Partial characterization of protease activity in squid (*Todaropsis eblanae*) mantle: modification by high- pressure treatment

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ABSTRACT

16 Optimum conditions for proteolytic activity in both, non-pressurized and pressurized (300 MPa, 7 °C, 20
min) squid (*Todaropsis eblanae*) muscle, occurred at acid pH levels (pH 3) over a broad range of
temperatures. Pressure treatment did not modify optimum pH and temperatures but did increase
proteolytic activity. The acid cysteine proteases and to a lesser extent the acid serine proteases were the
20 enzymes mainly affected by the high-pressure treatment. The SDS-PAGE was indicative of increased
protein hydrolysis by pressurization. Myosin heavy chain in both, non-pressurized and pressurized
squid, was degraded at all the temperatures tested, but actin was only susceptible to proteolysis in the
pressure-treated muscle at 7 °C and 40 °C. This behavior was not observed at 55 °C.

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Key words: Squid, high pressure, proteolysis, protease inhibitors

Introduction

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Cephalopods make up a significant fraction of living organisms that are fit for human consumption. They are normally consumed in the form of rings or whole when small. Nevertheless, in some cases they are not appropriate for consumption directly because on account of not having an adequate texture, or
32 size. In those cases they can be processed for use in various ways, one process being the manufacture of gels from their muscle. Because cephalopods have a low thermal gelling capacity due to their high levels of proteolytic activity, high-pressure gelling of the muscle protein could be considered as a possible alternative to conventional heat treatment (Montero and others 1997; Fernández-Martín and others
36 1998).

High-pressure treatments initially originated in food processing to prolong the shelf-life of foodstuffs during storage and retailing, mainly as a means of inactivating microorganisms and denaturing enzymes. High pressure may affect enzymatic reactions directly by altering the kinetic constants or by producing
40 conformational changes in the structure of the enzymes and/or substrates (Morild 1981; Dufour and others 1996). Such pressurization conditions as the amount of pressure, time, temperature, pH, etc. also play a role, and a given reaction may be activated or inhibited depending on the conditions (Defaye and
Ledward 1995). Additionally, studies have shown the enzymatic activity pattern to differ depending on
44 whether raw extract or whole muscle is used (Chung and others 1994). Earlier studies have contemplated using high pressure for enzyme denaturation, e.g., inactivation of aminopeptidases and carboxypeptidases at 500 MPa and at 400 MPa, respectively (Ohmori and others 1991); of the proteases
in spear squid (*Loligo bleekeri*) mantle at pressures greater than 800 MP (Nagashima and others 1993);
48 of collagenase and cathepsin B at a pressure of 300 MPa (Dufour and others 1996); and activation of the proteolytic reactions in meat at pressures of between 100-300 MPa (Kim and others 1992; Homma and others 1994).

In addition to interfering with gelling (Nagashima and others 1992; Gómez-Guillén and others 1996),
52 the action of the proteolytic enzymes in cephalopod muscle has been observed to lead to rapid

deterioration, resulting in a loss of quality (Sakai and Matsumoto 1981; Leblanc and Gill 1982; Konno and Fukazawa 1993; Ayensa and others 1999). Most studies on the role of proteases in the breakdown of seafood have dealt with post-mortem deterioration at chilled or high (50-70 °C) temperatures (Wasson 1992). Autolysis in frozen mantle and suitability for gelling has also been examined (Gómez-Guillén and others 2003).

Studies carried out using specific and semi-specific enzyme inhibitors have made it possible to characterize the proteases and classify them according to the configuration of their active site. Serine and cysteine proteases have proved to be the most closely involved in gel degradation. Marked reductions in the levels of the rheological properties of the mantle tissue in different cephalopod species has been described and associated with a peak in proteolytic activity related to serine protease activity at a temperature of around 40 °C (Ebina and others 1995; Gómez-Guillén and others 2002). Cathepsins are the most active cysteine proteases in the muscle, particularly cathepsins B and L, which interfere significantly with gelling because they are active at high temperatures. Ayensa and others (1999) reported activity by cathepsins B and L in the mantle in the squid *Todaropsis eblanae*.

Enzymatic activity is dependent on muscle pH. Proteolytic activity has been described in the mantle of various cephalopods at acid (Sakai and Matsumoto 1981; Hurtado and others 2002), basic and neutral (Rodger and others 1984; Ayensa and others 1999) pH levels. Similarly, different optimal temperatures have been suggested for different cephalopod species (Rodger and others 1984; Konno and Fukazawa 1993; Hurtado and others 2002).

The object of this experiment was to study changes by high pressure treatment in proteolytic activity in squid muscle (*Todaropsis eblanae*) at different pH and temperatures.

80 **Material and Methods**

Sample preparation and treatment

84 Squid (*Todaropsis eblanae*) was obtained from a local market within 24-48 hours of captured and transported in ice to the Instituto del Frío (CSIC), Madrid, Spain. The average weight and length were 113.55 g ($\pm$ 37.17) and 14.82 cm ($\pm$ 1.38). The squids were immediately gutted and washed upon arrival. Tentacles were removed and mantles were vacuum-packed in flexible bags (Cryovac BB4L, Barcelona, Spain). Half of the vacuum-packed mantles were subjected to pressure treatment at 300 MPa at 7 °C for 20 minutes (pressurized batch: P), while the other half were kept at 7 °C at atmospheric pressure (non-pressurized batch: NP). Conditions for the pressurization were chosen according to the results of previous assays (Pérez-Mateos and Montero 1997; Hurtado and others 2001). High-pressure treatments were performed in a high-pressure pilot unit (ACB 665, Gec Alsthom, Nantes, France) where the temperature of the immersion medium (distilled water) was controlled via a thermo-couple with a programmed thermostatization equipment (model IA/2230 AC, INMASA, Barcelona, Spain). Pressure was increased at 2.5 MPa/s. Both batches were then frozen at -30 °C for two hours in an air-blast tunnel freezer (AGA-Frigoscandia 0-6373, Helsingborg, Sweden). Frozen squids were skinned, cut into small pieces and mixed. Samples were vacuum-packed and stored at -54 °C to prevent any alterations during storage until analysis.

100 **Proximate analysis**

 The moisture and ashes content were determined by the method of A.O.A.C. (2000). The crude protein was measured by the method of Dumas following A.O.A.C. 992.15 (A.O.A.C. 2000), with the analyzer LECO (FP-2000 LECO Corporation, St. Joseph, USA). The crude fat determination was realized according to the method of Bligh and Dyer (1959) and later modified by Knudsen and others

(1985). Results were average of at least three determinations. The majority components of the raw squid mantle were 14.43 % ($\pm$ 0.13) of crude protein; 1.08 % ($\pm$ 0.03) of crude fat; 83.52 % ($\pm$ 0.22) of moisture and 1.14 % ($\pm$ 0.05) of ashes.

Determination of pH

The pH was determined according to the technique used by Vyncke (1981) in a pHmeter (MeterLab pHM 93, Radiometer Analytical, Copenhagen, Denmark). The result was average of at least three determinations.

Effect of temperature, pH and NaCl on proteolytic activity

To study the effect of temperature on proteolytic activity, the chopped muscle was homogenized in cold 0.2 M phosphate buffer (pH 6.5) at a ratio of 1:2 (w/v), at 10,000 rpm for 1 min in an iced water bath, using an Omnimixer (Model ES-231, Homogenizer, Omni International, Waterbury, USA). The homogenates were filtered through 4 layers of cheesecloth in order to remove connective tissue. The range of temperatures assayed were from 20 to 80 °C.

In the pH profile for proteolytic activity, the buffers used for the homogenization were McIlvaine buffer (0.2 M sodium phosphate and 0.1 M sodium citrate), for pH 2 to 7; and Tris-HCl buffer (0.2 M) for pH 8 and 8.5, all of them at 4 °C. The incubation temperatures were 40 and 55 °C.

In the study of NaCl effect on proteolytic activity, a cold solution of NaCl 0.15 M was used for the homogenization. The incubation temperatures were 40 and 60 °C. NaCl was supplied by PANREAC (Montplet & Esteban, S.A., Barcelona, Spain).

Proteolytic activity was assayed according to the modified method of Hurtado and others (1999), using at least three replicates, as follows. Three-gram aliquots of the homogenate, immediately after the preparation of crude homogenate, were incubated for one hour at the different experimental

132 temperatures. Following the incubation, adding 6 mL of cold 10 % trichloroacetic acid (TCA) the
proteolysis was stopped. The mixture was kept at 4 °C for 15 min to allow unhydrolyzed proteins to
precipitate, and then they were centrifuged at 6,100 x g for 15 min at 4 °C (Beckman J2-MC, JA-20
rotor, Calif., USA). The content of soluble oligopeptides in the supernatant was analyzed by the method
136 of Lowry and others (1951) using tyrosine as standard. Absorbance was measured in an UV-visible
Spectrophotometer (UV-1601 SHIMADZU, Tokyo, Japan). Activity was expressed as μ moles of
tyrosine released per gram of muscle per hour of incubation (μ mol Tyr/g/h). The results were average of
at least 3 determinations. In all cases the blank was the result got for a three-gram aliquot that was
140 introduced in an iced bath at 0 °C for incubation time.

Sample preparation for electrophoresis

144 The squid mantle muscle (NP and P batches) was finely cut manually. The chopped muscle, 1 g, was
incubated in a water bath at 40 or 55 °C for various periods: 0, 4 and 8 hours. Also 1 g of the chopped
muscle was held for 0, 8 and 24 hours at chilling temperature (7 °C). After the incubation, 9 mL of 20
mM of solubilization buffer (20 mM Tris-HCl, pH 8.0, containing 2 % β -mercaptoethanol (Merck), 2 %
148 SDS (Bio-Rad), and 8 M urea) was added and followed by continuous shaking for 16-24 h at room
temperature. Insoluble material in homogenates was centrifuged at 10,000 x g (MR 18.12 Centrifuges,
JOVAN, Saint Herblain, France) for 20 min at room temperature. The supernatant was analyzed for
protein concentration by the method of Bradford (1976), as well as for myofibrillar protein degradation
152 on sodium dodecyl sulfate gel electrophoresis (SDS-PAGE). For electrophoresis analysis, an aliquot of
the supernatant was taken and added to 200 μ L of the denaturing SDS-PAGE sample treatment buffer: 5
% β -mercaptoethanol, 2.5 % SDS, 10 mM Tris-HCl, 1 mM ethylenediaminetetra-acetic acid (EDTA)
(Sigma Chemical Co). and 0.002 % bromophenol blue (PANREAC), and finally was got a final
156 concentration of 2 mg/mL by adding distilled water. This mixture was boiled for 5 min at 100 °C.

SDS-PAGE

160 Electrophoresis was carried out by the method of Laemmli (1970) using 10 % polyacrylamide gels. The proteins were separated at a constant current of 30 mA. Separated protein bands were stained with brilliant Coomassie blue (R-250, Merck). The gel was de-stained in an aqueous solution of 30 % methanol and 10 % acetic acid. A high-molecular-weight standard (HMW, Pharmacia-Biotech) and
164 another low-molecular-weight standard (LMW, Amersham Biosciences) were used to calculate the molecular weights of the proteins. The HMW consisted of myosin (220 KDa), α_2 -macroglobulin (170 KDa), β -galactosidase (116 KDa), transferrin (76 KDa), and glutamic dehydrogenase (53 KDa). The LMW consisted of phosphorilase b (97 KDa), albumin (66 KDa), ovoalbumin (45 KDa), carbonic
168 anhydrase (30 KDa), trypsin inhibitor (20.1 KDa) and α -lactoalbumin (14.4 KDa).

Inhibition of proteolytic activity

172 Crude enzyme extract was prepared from chopped mantle muscle of both pressurized and non-pressurized squid. Chopped muscle was mixed with a 20 mM phosphate buffer, pH 6.5 at a 1:2 (w/v) ratio and homogenized in iced water for 1 min at 10,000 rpm. The homogenate was centrifuged (Beckman J2-MC, JA-14 rotor, Calif., CA, USA) at 20,000 x g for 40 min at 4 °C, and the supernatant
176 was used as a source of enzyme.

The following inhibitors were tested to classify proteinases in squid mantle muscle: 1 mM iodoacetic acid (IA) as a cysteine protease inhibitor; 1 mg/mL phenylmethyl sulphonyl fluoride (PMSF) as a serine protease inhibitor; 0.2 μ g/mL pepstatine A (Peps. A) as an aspartic protease inhibitor; and 10 mM
180 ethylenediaminetetra-acetic acid (EDTA) as a metallo-protease inhibitor. All the inhibitors tested were from Sigma Chemical Co. 20 mM NaCl was also assayed to study it as a possible activator of proteolysis. The concentrations were chosen based on previously reported proteinase classification

studies (Hameed and Hard 1985; An and others 1994; Seymour and others 1994; Ayensa and others
184 1999; Hurtado and others 1999). In all cases, 100 μ L of the stock solution of inhibitor was added to the
enzymatic extract (400 μ L) to give the final inhibitor concentrations. The enzyme-inhibitor mixtures
were preincubated for 15 min at room temperature prior to assay for residual proteolytic activity as
follows.

188 The preincubated enzyme-inhibitor mixture was mixed with 400 μ L of Mc Ilvaine buffer of different
pH depending on the conditions. The mixtures were then incubated in a thermostatic water bath for 1 h
at the temperature chosen. The conditions for the assay were pH 3, 40 $^{\circ}$ C and 55 $^{\circ}$ C; pH 6.5, 40 $^{\circ}$ C; and
pH 8.0, 40 $^{\circ}$ C.

192 After the incubation 100 μ L of 50 % (w/v) TCA was added to stop the reaction. The mixture was
incubated for 15 min at 4 $^{\circ}$ C to allow unhydrolyzed proteins to precipitate, followed by centrifugation at
5,700 x g for 10 min (MicroSpin 24S, Sorvall Instruments, DuPont, Wilmington, DEL., USA). TCA-
soluble peptides in the supernatant were analysed by the method of Lowry and others (1951). Residual
196 proteolytic activity was expressed as μ moles of tyrosine released per gram of sample per hour of
incubation (μ mol Tyr/g/h).

In all cases a blank and control were included. The result for the blank (without inhibitor and no
incubated) corresponds to existing peptides in 1 g of muscle no incubated. The control, without inhibitor
200 and incubated at the corresponding temperature, assumes 100 % of proteolytic activity.

Statistical analysis

204 Data were subjected to analysis of variance using SPSS computer program (SPSS 11.5, SPSS INC.,
Chicago, Illinois, USA), paired comparisons were made using Bonferroni test or Tamhane test, with
significance of difference set at $p \leq 0.05$, depending on variance homogeneity. Differences of means due
to treatment were using a T test.

Results and Discussion

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Determination of muscle pH

The pH of non-pressurized and pressurized squid muscle was $6.47 (\pm 0.01)$ and $6.69 (\pm 0.03)$,
216 respectively. Squid muscle pH rose slightly upon pressurization, a finding probably related to
denaturation of certain protein fractions. Angsupanich and Ledward (1998) reported similar behavior in
high pressure-treated cod (*Gadus morhua*) muscle.

220 The effect of temperature on proteolytic activity

Figure 1 depicts the proteolytic activity in a range of temperatures between 20 and 80 °C. Proteolysis
was observed over the entire range of temperatures studied, with proteolytic activity increasing
224 appreciably between 35 and 65 °C. This is consistent with the high level of enzymatic activity in
cephalopod muscle because of the high rate of protein turnover that goes hand in hand with high growth
rates (Sakai and Matsumoto 1981; Leblanc and Gill 1982; Konno and Fukazawa 1993).

	Temperature (°C)								
Batch	20-30	35	40	45	50	55	60	65	70-80
NP	a	b	bc	c	bc	bc	bc	bc	a
P	a	b	b	b	b	b	b	b	a

Figure 1. Proteolytic activity profile in non-pressurized (NP) and pressurized (P) squid mantle at different temperatures. Different letters (a, b, c) indicate significant differences ($p \leq 0.05$) for the same batch at different temperatures. (*) indicates significant differences ($p \leq 0.05$) between the batches at each temperature.

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Pressurization did not cause any variation in the proteolytic activity pattern of temperature, though enzymatic activity did increase significantly at 35-40 °C and 55-60 °C. The high level of activity at high temperatures indicates the presence of heat-stable, pressure-resistant proteases in the muscle. Various researchers have reported peak proteolytic activity levels in different cephalopod species at temperatures of around 40 °C. According to Konno and Fukazawa (1993) proteolysis in the muscle of the squid *Todarodes pacificus* increased with temperature up to 40 °C and then fell off sharply at higher temperatures. Rodger and others (1984) reported peak activity at 60 °C in the squid *Loligo forbesi* at pH 7.5. Working with the same species as in our study (*Todaropsis eblanae*), Ayensa and others (1999) observed two peaks in enzymatic activity at pH 7, at 40 and 65 °C, though they recorded much higher values than the values found here. These differences could be attributable to an eventual greater accumulation of degradation peptides in the squid used in the present work, greatly reducing proteolytic

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244 activity and also yielding high values for the blank readings. In contrast, Hurtado and others (2002)
found values for octopus (*Octopus vulgaris*) muscle at pH 6.5 that were much closer to the values
reported here and also observed two peaks in activity, one at 40 °C and the other at 60 °C. As we report
here for *Todaropsis eblanae*, they also reported that pressurization failed to alter the temperature profile
248 of proteolytic activity.

Profile of myofibrillar proteins

252 Figure 2 presents the electrophoretic pattern of the soluble protein fraction from non-pressurized and
high pressure-treated squid muscle after being held at 7 °C and incubated at 40 °C and 55 °C. In
descending order of molecular weight, myosin heavy chain (MHC), paramyosin (PM), and actin (Ac)
were recorded in all cases. Weaker bands were also observable between 45 and 15 KDa, largely
256 representing different kinds of troponins, tropomyosin and myosin light chain, along with bands for
unidentified proteins which probably come from the sarcoplasmic fraction (Kariya and others 1986;
Hurtado and others 1999). The band located between 220 and 97 KDa that is ordinarily associated with
protein C may also correspond to fragments produced by the breakdown of MHC, as has been observed
260 by different researchers during the degradation of cephalopod mantle (Rodger and others 1984; Konno
and Fukazawa 1993; Hurtado and others 2002).

Figure 2. SDS-PAGE pattern for non-pressurized (NP) and pressurized (P) squid mantle muscle held at 7 °C and incubated at 40 °C and 55 °C for various time periods, i.e., 0, 8, 24 h and 0, 4, and 8 h, depending on temperature. A: high molecular weight standard; B: low molecular weight standard; MHC: myosin heavy chain; PM: paramyosin; Ac: actin.

Observing the electrophoretic profiles for non-pressurized (NP) and pressurized (P) muscle stored at chilled temperature for 0, 8, and 24 h, the difference in the profiles for the two types of muscle at time 0 stands out. The number of bands increased on pressurized batch, indicative of hydrolysis with the formation of lower molecular weight peptides upon high-pressure treatment. The myosin heavy chain (MHC) in batch NP had undergone slight proteolysis after 24 h, but this was not yet perceptible at 8 h. MHC breakdown was higher in batch P. Additionally, the actin band in batch P was altered slightly after incubation for 24 h. Fernández-Martín and others (1998) pointed out that pressurization at non-denaturing temperatures resulted in unfolding of the actin in blue whiting (*Micromesistius poutassou*) muscle, the degree of unfolding increasing with pressure. In this sense, it may be that unfolding makes the actin more susceptible to degradation by the proteases.

At 40 °C the MHC band exhibited substantial degradation, to the point where this band was practically no longer visible after incubation for 8 h, especially in batch P. The higher degree of MHC breakdown in the pressurized muscle might be associated with the rise in proteolytic activity recorded at 40 °C (Fig.

1). As in the case of the muscle held at 7 °C, actin band intensity decreased in batch P after incubation for 8 h.

284 The electrophoretic profiles for batch NP at 55 °C were the same after incubation for 4 and 8 h, may be due to thermal inactivation of the relevant enzymes. Actin was not affected in either of the batches at this temperature, probably due to its higher thermostability. The myosin heavy chain in batch P underwent heavy proteolysis, quite considerable after incubation for 4 h and complete after 8 h. This
288 was consistent with the higher proteolytic activity observed following pressurization at 55 °C (Fig. 1). The enzymes responsible are probably lysosomal cysteine proteases like cathepsin B and cathepsin L, previously described in squid (*Todaropsis eblanae*) mantle (Ayensa and others 1999), which are active at neutral pH and have proved to be more active at temperatures above 40 °C. These enzymes are
292 probably released from the lysosomes as a result of the effect of high-pressure treatment. Homma and others (1994) reported increased cathepsin B, D, and L activity in pressurized bovine muscle extract. Alkaline proteases, which are capable of acting at neutral pH and are not activated at temperatures below 50 °C, are another factor to be taken into account (Makinodan and others 1987). Nevertheless,
296 Toyohara and others (1990) reported low levels of activity of heat-stable alkaline proteases on MHC in threadfin bream (*Nemipterus bathybius*) muscle at pH 7 and 8 at 65 °C. Cathepsin D, lysosomal aspartic protease, is another enzyme that may be involved (Ayensa and others 1999), and even though acid pH conditions are the optimal conditions for activity by this enzyme, it may nonetheless act on the different
300 myofibrillar proteins at physiological muscle pH levels. Jiang and others (1992) suggested that cathepsin D played a role in post-mortem breakdown of fish muscle observed in the form of increased hydrolysis of the myofibrillar proteins in tilapia (*Tilapia nilotica*) incubated with cathepsin D at pH 6 and 6.5 at low temperature.

304 Paramyosin, a characteristic protein in invertebrates, was unaltered in both batches under all the conditions tested in this study. This disagrees with the findings reported by Hurtado and others (2002), who observed unmistakable degradation of the paramyosin in non-pressurized octopus (*Octopus*

308 *vulgaris*) muscle after incubation at 40 °C, though not at 60 °C. They also found that pressure treatment made the paramyosin more susceptible to breakdown at both those temperatures.

Effect of pH on proteolytic activity

312 Figure 3A depicts the profile of proteolytic activity in batch NP at different pH levels at 40 and 55 °C. At 40 °C values were low, exhibiting a slight tendency to increase under acid pH conditions. In comparison, proteolytic activity displayed a significant increase at pH 3 and 55 °C, with another, minor peak in activity at pH 5.

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Figure 3. Proteolytic activity profile for non-pressurized (A) and pressurized (B) squid mantle muscle at different pH levels. Different letters (a, b, c) indicate significant differences ($p \leq 0.05$) between pH values at each temperature. (*) indicates significant differences ($p \leq 0.05$) between the two temperatures at each pH.

For the high pressure-treated batch (P) (Fig. 3B) at 40 °C, proteolytic activity was unchanged at all the pH levels tested. In contrast to the results at 40 °C, as in batch NP at 55 °C there was a significant peak in activity over the pH range of 2 to 4.5, with pH 3 being optimal for proteolytic activity.

Other researchers have also recorded high proteolytic activity in cephalopods at acid pH levels. Sakai and others (1981) found that activity in *Ommastrephes sloani pacificus* peaked at pH 2.9 at 35 °C. Leblanc and Gill (1982) observed two peaks in activity, one at pH 2.6 in *Illex illecebrosus* and another at pH 3.6 in *Loligo pealei*.

Hurtado and others (2002) reported two sets of optimal conditions for activity in non-pressurized octopus (*Octopus vulgaris*) muscle at acid pH levels, one at pH 2.5 at 40 °C and another higher peak at pH 4 at 60 °C. In pressure-treated muscle they found that proteolytic activity decreased at the first peak but increased at the second peak. These findings are consistent with the observations made in our experiment (Fig. 3), in that proteolytic activity decreased slightly at pH 3 at 40 °C and pH 4 at 55 °C but increased significantly at pH 5 and 55 °C ($p \leq 0.05$).

Effect of enzyme inhibitors and activators on proteolytic activity

The effect of specific enzyme inhibitors on proteolytic activity was study at the optimum pH level at the two critical temperatures of 40 °C (Fig 4A) and 55 °C (Fig. 4B), conditions at which peak activity had previously been observed (Fig. 1).

Figure 4. Effect of protease inhibitors and NaCl on proteolytic activity levels in non-pressurized (NP) and pressurized (P) squid mantle muscle incubated at pH 3 at 40 °C (A) and at 55 °C (B). Different letters (a, b, c, d) indicate significant differences ($p \leq 0.05$) between the inhibitors in each batch. (*) indicates significant differences ($p \leq 0.05$) between the batches, NP and P, for each inhibitor.

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The figure plots percentage residual activity following application of specific inhibitors and NaCl compared with a control with no additives representing 100 % activity. PMSF, which mainly inhibits serine proteases, produced the greatest decrease in activity levels (> 95 %) in both batches. Significant reductions were also observed for the cysteine and aspartic proteases, with about 70 % residual activity recorded in both cases. Accordingly, the acid serine proteases active at 40 °C were unaffected by the high-pressure treatment, unlike the acid cysteine proteases, which exhibited around 20 % more inhibition in batch P than in batch NP. This suggests that pressurization either led to an increase in acid cysteine proteases by rupturing the lysosomes or resulted in greater accessibility of the substrate.

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Pepstatin A brought a slight significant reduction in aspartic protease activity, but not after pressure treatment at this pH and temperature. EDTA and NaCl did not significantly alter enzymatic activity. Hurtado and others (2002) found that a concentration of 20 mM NaCl brought about an increase in proteolytic activity in octopus (*Octopus vulgaris*) muscle at pH 2.5 at 40 °C and that pressure treatment inhibited the enzymes responsible for the increase. They also found that adding NaCl increased activity levels in pressurized octopus muscle at pH 4 at 60 °C.

Figure 4B depicts the effects at pH 3 and at 55 °C, the conditions at which peak activity had previously been observed. At this pH and temperature the inhibitory effect of PMSF was very high in both batches, as it was at 40 °C. However, acid serine protease activity in the pressure-treated batch was higher than in the control batch, suggesting the release of these enzymes from the lysosomes or greater accessibility of the substrate due to structural changes brought about by pressurization. Inhibition of aspartic proteases by pepstatin A was nearly 95 % in both batches, hence it follows that high pressure did not affect the activity of these enzymes at pH 3 at either of the temperatures considered, though 55 °C is optimum for this enzymatic activity. Pepstatin A is known to be a good inhibitor of cathepsin D. Hence, the maximum proteolytic activity recorded at pH 3 and 55 °C might be ascribable to the presence of cathepsin D. In this sense Gildberg (1987) characterized a cathepsin D from the digestive gland of squid (*Todarodes sagittatus*) with an optimum temperature found at 55 °C.

Inhibition of acid cysteine proteases by iodoacetic acid was higher at 55 °C than at 40 °C, nearly 60 % in batch NP and significantly greater in batch P. Thus, the response of these enzymes to pressurization at pH 3 was similar at both temperatures tested, though enzymatic activity was higher at 55 °C. Because of the acid pH used in the study, in all likelihood these enzymes were principally cathepsins. Ayensa and others (1999) reported that certain cysteine proteases were present in squid (*Todaropsis eblanae*) mantle, specifically confirming the presence of cathepsins B and L, which they observed to be more active at temperatures higher than 40 °C. The inhibitory effect produced by pepstatin A showed cathepsin D to be present in the squid mantle muscle, and the peak in proteolytic activity at pH 3 at 55 °C was probably attributable to this protease. The high inhibitory effect of PMSF was indicative of

the presence of serine proteases, but since PMSF also partially inhibits cysteine proteases, it will also be responsible for a certain proportion of the inhibition of cysteine protease activity.

In addition to studying enzyme activity at the optimum pH level, enzyme activity was also characterized at physiological pH and at a basic pH, both at 40 °C, in order to establish whether neutral and alkaline proteases were present in addition to the acid proteases. At pH 6.5 and 40 °C (data not shown) the proteolytic activity was quite low, with very little difference in activity between the blank and the control in both, the non-pressurized and pressurized muscle. For batch NP, moreover, there were no significant differences ($p \leq 0.05$) between the control and the samples for any of the inhibitors tested. Pressurization, by the same token, likewise did not seem to exert any influence on the neutral proteases in the squid muscle. In contrast, working with the same species used in this study (*Todaropsis eblanae*), Ayensa and others (1999) observed appreciable inhibition by PMSF and pepstatin A at pH 7 at 40 °C, which they attributed to the neutral serine and aspartic proteases active at that pH. These dissimilar results might be related to dissimilarities in initial muscle quality.

At pH 8 and 40 °C (data not shown), there was, first of all, a significant difference ($p \leq 0.05$) in the concentrations of soluble oligopeptides between the blanks for the untreated and pressure-treated muscle. This difference may have been caused by protein hydrolysis taking place on pressurization, and a similar difference was also observed in the electrophoretic analysis results (Fig. 2). In the non-pressurized muscle adding inhibitors and NaCl did not give rise to any significant differences in proteolytic activity between the control and the samples, indicative of little alkaline protease activity in the conditions of the experiment. By comparison, addition of PMSF or EDTA to the pressure-treated muscle resulted in a significant decrease in proteolytic activity with respect to the control, respectively indicative of increased activity by the alkaline serine proteases and alkaline metallo-proteases. Ayensa and others (1999) reported the presence of alkaline cysteine proteases in squid (*Todaropsis eblanae*) muscle at pH 9 at 40 °C and 65 °C. Further study at higher pH levels and temperatures would be interesting with a view to confirming the presence of alkaline proteases and to profiling how their activity might be altered by high-pressure treatment.

Influence of NaCl on proteolytic activity in squid muscle

Earlier in this study a concentration of 20 mM NaCl was observed to have no effect on proteolytic activity in the squid muscle in any of the conditions tested. Even so, since NaCl is an ingredient widely used in processing cephalopods, alterations in proteolytic activity at a concentration of 0.15 M NaCl were also studied. This concentration was chosen because it is commonly employed in making gels from muscle. Figure 5 depicts the results for proteolytic activity in squid muscle homogenized with 0.15 M NaCl and then incubated at 40 and 60 °C for 1 h compared with muscle homogenized in phosphate buffer at pH 6.5.

NaCl inhibited proteolytic activity at 40 °C (Fig. 5A) was greater in the non-pressurized muscle, attaining nearly 70 % inhibition. The overall decrease in proteolytic activity at high concentrations of NaCl is most likely due to the inhibition of certain alkaline proteases (principally cysteine and serine proteases) that are active at neutral pH levels. Toyohara and others (1990) previously showed that heat-stable alkaline proteases that were inactivated by NaCl were present in threadfin bream (*Nemipterus bathybius*).

Figure 5. Effect of 0.15 M NaCl on proteolytic activity in non-pressurized (NP) and pressurized (P) squid mantle muscle incubated at 40 °C (A) and 60 °C (B). Different letters (a, b) indicate significant differences ($p \leq 0.05$) between the NaCl-containing and control batches for each treatment (NP and P).

(*) indicates significant differences ($p \leq 0.05$) between the NP and P batches at each condition (NaCl-containing or control).

432 Proteolytic activity increased significantly with pressure for both the muscle homogenized with NaCl and the muscle homogenized in pH 6.5 phosphate buffer (control), with a higher increase in the presence of NaCl. This could be ascribed to the presence of cathepsin C, a chloride ion dependent lysosomal cysteine protease presumably released by high-pressure treatment. The chloride ion has been reported to
436 be required for proteolytic activity through activation of cathepsin C present in short-finned squid (*Illex illecebrosus*) muscle (Hameed and Haard 1985). Konno and Fukazawa (1993) also observed an increase in proteolytic activity in squid (*Todarodes pacificus*) mantle at higher concentrations of NaCl, with peak activity taking place at a concentration of 0.3 M at pH 7 at 40 °C. They related the increased proteolytic
440 activity to NaCl-induced unfolding of the myosin, which then became more susceptible to the action of the proteases. Kinoshita and others (1990) characterized two serine proteases in crucian carp (*Carassius auratus cuvieri*) muscle that were only active at high temperatures (50 °C and 60 °C) and an NaCl concentration of at least 1 %.

444 NaCl inhibited proteolysis by about 70 % in both the pressure-treated and untreated muscle at 60 °C (Fig. 5B). On the other hand, proteolytic activity was not observed to increase in the presence of NaCl on pressurization. This difference with respect to incubation at 40 °C (Fig. 5A) is probably the result of inactivation of cathepsin C at the higher temperature. Carp cathepsin C has been reported to remain
448 stable at 60 °C for only 20 min (Makinodan and Ikeda 1971).

In conclusion, pressurization did not bring about any appreciable alteration in proteolytic activity, possibly because of the low initial proteolytic activity levels in the raw material. In consequence,
452 changes in the gelling capacity of pressure-treated and untreated muscle should not be expected when manufacturing restructured products from this particular squid lot. Moreover the addition of salt at

concentration used as ingredient in restructured product would favor the inhibition of proteolytic activity.

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