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Role of cathepsin D activity in gelation of chicken meat heated under pressure

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Abstract

The role of cathepsin D activity in gelation of chicken meat batters (400 MPa/30 min/70 °C) heated-under pressure was investigated, using a specific inhibitor, pepstatin, dissolved in dimethyl sulphoxide (DMSO)/acetic acid (9:1 v/v). Thermal treatment (70 °C/30 min) produced less thermal inactivation of cathepsin D activity at 400 MPa than at atmospheric pressure. Heating, under pressure conditions, produced gels which were less hard and chewy than those produced at atmospheric pressure. Irrespective of the pressure, the presence of the inhibitor solvent influenced the thermal gelation of meat batters, facilitating the formation of harder, chewier gels.

Keywords: Meat batter; High pressure; Gelation; Cathepsin D activity; Pepstatin

1. Introduction

One of the various applications of high pressure processing (HPP) of food systems is that it affects protein gel-forming properties, which suggests interesting possibilities for the development of processed muscle-based food. Pressure-assisted gelation depends on pressure/temperature combinations, so that not only are the levels of pressure and temperature important, but also the sequence in which they are applied.

Heating (> 40 °C) under high pressure conditions limits the gelling of meat systems. When pork and chicken meat batters were heated at 60–80 °C/30 min at 200–400 MPa, the resulting structures were weaker and had better water-binding properties than gels made by heating (under atmospheric conditions) or pressurized prior to heating (Cofrades, Fernández, Carballo, & Jiménez-Colmenero, 1998; Fernández-Martín, Fernández, Carballo, & Jiménez-Colmenero, 1997; Jiménez-Colmenero, Cofrades, Carballo, Fernández, & Fernández-Martín, 1998). Similar results have been reported in chum salmon and Alaska pollack surimi (500 MPa/10 min/60 °C; Okazaki, Ueda, Kusaba, Kimura, Fukuda, & Arai, 1997), Pacific whiting surimi (0.1–0.24 MPa/60 min/50 °C; Chung, Gebrehiwot, Farkas, & Morrissey, 1994) and blue whiting (200–420 MPa/10–30 min/0–75 °C; Peréz-Mateos, Lorenço, Montero, & Borderías, 1997). This behaviour has been associated with increased protease activity in meat protein, due to high pressure (Jiménez-Colmenero et al., 1998), observed during heating-under-pressure treatments (Boton, Harris, Macfarlane, & O'Shea, 1977; King, Kurth, & Shorthose, 1981; Locker & Wild, 1984; Macfarlane, 1985; Macfarlane, Mackenzie, & Turner, 1986). Under these experimental conditions, proteolytic myofibrillar protein breakdown caused formation of various molecular fragments (Jiménez-Colmenero et al., 1998; Macfarlane et al., 1986). This may help to improve water binding properties, but the resulting 3-dimensional gel is less complete and orderly, so that the gel matrix is less rigid than when meat batters are heated under atmospheric conditions (Jiménez-Colmenero et al., 1998).

Many endogenous proteases are recognized as important in muscle proteins during storage and processing. Of these, cathepsin D was considered to be one of most important in post-mortem degradation of muscle and could therefore play an important role in meat

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processing. Cathepsin D from different sources (bovine, chicken, ostrich, pork) has been reported to be active at elevated temperatures, 33–60 °C (Draper & Zeece, 1989; Przysiezna & Skarabka-Blotnicka, 1996; van Jaarsveld, Naudé, & Oelofsen, 1997) and even to retain high levels of activity at close to 70 °C (Spanier, McMillin, & Miller, 1990; Toldrá, Rico, & Flores, 1992). At the same time, a considerable pressure-induced increase has been reported in cathepsin D activity up to 400 MPa (at low temperature, 2–10 °C) in both purified enzyme and meat (Homma, Ikeuchi, & Suzuki, 1994; Jung, Lamballerie-Anton, Taylor, & Ghoul, 2000). The broad temperature range (33–70 °C) of cathepsin D activity, the optimum temperature (45–55 °C) and its behaviour during HPP were compatible with the proposition that some extra proteolytic activity could occur during heating under pressure conditions, limiting pressure-assisted gelation. However, there are no references to studies that address this possibility.

Specific inhibitors have classically been used to investigate enzyme activity and their mechanism of action. Pepstatin is a specific and powerful inhibitor of cathepsin D, which has been used to study the effect of endogenous proteases on meat aging (Alarcon-Rojo & Dransfield, 1995), heat-induced tenderization (King & Harris, 1982; King et al., 1981), thermal scanning rheology of myofibrillar proteins (Young, Torley, & Reid, 1992) and pressure/heat-induced changes in meat (Macfarlane et al., 1986).

Very little is known about the possible roles played by certain proteases in thermal gelation of pressurized meat batters and how they relate to gel rheological properties. The object of this study was to examine how proteolytic phenomena, produced by cathepsin D activity, influence the gelation process during heating of myosystems under pressure.

2. Materials and methods

2.1. Preparation of meat batters

Fresh chicken breast (10 kg) was obtained from a local meat market. The meat was trimmed to remove visible fat and connective tissue, preground through a 3-mm plate (FTS111, Van Dall SRL. Milano, Italy) to obtain a homogeneous mass, vacuum-packaged and kept frozen at (−20 °C) prior to use, which took place within 2 weeks.

Sufficient amounts of meat (previously thawed in air at 2–3 °C for 20 h, to reach between −3 and 0 °C) and water and 1.5% NaCl were combined to formulate a meat batter (500 g) containing approximately 16% meat protein (control sample, C). A similar meat batter was prepared, treated with 4 mg of pepstatin A (isovalerylpepstatin) (Sigma-Aldrich Chemical Co, St Louis, MO) per 100 g of sample (inhibitor sample, I). The pepstatin was dissolved in 8.9 ml of a dimethyl sulphoxide (DMSO)/acetic acid solution (9:1 v/v), prepared according to Sigma brand instructions. The concentration of pepstatin used was similar to those of King and Harris (1982) and Macfarlane et al. (1986). To determine the real effect of pepstatin, a third meat batter was formulated, containing the solvent (DMSO/acetic acid) but without the inhibitor (solvent sample, S). To prevent changes in final sample volume, the amount of solution used in meat batters I and S (with and without inhibitor) was subtracted from the volume of water required by the formulation.

The batters were prepared as follows: raw meat material was homogenized and ground for 60 s in a chilled cutter (2 °C) (Stephan Universal Machine UM5, Stephan u. Sóhne GmbH & Co., Hameln, Germany); then half the water plus NaCl were added and homogenization continued for 30 s. The rest of the water, plus pepstatin in DMSO/acetic acid or DMSO/acetic acid solution, was then added and the mixture homogenized again under chilled vacuum (2 °C, 610 mm Hg) for 60 s. In every case the final chopping temperature of the batters was 7–10 °C.

Each meat batter was packed into two different flexible plastics jars, filling them without entrapping air. Jar dimensions were height = 7 cm, diameter = 3.3 cm (60 ± 1g) and height = 5.6 cm, diameter = 2.2 cm (20 ± 1g). The jars were hermetically closed with threaded caps and placed in 8 × 30 cm Ultra-Cover latex bags (Amevisa S.A., Madrid, Spain).

2.2. Pressure and thermal treatment

Pressurization was carried out in an ACB model AGIP No. 665 high-pressure pilot unit (GEC, Alsthom, Nantes, France). Two different pressure/thermal treatments were used: (1) 60-g jars were heated under pressure (400 MPa, 30 min) using water at 70 °C as the heating and pressurizing medium (HUPC sample); (2) 20-g jars were pressurized at low temperature (400 MPa, 30 min), using water at 10 °C, as the pressurizing medium (P sample).

Pressure was increased to 2.25 MPa/s and released in 10 s. The heating conditions, required to attain a temperature of 70 °C, were determined beforehand by inserting thermocouples connected to a temperature recorder (Yokogawa Hokushin Electric YEW, model 3087, Tokyo, Japan). The temperature in the pressurizing medium increased by 5 °C during pressurization, returning to the initial temperature (10 or 70 °C) after 3.5 min. During depressurization the temperature of the pressurizing medium dropped by 8 °C in samples pressurized at 70 °C and by 5 °C in samples pressurized at 10 °C.

For each formulation, two non-pressurized control samples were made under the same conditions as the

pressurized samples: heated (30 min/70 °C; NPH sample) and treated 30 min/10 °C; NP sample) .

After pressurizing, the samples were chilled and stored for 18 h at 0–4 °C for analysis.

Since high pressure/temperature processing could affect the activity of the enzyme inhibitors (Jung et al., 2000), the ability of pepstatin (treated using the same processing conditions and pressure/thermal treatments as in this experiment) to inhibit cathepsin D activity had been verified in a previous experiment (Cofrades, Bañon, Carballo, & Jiménez Colmenero, 2002).

2.3. Proximate analysis, pH and weight loss

Moisture, protein, fat and ash of uncooked meat batters and pH and weight loss (WL, as% fluid released) of heated samples were evaluated according to Fernández, Cofrades, Solas, Carballo, and Jiménez Colmenero (1998).

2.4. Assay of cathepsin D activity

The assay of cathepsin D activity was performed according to procedures described by Rico, Toldrá, and Flores (1991). The activity was defined as the increase of absorbance at 280 nm per hour at 45 °C and pH 3.7 (A_{280} h^{-1}). Relative activity was expressed as the percentage difference in specific activity between treated sample and untreated meat batter (control sample non-pressurized, C/NP).

2.5. Dynamic rheological measurement

Rheological changes in unheated and non-pressurized samples (C, I, S) during thermal gelation were studied using a Bohlin CSR rheometer (Bohlin Instruments, Inc., Cranbury, NJ) operating in the small-amplitude oscillatory mode. After equilibration at room temperature (20 °C), thermal gelation was induced by heating samples from 20 to 70 °C at 1 °C/min using a Bohlin temperature control unit. Samples were sheared at a fixed frequency of 1.0 Hz with a strain of 0.02. The gap between the plates was set at 1 mm. The sample perimeter was covered with a thin layer of silicon oil to prevent dehydration. The storage modulus (G') data were collected every minute during shearing measurements.

2.6. Rheological assessment

A penetration test was carried out (in quintuplicate) on the unheated samples (NP, P) in their containers (20-g jars) once they attained ambient temperature. This was performed with a cylindrical stainless-steel plunger (diameter 0.5 cm) attached to a 100 N cell connected to the crosshead of the Instron machine; crosshead speed was 1.0 cm/min. Gel strength (J) was measured from the force-deformation curves as the area enclosed by the curve from the first moment of contact with the surface to a depth of 10 mm.

Texture profile analysis (TPA) of heated samples (60-g jars) was performed as described by Bourne (1978). Five cores (diam. = 3.3 cm, height = 2.0 cm) were axially compressed to 40% of their original height. Force-time deformation curves were derived with a 5 kN load cell applied at a crosshead speed of 50 mm/min. Attributes were calculated as follows: hardness (Hd) = peak force (N) required for first compression; cohesiveness (Ch) = ratio of active work done under the second compression curve to that done under the first compression curve (dimensionless); springiness (Sp) = distance (mm) the sample recovers after the first compression; chewiness (Cw) = Hd × Ch × Sp (N × mm).

A Universal Testing Machine (model 4501 Instron Engineering Corp., Canton, MA) equipped with an Hewlett Packard Vectra ES/12 computer (Hewlett Packard Company, WA, USA), was used.

2.7. Statistical analysis

One-way analysis of variance by an F test and least squares differences by Statgraphics 5.0 (STSC Inc., Rockville, MD) were used to compare mean values and to identify significant differences ($P < 0.05$) among treatments.

3. Results and discussion

The previous studies (Cofrades et al., 2002) showed that the ability of pepstatin to inhibit cathepsin D activity was unaltered by the same processing conditions (pressure and thermal treatments) as were used for meat batters in the present experiment.

3.1. Proximate analysis, pH and weight loss

The composition of the formulated meat batters was: moisture 79%, protein 16.3%, fat 1.9% and ash 2.1%. The presence of the added compounds (inhibitor and/or solvent) appreciably ($P < 0.05$) reduced the pH in untreated (C, 6.1, I and S, 5.2) and heated samples (Table 1). Pressurization had very little effect ($P > 0.05$) on the pH .

In both nonpressurized and pressurized samples, WL increased ($P < 0.05$) with the addition of solvent and/or inhibitor (Table 1). In the control (C), pressurization reduced ($P < 0.05$) WL (Table 1). A similar pattern has been described by Fernández-Martín et al. (1997), Fernández et al. (1998) and others. Whereas WL was unaltered ($P > 0.05$) by pressurization in the sample with inhibitor, HPP enhanced binding properties in samples with solvent only. The inhibitor had no effect on WL in pressurized meat batters.

Table 1
pH and weight loss (WL) for the different heated samples

Sample[a]	pH	WL (%)
C/NPH	6.3a	2.8a
S/NPH	5.7b	6.3b
I/NPH	5.7b	4.7c
C/HUPC	6.4a	0.4d
S/HUPC	5.8b	4.3c
I/HUPC	5.8b	4.1ac
SEM	0.0	0.3

[a] C, control sample; I, batter with inhibitor in DMSO/acetic acid; S, batter with solvent only (DMSO/acetic acid); /NPH, nonpressurized-and-heated (30 min/70 °C); /HUPC, heated under pressure conditions (400 MPa/30 min/70 °C). SEM, standard error of means. Different letters in the same column indicate significant differences ($P < 0.05$).

3.2. Enzymatic activity

As expected, under the experimental conditions, pepstatin appreciably inhibited cathepsin D activity. However, inactivation was not complete, the I/NP sample retaining just under 10% of the activity recorded in absence of pepstatin (sample C/NP; Fig. 1). Given the pH of the sample and the pepstatin concentration, this percentage is consistent with the findings of Knight and Barrett (1976). Heating at 70 °C (at atmospheric pressure) completely halted enzymatic activity in the control (C/NPH), but not in the other two samples, where low but appreciable activity (3–5%) persisted. These results are consistent with the reports of other authors on the effect of temperature on cathepsin D activity (Draper & Zeece, 1989; Przysiezna & Skarabka-Blotnicka, 1996; van Jaarsveld et al., 1997). Spanier et al. (1990) found 12% of initial activity persisting at 70 °C

Some authors have observed a gradual increase of cathepsin D activity with increasing pressure up to 400 MPa at low temperature (Homma et al., 1994; Jung et al., 2000), but no such behaviour was observed in the present case following pressurization at 400 MPa/30 min/10 °C (Fig. 1). There are two factors that possibly account for the difference: (1) thermal denaturation under high pressure conditions used in the present experiment, and (2) the methodology followed in the assay of cathepsin D, where the use of a blank containing pepstatin eliminated the effects of acid proteases other than cathepsin D (Rico et al., 1991). In all cases, residual cathepsin D activity was higher in samples heated under pressure than in those heated at atmospheric pressure (Fig. 1), indicating less thermal inactivation at 400 MPa. In fact there was an apparent tendency for activity to increase in the sample with inhibitor (Fig. 1). Variations in enzymatic activity during HPP have been related to changes in specific enzyme activity and the concentration of the enzyme resulting from its release into the medium (Homma et al., 1994; Jung et al., 2000). Kurth (1986) reported that, not only

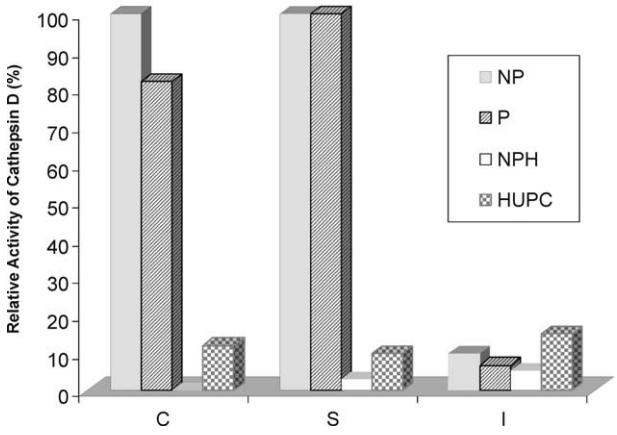

Fig. 1. Relative cathepsin D activity for the different samples. C, control sample; I, batter with inhibitor in DMSO/acetic acid; S, batter with solvent only (DMSO/acetic acid); NP, nonpressurized; P, pressurized (400 MPa/30 min/10 °C); NPH, nonpressurized-and-heated (30 min/70 °C); HUPC, heated under pressure conditions (400 MPa/30 min/70 °C).

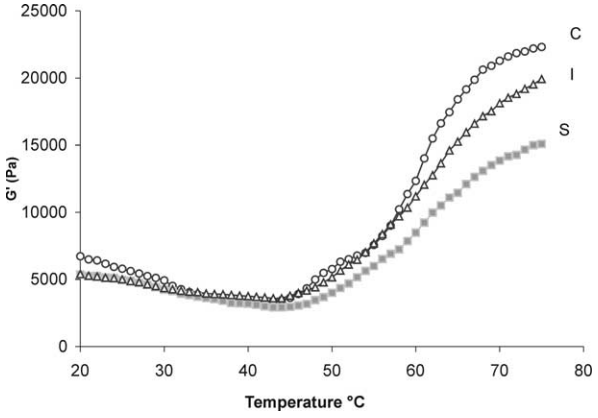

Fig. 2. Storage modulus as a function of heating temperature. C, control sample; I, batter with inhibitor in DMSO/acetic acid; S, batter with solvent only (DMSO/acetic acid).

was the activity of cathepsin B1 (in solution) retained under the elevated pressures used (150 MPa), but in some pressure-heat combinations it was greatly enhanced. As far as the authors are aware, there are no data, in the literature, on the effect of heated-under-pressure conditions on residual cathepsin D activity in meat batters.

3.3. Dynamic rheological measurement

Fig. 2 shows storage modulus as a function of temperature for different non-pressurized samples. The rheological thermogram of the control meat batter presents the features normally seen in minced meat with low added salt (Egelandsdal, Martinsen, & Autio, 1995). The presence of pepstatin and/or solvent produced some differences in the rheological behaviour of the samples during heating. In earlier stages of heat processing, all three samples behaved similarly, G'

values falling to a minimum at around 45–48 °C (Fig. 2). Further heating produced a sharp increase of G', indicating the formation of a stiff, elastic matrix structure, typical of heat induced protein gels. Rheological behaviour of samples was different above 55–60 °C; the presence of the solvent limited the formation of a gel with lower rigidity values, and values of G' were closer to those of the control. This seems to suggest that, although the presence of chemicals alters the meat system, inhibition of cathepsin D enhances gel formation.

3.4. Rheological assessment

The presence of inhibitor and/or solvent had no observable effect on the gel strength ($P > 0.05$) of uncooked and unpressurized meat batters (Fig. 3). Nevertheless, pressurization caused certain alterations in the characteristics of the protein matrix, as shown by a significant increase of gel strength (Fig. 3), which was greater ($P < 0.05$) in samples S/P and I/P. However, the inhibitor had no additional effect on gel strength over and above that of the solvent.

At atmospheric pressure, the samples with inhibitor and/or solvent (I/NPH and S/NPH) produced gels all of very similar TPA characteristics to one another but lower ($P < 0.05$) Hd, Ch and Cw than the control batter (C/NPH) (Table 2). This indicates that the solvent by itself induced changes in the properties of the gel while the pepstatin had no apparent effect. King and Harris, (1982) observed no effect on Warner-Braztler measurements from injections of DMSO (without inhibitor) in lambs. However, when pepstatin (dissolved in DMSO) was injected *pre-rigor*, the meat was found to be harder, a fact attributed to inhibition of cathepsin D activity by the pepstatin. No such behaviour was observed when injection was performed *post-rigor* (King & Harris, 1982). In other cases where the role of pepstatin has been studied, no attempt was made to assess the possible effect of the solvent on textural properties of meat systems.

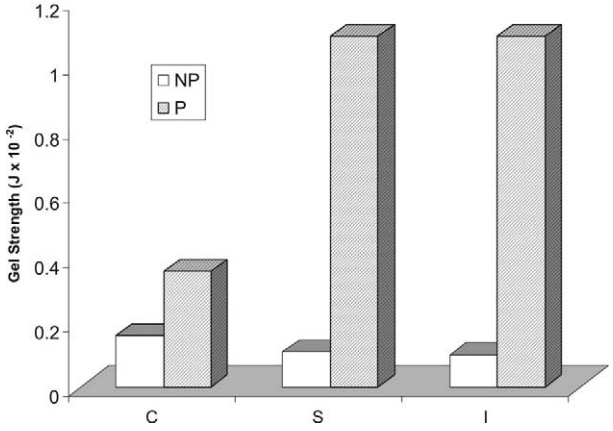

Fig. 3. Gel strength of uncooked meat batters. C, control sample; I, batter with inhibitor in DMSO/acetic acid; S, batter with solvent only (DMSO/acetic acid); NP, nonpressurized; P, pressurized (400 MPa/30 min/10 °C). Different letters indicate significant differences ($P < 0.05$). Standard error of means 0.017.

Table 2
TPA parameters (hardness, Hd; Springiness, Sp; Cohesiveness, Ch and Chewiness, Cw) for the different heated samples

Sample[a]	Hd (N)	Sp (mm)	Ch	Cw (N×mm)
C/NPH	53.4a,d	7.1a	0.62a	233.4a,d
S/NPH	40.5b	7.3a	0.58b	171.0b
I/NPH	41.6b	7.2a	0.58b	173.5b
C/HUPC	30.5c	7.0a	0.57b	122.1c
S/HUPC	56.5a	7.0a	0.64c	253.7a
I/HUPC	49.5d	7.1a	0.65c	227.2d
SEM	1.1	0.1	0.00	5.2

[a] C, control sample; I, batter with inhibitor in DMSO/acetic acid; S, batter with solvent only (DMSO/acetic acid); /NPH, nonpressurized-and-heated (30 min/70 °C); /HUPC, heated under pressure conditions (400 MPa/30 min/70 °C). SEM, standard error of means. Different letters in the same column indicate significant differences ($P < 0.05$).

Pressurization influenced TPA parameters differently in each of the three samples (Table 2). In the control batter (sample C/HUPC), HPP caused the formation of gel structures with lower ($P < 0.05$) Hd, Ch and Cw than the samples treated at atmospheric pressure. Similar results have been reported elsewhere for this type of HPP (Jiménez Colmenero et al., 1998), and it has been suggested that such behaviour could be related to a higher degree of proteolytic protein breakdown.

In the samples with inhibitor and/or solvent, the effect of pressurization on TPA parameters was the opposite to the effect on the controls (Table 2). HPP produced gels with higher ($P < 0.05$) Hd, Ch and Cw (Table 2) than the corresponding samples heated at atmospheric pressure. There were some differences in Hd and Cw due to the presence of pepstatin (Table 2). Generally, samples S/HUPC and I/HUPC exhibited very similar gel properties to those of sample C/NPH. Under the experimental conditions, there was no clear tendency for the inhibitor to affect TPA parameters. Macfarlane et al. (1986) reported that pressurized (150 MPa/60 °C/10 min) cold-shortened *post-rigor* muscle (injected with DMSO, 1 ml/100 g y DMSO + pepstatin, 4 mg/100 g) produced lower Warner-Braztler shear values than samples heated at atmospheric pressure (60 °C/10 min). That study did not attempt to compare shear values against samples without DMSO and therefore it is not possible to evaluate the role of the solvent; however, the results do suggest that pepstatin did not affect texture under the given conditions.

3.5. Conditions of use of inhibitor

Various protease inhibitors have been used to study the effect of muscle endogenous proteases on thermal rheological properties of muscle protein gels (Liu & Xiong, 1997). Pepstatin was used to study the effect of

muscle endogenous proteases on different meat processes, among them thermal gelling ability (Young et al., 1992). Because of its solubility characteristics, pepstatin is generally used dissolved in DMSO (King & Harris, 1982; Macfarlane et al., 1986; Uytterhaegen, Claeys, & Demeyer, 1994; van Jaarsveld et al., 1997). Given that, it is essential to estimate what effect if any is induced by the solvent, by comparing the results for sample with pepstatin (inhibitor plus inhibitor solvent) to the results for a sample containing inhibitor solvent only (King & Harris, 1982; Macfarlane et al., 1986; Uytterhaegen et al., 1994). There are few references to the use of pepstatin to analyse the impact of muscle endogenous proteases on thermal gelation processes. Also, to the authors' knowledge there has been no attempt to evaluate the influence of the inhibitor solvent on the gel properties of meat batters and to ascertain how they compare with samples without any of these compounds. Mestre-Prates, Ribeiro, and Dias Correia (2001) observed no effect of DMSO on shear force in several injected rabbit muscles; however, in the present experiment the presence of the inhibitor solvent clearly affected thermal gelling properties of meat batters (Table 1). This effect may be related to the changes that it induces in meat batter conditions, which were reflected in a reduction ($P < 0.05$) of pH (Table 1). DMSO is known to modify the characteristics of different biological systems where it has been used as a cryoprotectant (Walicka, Ding, Adelstein & Kassiss, 2000), but in this experiment the observed behaviour was due to the heating process.

The results illuminate some shortcomings in the model systems used to study the effects of certain enzymes on meat processing and point to the need for new approaches for the purpose of analyzing the effects of endogenous proteases (e.g. cathepsin D) on the thermal gelation process. Data, such as those reported here, are useful as a starting point for further examination of the role played by enzymatic phenomena in pressure-assisted gelation. In this connection we would stress the desirability of evaluating enzymatic activity in assay conditions rather than assessing their effect on the basis of post-treatment residual enzymatic activity in the meat system, as is often the case. If inhibiting mechanisms are to be used for that purpose, we need to investigate various aspects, such as the type of inhibitor, the concentration required, the solvent used for solubilization, HPP effect on inhibitor activity, to identify those that adequately inhibit enzymatic activity without interfering with gelation under the given processing conditions.

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