7$, $P < 0{\cdot}0001$).

INTRODUCTION

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

Muscle is a complex mixture of many different compounds. Heating of muscle during processing or cooking results in both chemical and physical changes in these compounds. With muscle proteins, this is evidenced by denaturation-coagulation (Davis & Anderson, 1984).

Studies of the antigenic structures of globular proteins have shown that antibodies to the native proteins are directed mainly against conformational rather than sequential sites (Iwabuchi & Shibasaki, 1981), now known as discontinuous and continuous epitopes. Consequently, only poor cross-reaction is observed between denatured proteins and antibodies to the same proteins in their natural forms.

An immunological technique provides a sensitive tool for the detection of structural changes produced in the antigen by the application of various treatments. In this way, a competitive inhibition enzyme-linked immunosorbent assay (ELISA) has been used for the detection of residual antigenic milk proteins after graded heat treatments (Kilshaw *et al.*, 1982; Heppell *et al.*, 1984).

The objective of the present work was to study the effects of graded heat treatments on meat protein denaturation measuring the remaining antigenic activity in heated meat proteins by an ELISA.

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MATERIALS AND METHODS

Preparation of meat extracts

Salt-soluble protein extracts of beef (semi-membranosus and adductor muscles) were prepared in PBS (0·01 M sodium phosphate buffer (pH 7·0), containing 0·6 M NaCl and 1 mM $MgCl_2$), as previously described by Li-Chan *et al.* (1984). Extracts were analyzed for protein concentration using the Lowry method (Lowry *et al.*, 1951).

Heat treatment

Salt-soluble protein extracts in the above-mentioned buffer were subjected to the heat treatment in a water bath with constant stirring to reach internal temperatures from 40 to 100°C for 30 min. After achieving the desired temperatures, the solutions were rapidly cooled in an ice water bath, then homogenized in a Sorwall Omni-mixer blender (DuPont, Newtown, Connecticut, USA) at approximately 369*g* for 15 s.

Preparation of the antiserum

Antibodies against salt-soluble meat proteins (anti-SSMP) were produced in two New Zealand male white rabbits. Immunization commenced 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 Adjuvant (Difco Laboratories, Detroit, MI). Ten booster doses were applied subcutaneously every five days. After 50 days, 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. Antisera obtained from two rabbits was pooled and stored frozen at –20°C.

Immunodiffusion analysis

The microtechnique of Ouchterlony's double diffusion method as described by Crowle (1973) was used. One per cent (w/v) agarose in PBS (0·01 M phosphate buffer, pH 7·2) was used as the medium for double diffusion; 0·1% sodium azide was added as a preservative.

Indirect-ELISA procedure

Salt-soluble protein extracts were initially diluted in the coating buffer (0·05 M carbonate-bicarbonate, pH 9·6) to contain 50 μg/ml of protein concentration. These were further diluted in doubling dilutions in the same buffer. Twelve serial dilutions were used to coat wells of a microtitre plate (Costar, Cambridge, MA) using 100 μl per well of each antigen dilution. The coated plates were incubated for 1 h at 37°C. The wells were washed five times with PBST (PBS containing 0·5 ml of Tween 20). In each cycle, the plates were usually flooded with PBST and dried to air. To block non-specific binding, 100 μl of 1% gelatine dissolved in PBS were added to every well and the plates incubated at 37°C for 1 h. After another five washes with PBST, 0·1 ml of anti-SSMP antiserum diluted 1/20 in PBS were added on the wells and the plates were incubated for 1 h at room temperature (19–21°C). Following a new washing with PBST, 0·1 ml of horseradish peroxidase–protein A conjugate (HRPO–protein A) (Sigma Chemical Co., St Louis, MO) at 1/5000 were added to the wells and the plates incubated for 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 (ABTS at 15% in citric acid, buffer pH 3·9) were added to each well and the reaction allowed to proceed for 30 min, before termination with 3 N sulphuric acid. Then the optical density (O.D.) at 405 nm was measured with a Titertek Multiskan spectrophotometer (Flow Laboratories, McLean, VA).

ELISA calibration curve

The calibration standards were prepared from a standard solution of unheated salt-soluble protein extract which was serially diluted (12 doubling dilutions). The observed optical densities were plotted against $\log_{10}$ concentrations of sample dilutions. Then, the equation of regression line correspondent to the linear part of the curve was calculated.

Calculation of original antigenic concentration in heated samples

The concentration of original antigen in heated samples, which reacted with the antiserum anti-SSMP, can be read directly from the calibration curve by choosing an appropriate dilution which gives an optical density value which lies on the linear part of the curve. However, more accurate results were obtained using a regression line formula.

Estimation of the remaining antigenicity percentage in each heated sample was done according to the equation: % remaining antigenicity = $(A/A_0) \times 100$, where A is the amount of remaining antigenic meat proteins after heating, and A_0 is the original amount of these untreated meat proteins.

RESULTS AND DISCUSSION

We have used an enzyme-linked immunosorbent assay (indirect-ELISA) to study the effects of heat treatment on meat proteins. This method was used to measure the amount of meat proteins that remained antigenic after heating for 30 min at temperatures from 40–100°C.

The double immunodiffusion test confirmed that the undiluted antiserum anti-SSMP gave a strong reaction of identity (showing one or more fused precipitating lines) with the unheated salt-soluble protein extract. However, a decrease in the intensity of the precipitation lines was observed when the heated extracts were tested against the same antiserum. These results show that meat extracts maintained their antigenic activity after a heat treatment but this activity decreased when the temperature increased.

To optimize the indirect-ELISA method, an exhaustive checkerboard titration of antibodies (anti-SSMP) and conjugate (HRPO–Protein A) was carried out. Three dilutions of antiserum and conjugate were tested: 1/10, 1/12 and 1/40 for antiserum and 1/2500, 1/5000 and 1/7500 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 30 min of substrate incubation time were used for every subsequent test. In the authors' case, the optimum dilutions were 1/20 and 1/5000 for antiserum and conjugate, 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 antibody to the 96 wells of the plate.

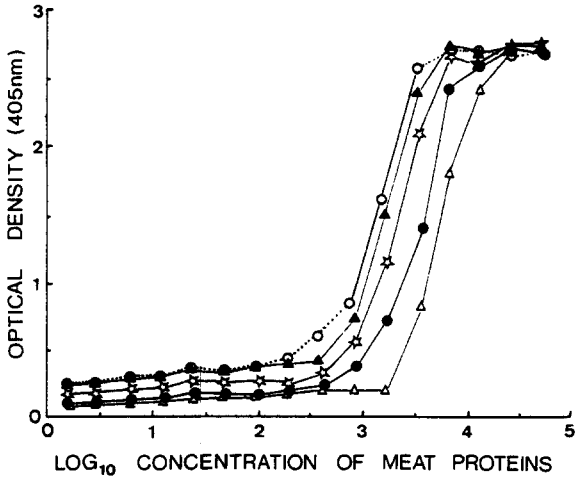

Fig. 1. Assay of meat proteins receiving several levels of heat treatment by the indirect-ELISA method. (○) Calibration curve (unheated meat proteins); (▲) meat proteins heated at 40°C for 30 min; (☆) at 60°C for 30 min; (●) at 80°C for 30 min; (Δ) at 100 °C for 30 min.

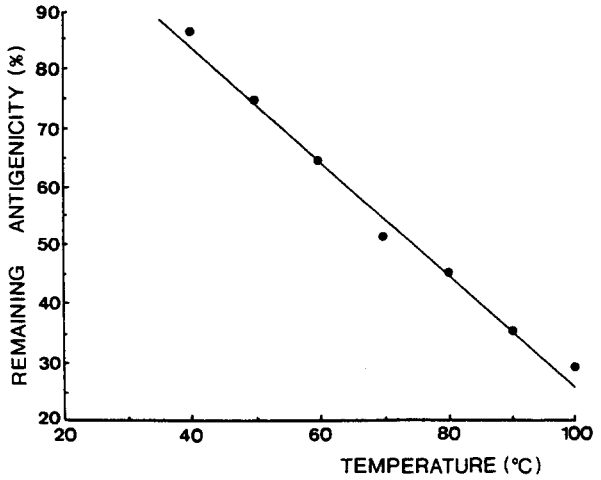

Fig. 2. Relationship between the remaining antigencity percentage in heated meat proteins and the heating temperature.

Figure 1 shows the behaviour of meat proteins against heating. As reference to the calibration curve, it was necessary to have a higher concentration of meat proteins after heating for reaching a given absorbance. The concentration of original antigen in heated samples which reacted with the antiserum anti-SSMP was calculated according to the equation of the regression line correspondent to the linear part of the calibration curve. This equation was $y = -7{\cdot}094 + 2{\cdot}731x$.

Table 1 shows the remaining antigenicity percentage in the heated meat proteins. This term signifies the relative amount of meat proteins that remain antigenic after certain heat treatment in relation to a control unheated sample which has been considered as 100% antigenic. The amount of antigenic meat proteins diminishes by heating. Approximately 14% of the proteins lost their antigenicity by heating up to 40°C, 50% up to 70°C and 70% at 100°C.

Figure 2 shows the correlation between temperature of heating and remaining antigenicity percentage in heated meat proteins. Linear regression analysis showed that the remaining antigenicity percentage in the heated meat proteins was significantly correlated ($r = 0{\cdot}993$; $n = 7$; $P < 0{\cdot}001$) with the temperature of heating. The following regression equation was obtained: $y = 122{\cdot}768 - 0{\cdot}968x$.

Table 1. Remaining antigenicity percentage in the meat proteins receiving several levels of heat treatment

Heat treatment	Concentration of original antigen (ng/50 μg)	Remaining antigenicity (%)
None	1622	100·0
40°C–30 min	1401	86·3
50°C–30 min	1210	74·6
60°C–30 min	1045	64·4
70°C–30 min	826	50·9
80°C–30 min	729	44·9
90°C–30 min	567	35·0
100°C–30 min	469	28·9

Several heat-stable antigens from muscle tissues have been reported by Sherikar *et al.* (1988) and Kang'ethe and Gathuma (1987). However, it has been demonstrated that the relative reduction in immunological activity is dependent on the temperature of heating. So, according to Berger *et al.* (1988), these antigens are not completely heat-stable because increased heating results in a concomitant reduction of reaction intensities. On the other hand, antigenic sites can be divided into two structural categories. A sequential (continuous) site exists wholly within a continuous segment of the amino acid sequence. An assembled topographic site (conformational) consists of amino acid residues far apart in the primary sequence but brought together in the surface topography of the native protein by the way it folds in three dimensions (Berzofsky, 1985). The relative reduction of antigenic activity could be explained by the possible destruction of some antigenic sites as a consequence of heat treatment. However, it is not yet possible to say if this reduction may be associated with the disruption of sequential or conformational antigenic sites.

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