A comparative study of the effects of high pressure on proteolytic degradation of sardine and blue whiting muscle

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ABSTRACT: High-pressure technology is used as an alternative to heat processing because of its inactivating effect on microorganisms and enzymes. However, it can also alter the structure of other muscle proteins. The present study compares the effects of high pressure (300 MPa, 7°C, 20 min) on the proteolytic degradation and alterations in the myofibrillar proteins of sardine and blue whiting muscle. Also, muscle homogenates and enzyme extracts were pressurized in order to evaluate the high-pressure effects on unprotected proteolytic enzymes outside the whole muscle structure. Peak proteolytic activity was found to occur at 55°C in both species. The peak activity pH was pH 3 for the sardine and pH 8 for the blue whiting; the main enzyme families being aspartic proteases in the former and alkaline serine proteases in the latter. Pressurization lowered activity levels at the peak activity pH and temperature in the fish muscle (by 30.8% in the sardine and by 9.5% in the blue whiting) and also slightly in the enzyme extracts (by 16.8% in the sardine and by 19.4% in the blue whiting). The electrophoretic profiles disclosed higher protein degradation in the pressurized muscle. Overall, the observed changes in proteolytic activity can be attributed not only to the effect of high pressure on the enzymes, but also and mainly, to the effect on other muscle proteins.

KEY WORDS: enzymes, high pressure, muscle, myofibrillar proteins, proteolytic

INTRODUCTION

Enzymatic activity plays a particularly important role in post-mortem degradation in fish muscle tissue. Lysosomal cathepsins and calpains are regarded as the major groups responsible for post-mortem changes in fish muscle texture,¹ but metalloproteases are also involved.² Proteases also play an important role in the degradation of myofibrillar proteins and may affect the suitability of the muscle for technological processing. For instance, proteases can hinder traditional heat-induced gelation, a process which may vary considerably according to species and attributable to, among other factors, the action of heat-stable, myosin-degrading.³⁻⁵

It therefore follows that regulating enzymatic activity should help maintain or improve product quality and shelf life. A number of methods of inhibiting the action of muscle proteases have been employed, for example, washing the muscle, adding food-grade inhibitors,⁶ and using fast heating methods such as ohmic heating⁷ or microwaving.⁸ High hydrostatic pressure technology has gained popularity as an alternative to heat processing for use as a food processing tool in controlling microbial activity and altering proteins, enzymes and other food macromolecules. The literature dealing with changes in proteolytic activity in fish muscle indicates that the effects of high pressure depends on the species concerned, and the duration, temperature and degree of pressurization, the pH, and enzyme.⁹⁻¹¹

There has been ongoing disagreement as to whether high pressure in fact activates or inactivates muscle enzymes. Leaving aside possible differences due to enzyme type and processing conditions, the pattern of enzymatic activity appears to differ according to whether studies are conducted on extracts or on actual muscle tissue. In extracts, enzymes are unprotected, and for that reason intramolecular changes in enzyme structure/conformation are the main cause of pressure-induced inactivation. However, pressurization of the muscle entails changes in the conformation of the myofibrillar proteins, thereby influencing the specificity of the enzyme/substrate interaction, and disrupts cell membranes, releasing enzymes, which may in turn increase proteolytic activity, depending on the level of pressure employed. Simpson¹² compared residual activity in enzyme extracts from pressurized fish muscle and in pressurized enzyme extracts from non-pressurized muscle, and found the extent of inactivation of the proteases to be generally higher in the enzyme extracts from the pressurized tissue. The explanation posited was that other components naturally present in the intact fish tissue may act in concert with pressure to bring about greater inactivation of the enzymes. Conversely, Lakshmanan *et al.*¹³ reported that the effect of high pressure on proteolytic activity was lower in pressurized cold-smoked salmon muscle than in pressurized enzyme extracts, suggesting that enzymes are more resistant to pressure in the muscle tissue.

Sardine and blue whiting are two small fish species, the former being a fatty species, the latter a lean species. Both species spoil rapidly and are undervalued and underused. In these circumstances, using high pressure to inactivate

proteolytic enzymes could enhance shelf life, while at the same time producing alterations in muscle proteins which could improve the usefulness of these species in processing restructured seafood products, thereby upgrading their marketability. The object of this study was to determine the effects of high pressure on proteolytic enzymes in sardine and blue whiting muscle, and to assess alterations in the myofibrillar proteins. These aspects offer interest to understanding the degradation in protein quality taking place during product shelf life, as well as the suitability of pressurized raw muscle for processing.

MATERIALS AND METHODS

Sample preparation and treatment

Sardine *Sardina pilchardus* and blue whiting *Micromesistius poutassou* were purchased at a local market, within 24–48 h of capture, and transported in ice to the laboratory. Mean individual weight and length were (89.8 ± 9.1) g / (17.9 ± 0.8) cm for the sardine and (68.1 ± 10.0) g / (19.7 ± 1.0) cm for the blue whiting.

Muscle samples

The muscle samples were prepared and pressurized as described in Hernández-Andres *et al.*¹⁴ The fish were gutted and washed immediately upon arrival and vacuum packed in flexible bags (Cryovac BB4L, Barcelona, Spain). Half of the vacuum-packed units were subjected to pressurization at 300 MPa at 7°C for 20 min (pressurized batch: P), while the other half were held unpressurized (atmospheric pressure) at 7°C (non-pressurized batch: NP). Pressurization conditions were selected based on the results of previous enzyme inactivation assays, seeking the least possible alteration of the muscle with a view to further technological processing.^{9,12,15,16} Pressurization was carried out in a pilot high-pressure unit (ACB 665; Gec Alsthom, Nantes, France), where the temperature of the immersion medium (distilled water) was regulated by a thermocouple and programmed thermostating equipment (model IA/2230 AC; INMASA, Barcelona, Spain). Pressure was increased at 2.5 MPa/s.

Pressurized and non-pressurized muscle samples were then frozen in an air-blast tunnel freezer (AGA-Frigoscandia 0–6373, Helsingborg, Sweden) at –30°C for 2 h. For each batch, the frozen fish were skinned and chopped into small pieces, and the pieces were all mixed together. The sample material was then again bagged as before, that is, vacuum packed in flexible bags, and stored for later analysis at –54°C to minimize any alterations during storage.

The pH readings for *muscle* (1:10 [muscle : water]) were taken as described by Vyncke.¹⁷ The pH of the non-pressurized and pressurized muscle was, respectively, 6.09 ± 0.01 and 6.19 ± 0.03 in the sardine and 6.98 ± 0.03 and 7.03 ± 0.06 in the blue whiting.

Homogenate samples

Homogenates were prepared and pressurized as described in

Hernández-Andres *et al.*¹⁴ The *homogenates* were obtained from sardine chopped muscle (1:3 [muscle : buffer]) and from blue whiting chopped muscle (1:2 [muscle : buffer]) at the peak activity pH (pH 3 for the sardine and pH 6 for the blue whiting) and at muscle pH (pH 8 for the sardine and pH 7 for the blue whiting). The chopped muscle was homogenized in cold buffer at 10 000 r.p.m. for 1 min in an iced-water bath, using an Omnimixer (Model ES-231, Homogenizer; Omni International, Waterbury, USA). The homogenates were filtered through four layers of cheesecloth in order to remove connective tissue. The homogenization buffers were McIlvaine buffer (0.2 M sodium phosphate, 0.1 M sodium citrate) for acid pH; 0.2 M phosphate buffer for pH 6 and 7; and 0.2 M Tris-HCl for alkaline pH. Half of homogenates (for each species and pH) were pressurized (P: 300 MPa at 7°C for 20 min), while the other half were held without pressurization (NP: atmospheric pressure at 7°C). Pressurized and non-pressurized *homogenates* were stored at –80°C prior to analysis.

Enzyme extracts

The enzyme extracts were obtained as described by Lakshmanan *et al.*¹³ Chopped muscle (10 g) was homogenized with 50 mL ice-cold double-distilled water for 2 min and the homogenate allowed to stand in ice for 30 min with occasional stirring. After 30 min, the homogenate was centrifuged at $14\,600 \times g$ at 4°C for 20 min. The supernatant was then filtered through a 0.45 µm sterile filter unit (Sartorius 11306–090 G; Filtros Anioia, S.A., Barcelona, Spain). Half of all the extracts were pressurized (P: 300 MPa at 7°C for 20 min), while the other half were held without pressurization (NP: atmospheric pressure at 7°C). Enzymes extracts were stored at –80°C prior to analysis.

Determination of proteolytic activity

Proteolytic activity in the *muscle* and *homogenates* was determined according to the method described by Hernández-Andrés *et al.*¹⁴ For the *enzyme extracts*, proteolytic activity was assayed using the method of Lakshmanan *et al.*,¹³ with incubation in a 5% (w/v) hemoglobin solution for 1 h. In all cases, proteolytic activity has been expressed as µmoles of tyrosine released per gram of muscle per hour of incubation (µmol Tyr/g/h).

First, the effect of temperature (20–80°C) on proteolytic activity in the *muscle* was examined at muscle pH. Next, proteolytic activity was assayed over a range of pH values, that is, pH 2.5–8 at 40 and 55°C in the sardine muscle and pH 3–10 at 55°C in the blue whiting muscle. For this assay, homogenates were prepared at each experimental pH using the buffers described above. The conditions of optimum proteolytic activity in the muscle were known from the results of this study. Proteolytic activity in the *homogenates* and *enzyme extracts* of both species was determined at muscle pH and at the peak activity pH at 55°C. These pH and temperature conditions were chosen in order to ascertain how high pressure affected proteolytic activity, since under these conditions of muscle pH and peak activity pH,

changes brought about by high pressure processing should be more appreciable at the maximum temperature.

Sample preparation for electrophoresis/sodium dodecylsulfate–polyacrylamide gel electrophoresis

An amount of 1 g of *muscle* was first incubated in a water bath at 40°C or 55°C for 0, 4 or 8 h, and held at 7°C for 0, 8 or 24 h. The electrophoresis sample was then prepared according to the method described by Hernández-Andrés *et al.*¹⁴

For the *homogenates*, proteins were precipitated with 10% trichloroacetic acid (TCA) in a proportion of 1:3 (v/v) before and after incubation at 55°C for 1 h, and the TCA-soluble peptide composition was determined.

The denaturing sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer for the proteins from the muscle or the homogenates of both species was 25% (v/v) β -mercaptoethanol, 12.5% (w/v) SDS, 50 mM Tris-HCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 10% (w/v) glycerin, and 0.01% (w/v) bromophenol blue (PANREAC). Buffer composition for the *enzyme extracts* was the same, except no β -mercaptoethanol was used. Electrophoresis SDS-PAGE (10%) was carried out in the conditions described by Hernández-Andrés *et al.*¹⁴ An amount of 30 μ g of protein was loaded per lane for both the *muscle* and the *enzyme extracts*. Table 1 sets out the protein content of the *homogenates* for electrophoresis.

Inhibition of proteolytic muscle enzymes

The assay was performed according to Hernández-Andrés *et al.*¹⁴ The following inhibitors were tested to classify the proteinases in the muscle: 1 mM iodoacetic acid (IA), as a cysteine protease inhibitor; 1 mg/mL phenylmethyl sulfonyl fluoride (PMSF), as a serine protease inhibitor; 0.2 μ g/mL pepstatin A (Peps. A), as an aspartic protease inhibitor; and 10 mM (EDTA), as a metalloprotease inhibitor. All the inhibitors used were from Sigma Chemical (St. Louis, MO, USA). The assay conditions were pH 3 and pH 6 at 55°C for the sardine and pH 7 and pH 8 at 55°C for the blue whiting, these being the optimum pH and temperature conditions to maximize enzyme activity.

Determination of cathepsin D-like activity in the blue whiting muscle

The determination was carried out according to the method described by Gómez-Guillén and Batista¹⁸ at pH 3 and pH 7 at 55°C for 1 h. The reaction was stopped with 10% (w/v) trichloroacetic acid (TCA) in a proportion of 1:1 (v/v). The test tubes were then held at 4°C for 15 min to allow precipitation of unhydrolyzed proteins and centrifuged (MicroSpin 24S, Sorvall Instruments, DuPont, Wilmington, DE, USA) at 5700 $\times g$ at 4°C for 15 min. The results have been expressed as μ moles of tyrosine equivalent released from bovine Hb per gram of muscle per hour.

Statistical analysis

Statistical analysis was carried out separately for each

species. Analysis of variance (ANOVA) was performed using the SPSS computer program (version 11.5; SPSS Inc., Chicago, IL, USA). Pairs of mean values were compared using the Bonferroni test, Tamhane test, or Tukey's test, depending on the homogeneity of the variance, with the significance level set at $P \leq 0.05$. A *t*-test was used to compare the differences between the means for variables that discriminated between groups.

RESULTS AND DISCUSSION

Influence of high-pressure processing of fish muscle on protease activity

The effect of temperature and pH were evaluated to establish the influence of high-pressure processing of fish muscle on protease activity. Proteolysis activity, expressed as μ mol Tyr/g/h, was considerably higher in the blue whiting muscle than in the sardine muscle, especially at the peak activity temperature of 50–60°C (Fig. 1a). Pressurization appreciably increased ($P \leq 0.05$) the proteolysis values for the blue whiting muscle at temperatures below the peak activity temperature. In contrast, pressurized sardine muscle exhibited a significant decrease at the peak activity temperature (55–60°C). Hernández-Andrés *et al.*¹⁴ reported that pressurization in the same conditions as those used in the present study significantly increased enzymatic activity in squid *Todaropsis eblanae* mantle muscle at 35–40°C and at 55–60°C.

The effect of incubation at different pH levels on proteolytic activity was studied at 55°C, the peak activity temperature for both species. Proteolysis values in the blue whiting muscle peaked at pH 8–9, substantially higher than in the sardine (Fig. 1b). The sardine muscle, by contrast, displayed higher values under acid conditions, with activity values peaking at pH 3, probably a consequence of cathepsin D-like activity.¹⁸ In general, high-pressure processing did not significantly alter the proteolytic activity curve with pH in the blue whiting muscle, although slight differences ($P \leq 0.05$) were observable over the range of peak activity (Fig. 1b). Angsupanich and Ledward¹⁵ reported three activity peaks in cod, the highest at pH 6.6, the next at pH 9.0 and the last at pH 3.3. The differing profile with respect to blue whiting is probably due to interspecific differences, even though both species belong to the same family, Gadidae. In the sardine muscle, pressurization caused a significant reduction ($P \leq 0.05$) in proteolytic activity at acid pH levels. The effect of pressurization on proteolytic activity in the sardine muscle at different pH values was also tested at a lower temperature (40°C) (data not shown), because as illustrated in Figure 1a, the differences with respect to the values recorded at 55°C were not as large as those observed in the blue whiting. The curves for proteolytic activity in both the pressurized and non-pressurized batches of sardine muscle on pH at 40°C were similar to the curves at 55°C, though values were somewhat lower. As in the case of the sardine muscle, the

pH for peak proteolytic activity in pressurized squid *Todaropsis eblanae* mantle (300 MPa, 7°C, 20 min) was also acidic, and pressure likewise did not alter the pH.¹⁴

Influence of high-pressure processing on fish muscle protease

In order to determine the influence of high-pressure processing on enzymatic activity in the fish muscle, the enzyme families were identified by adding specific protease inhibitors under both natural *post-mortem* pH conditions as well as peak activity conditions in order to intensify the effects.

Figure 2 depicts the effects of specific enzyme inhibitors on proteolytic activity in the sardine and blue whiting muscle at 55°C at different pH values, namely, the peak activity level in both species and also the natural post-mortem pH (pH 6 in the sardine, pH 7 in the blue whiting). At the natural post-mortem pH (Fig. 2a), the high degree of inhibition of proteolytic activity (low residual activity), achieved by PMSF and pepstatin A, suggests that serine and aspartic proteases predominated at the peak activity temperature (55°C). In the non-pressurized batches, inhibition by pepstatin A was significantly higher in the sardine muscle than in the blue whiting, as was to be expected from the activity curves with pH for both species (Fig. 1b). In the sardine muscle, no significant differences in serine protease activity were observed after high-pressure processing, but aspartic protease activity was lower (higher residual activity) in the pressurized batch ($P \leq 0.05$). The behavior of the serine and aspartic protease populations in the blue whiting muscle differed from that in the sardine muscle, with lower residual activity levels being observed for both these enzyme populations following pressurization.

Cysteine proteases inhibited by iodoacetic acid were partially activated by high pressure in the sardine muscle, in opposition to what happened in the pressurized blue whiting muscle, in which residual activity levels remained higher than in the non-pressurized batch. These neutral cysteine proteases, partially inactivated by high pressure, were probably calpains, cytoplasmic cysteine proteases with peak enzymatic activity at pH 7 sensitive to high pressures.¹⁹ The above-mentioned pressure-induced increase in cysteine protease activity in the sardine muscle could be explained by the release of enzymes from the lysosomes, that is, high pressure would appear to break down the lysosome membranes, resulting in leakage of the lysosomal enzymes.^{20–22} Chéret *et al.*²³ showed that high-pressure processing up to 500 MPa (5 min, <10°C) increased cysteine protease (cathepsins B, H, L) activity in sea bass *Dicentrarchus labrax* fillets, whereas cathepsin D-like activity increased up to 300 MPa.

Inhibition of the neutral metalloproteases by EDTA showed the contribution of these enzymes to be slight, perceptible only in the case of the sardine muscle. The metalloproteases were unaffected by high-pressure processing.

Incubation with pepstatin A at the peak activity pH for the sardine (pH 3) yielded complete inactivation in both the

pressurized and non-pressurized batches (Fig. 2b). This result is indicative of the predominance of aspartic proteases, with cathepsin D being the main lysosomal pepstatin-sensitive aspartic protease involved in the degradation of cell proteins. Gómez-Guillén and Batista¹⁸ reported a peak activity pH of 3.2 and a peak activity temperature of 55°C for cathepsin D-like activity in sardine muscle. It therefore seems likely that the peak proteolytic activity recorded at pH 3/55°C in this study should be ascribable to the presence of this enzyme. The high degree of inhibition achieved by PMSF and iodoacetic acid denoted significant contributions to proteolytic activity by acid serine and cysteine proteases in the sardine muscle. Proteolytic activity by these two families of proteases increased slightly upon pressurization.

Incubation at the peak activity pH for the blue whiting (pH 8) did not test pepstatin A as an inhibitor (Fig. 2c). Alkaline serine proteases were the main family of enzymes involved in muscle proteolysis and were activated slightly by high pressure. No significant contributions by cysteine proteases or by metalloproteases were recorded in either of the two batches (pressurized and non-pressurized) in these experimental conditions.

According to the present findings, aspartic and serine proteases predominated in both the sardine and the blue whiting muscle, and were affected differently by pressurization. In squid mantle,¹⁴ in contrast, acid cysteine proteases and to lesser extent acid serine proteases, were the principal enzymes present and the main enzymes affected by high pressure.

The assay performed using specific enzyme inhibitors at pH 7 at 55°C in blue whiting muscle (Fig. 2a) resulted in strong inhibition of aspartic protease activity by pepstatin A. In view of this finding, it was decided to examine cathepsin D-like activity at pH 7 as well as at pH 3, within the range of peak activity for cathepsin D-like enzymatic activity.^{18,24} The assay was carried out at 55°C, the same temperature used previously for the specific inhibitor assay. Cathepsin D-like activity (0.07 $\mu\text{mol Tyr/g/h}$) was recorded for batch NP at pH 7, but a higher value (0.16 $\mu\text{mol Tyr/g/h}$) was recorded for the batch P. As was to be expected, activity levels were higher at the peak activity pH (pH 3), and again the value was higher for batch P (NP: 0.22 $\mu\text{mol Tyr/g/h}$; P: 0.39 $\mu\text{mol Tyr/g/h}$). The higher activity rate brought about by the effects of pressurization might be attributable to the release of lysosomal enzymes.^{20–22} Kubo *et al.*²⁵ subjected rabbit muscle to high pressures (100–400 MPa, 5 min, chilled temperature) and showed that 100 MPa sufficed to alter lysosome shape, and that the release of cathepsin D from the lysosomes intensified as pressure increased; after release, the cathepsin D was absorbed by the myofibrils.

Influence of high-pressure treatment on myofibrillar proteins at chilled storage and fish cooking/processing

Basically, what was being studied here was the effect of high pressure on enzymes, and this may involve an effect on muscle proteins, both as a result of direct action by pressure

and by enzymatic activity following pressurization.

The influence of enzymes and/or high pressure on muscle proteins (aggregation and proteolysis) at chilled storage was therefore examined using the electrophoretic profile. The pressurized muscle was held at 7°C (chilled storage temperature) for 0, 8, or 24 h (Fig. 3) in order to study the proteolytic degradation taking place under the actual conditions of chilled-fish storage. The results showed that the myosin heavy chain (MHC) band gradually faded with the time of incubation in the sardine batch P (Fig. 3a), and slightly after 24 h in the control bath. Actin (Ac) was hydrolyzed only in batch P, the breakdown being more apparent after 24 h. However, in the blue whiting muscle (Fig. 3b), no appreciable proteolysis took place at this temperature over the 24-h study period in batch P. Similarly, Angsupanich and Ledward¹⁵ recorded no changes in the electrophoretic patterns for pressurized and non-pressurized cod refrigerated at 4°C for 7 days.

In general, Ac was more susceptible to proteolysis in the pressurized batches, primarily in the sardine. In a study using differential scanning calorimetry, Fernández-Martín *et al.*²⁶ also reported different behavior of batches of blue whiting muscle homogenate with NaCl following pressurization at 7°C, attributed to unfolding of the Ac.

Furthermore, the influence of enzymes and/or high pressure on muscle proteins (aggregation and proteolysis) on cooking or later thermal processing was also determined by the electrophoretic profile in order to evaluate the final quality of the fish protein. The pressurized muscle was held at 40°C and 55°C for 0, 4 or 8 h. Figures 4 and 5 show the protein composition of the non-pressurized and pressurized sardine (Fig. 4) and blue whiting (Fig. 5) muscle incubated at 40°C and at 55°C for 0, 4 and 8 h. The main bands observable at 0 h were, in all cases, MHC and Ac. Other bands between 45 and 15 kDa were different tropomyosin, tropomyosin and myosin light chains, along with sarcoplasmic proteins. The electrophoretic profiles for the non-pressurized sardine muscle were the same after incubation at 40°C for 4 h and 8 h (Fig. 4a). The MHC band was slightly visible after incubation for 8 h. In contrast, in batch P, the MHC had undergone complete proteolysis after 8 h and was not visible. Incubation at 55°C yielded a similar profile (Fig. 4b). The lower proteolytic activity values for batch P at temperatures ranging from 40°C to 80°C, described in previous sections (Fig. 1a), which were incubated during 1 h, were not visible in the SDS-PAGE results, very likely because of the heat stability of the enzymes involved (with enzymatic activity peaking at around 55°C) and because of the time-dependent nature of the degradation of the myofibrillar proteins by endogenous proteinases. Ac was only slightly affected by pressurization after incubation at 40°C for 4 h and 8 h; at 55°C, it was unaltered in both batches, probably due to inactivation, at high temperatures, of the enzymes involved in Ac degradation.

The MHC band grew fainter with incubation time in the non-pressurized blue whiting muscle incubated at 40°C (Fig. 5a), and ceased to be visible after 8 h. Degradation was

faster in batch P, and the MHC band ceased to be observable after incubation for 4 h. Bands resulting from MHC breakdown (with weights between 220 and 97 kDa) did not disappear in batch P with incubation time. Ac was only slightly affected in the pressurized batch after incubation for 4–8 h.

The electrophoretic patterns for the two blue whiting batches at the incubation temperature of 55°C are shown in Figure 5b. Heavy proteolysis of the MHC, leading to the appearance of MHC breakdown bands, took place in both batches after incubation for 4 h. In this connection, high degradation of myofibrillar proteins, in particular myosin, by heat-stable alkaline serine proteases has previously been described.^{5,27} Upon pressurization, numerous high-intensity MHC degradation bands appeared after incubation for 4 and 8 h, as was also observed at 40°C (Fig. 5a). Ac was slightly affected at 55°C, though less than at 40°C.

Influence of high-pressure processing on muscle protein specificity as a protease substrate

The first part of this article dealt with the effect of pressure on the enzymes in whole or minced muscle, but it should be borne in mind that in certain processes (e.g. gelation), the muscle completely disintegrates, releasing the content of cell organelles into the surrounding medium. These organelles include lysosomes, which collect large quantities of proteolytic enzymes. Therefore, assays were carried out using homogenates to evaluate the effect of pressure in conditions in which the muscle has been homogenized and consequently there is greater enzyme availability. As further shown, these assays were intended to gauge extant levels of autolysis, that is, enzymatic activity in homogenates containing muscle proteins and muscle enzymes at actual muscle pH and at peak activity pH, though to speed up the process the assays were carried out at optimum temperature in both cases. Extracts were also prepared. That is, after the muscle had been homogenized, most of the myofibrillar proteins were removed, leaving behind mainly enzymes, in order to assess the stability of the enzymes (enzymatic activity) when they were unprotected from the surrounding medium.

Table 2 summarizes the results of proteolytic activity at 55°C for pressurized and non-pressurized homogenates, and extracts from sardine and blue whiting muscle at the natural post-mortem muscle pH (pH 6 in the sardine; pH 7 in the blue whiting), and also at the peak activity pH (pH 3 in the sardine; pH 8 in the blue whiting). Pressurization of the sardine muscle homogenates sharply increased proteolysis at the two pH levels tested as compared to the non-pressurized homogenates (Table 2). This was probably caused by greater accessibility of the proteins acting as substrates, ensuing from structural changes brought about by pressurization as well as by disruption of lysosomal membranes and the resulting release of enzymes. However, direct pressurization of the enzyme extracts, with their low contents of both myofibrillar proteins and other cell structures, reduced proteolytic activity only very slightly at both pH 6 and pH 3, suggesting that the enzymes were quite resistant to pressure.

The behavior of the pressurized blue whiting homogenates was the converse of that of the sardine, that is, proteolytic activity declined ($P \leq 0.05$) after pressurization at both pH levels tested (pH 7 and 8). Therefore, there seemed to be no apparent contribution of lysosomal leakage to global proteolytic activity values in the pressurized blue whiting homogenates. Proteolytic activity in the pressurized blue whiting extracts decreased very slightly at pH 8, as was observed for the sardine extracts, whereas activity at pH 7 was practically unchanged.

The results of electrophoresis of the TCA-soluble peptides in the sardine and blue whiting homogenates, before and after incubation at 55°C for 1 h, are depicted in Figure 6. A combination of faded bands and an accumulation of low-molecular-weight proteins (Fig. 6a), probably caused by a conjunction of protein rupture and aggregation, was observed after pressurization of the sardine homogenate at pH 6. A clearer increase in low-molecular-weight bands, with a concomitant loss of higher-molecular-weight bands, was observed in batch P after incubation at 55°C for 1 h. This was probably related to increased proteolytic activity after pressurization at pH 6 (Table 2).

The electrophoretic profiles for the sardine homogenate at pH 3 (Fig. 6b) generally had fewer bands than the profiles obtained at pH 6 as a result of the protein aggregating effect exerted by the acid pH. Pressurization led to an increase in the number of high and, especially, low-molecular-weight proteins (Fig. 6) and was associated with an increase in the concentration of TCA-soluble peptides (Table 3). This finding could suggest that the appreciable pressure-induced rupture of proteins occurred, outweighed the aggregating effect. Furthermore, compared to the unincubated batches, incubation at 55°C resulted in a larger accumulation of breakdown peptides in the pressurized homogenate than in the non-pressurized homogenate, as a result of the higher level of proteolysis described in Table 2.

Various bands disappeared or grew fainter in the blue whiting homogenate at pH 7 (Fig. 6c), but unlike the case of the sardine, this was not accompanied by an increase in breakdown peptides. Instead, there was a decline in TCA-soluble peptides after pressurization (Table 3), hence it follows that protein aggregation brought about by the high-pressure processing was the predominant effect in this case. When the homogenates were incubated, protein degradation was again distinctly discernible and was more pronounced in batch P. Pressurizing the homogenate at pH 8 (Fig. 6d) altered the electrophoretic profile drastically, with no medium and high-molecular-weight bands being present, and again without any concomitant increase in low-molecular-weight peptides to speak of. This suggests a significant degree of thermal aggregation yielding polymers that were too large to enter the polyacrylamide stacking gel, which would also account for the lower TCA-soluble peptide concentrations (Table 3). Incubation resulted in virtually complete elimination of the principal bands in both the non-pressurized and pressurized homogenates, accompanied by the formation of lower-molecular-weight peptides. Table 2 shows pressure reduced proteolytic

activity, but the higher activity recorded at pH 8 than at pH 7 indicated that pressure did not inactivate the alkaline serine proteases.

No changes in the electrophoretic profiles for the enzyme extracts from the sardine and blue whiting were observed following high-pressure treatment (Fig. 7). The principal bands remained discernible and did not undergo any alterations in intensity, indicating that no pressure-induced rupture of the proteins took place. Forces that stabilize the quaternary structure of proteins may have been altered by the high-pressure processing; but if so, this was not accompanied by changes in molecular weight. This finding was in accordance with the results for proteolytic activity (Table 2), which remained practically unaltered, indicating that the proteases were highly resistant to high pressure.

CONCLUSIONS

Peak proteolytic activity was recorded at 55°C for both species, at an acid pH in the sardine and at an alkaline pH in the blue whiting; the predominant enzyme families involved being aspartic proteases in the case of the sardine, and alkaline serine proteases in the case of the blue whiting.

High pressure decreased proteolytic activity in the sardine muscle, especially at the peak activity pH and temperature, by reducing the activity of the aspartic proteases. Proteolytic activity in the pressurized blue whiting was barely affected under the peak activity conditions, but increased slightly at 35–50°C at the natural post-mortem muscle pH. In this species high pressure gave rise to a small increase in serine and aspartic protease activity and to a decrease in cysteine protease activity.

Consequently, in view of the small direct effect of high pressure on the proteases in the conditions of this study, the changes in proteolytic activity that took place in the muscle can be attributed largely to the effects of pressure on other muscle components, in particular the myofibrillar proteins, the main substrate for enzymatic reactions. This finding agrees with the higher level of protein degradation observed on incubating the pressurized muscle and the low level of inactivation of the enzyme extracts on applying high pressure.

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Fig. 1 Proteolytic activity in the non-pressurized (NP) and pressurized (P) sardine (circles) and blue whiting (squares) muscle: (a) for the natural post-mortem muscle pH (pH 6 in the sardine, pH 7 in the blue whiting) at different temperatures, (b) for different pH levels at the peak activity temperature (55°C).

Fig. 2 Effect of protease inhibitors on proteolytic enzymes in the non-pressurized (NP) and pressurized (P) sardine (dots) and blue whiting (lines) muscle incubated at the peak activity temperature (55°C) at (a) post-mortem muscle pH (pH 6 in the sardine, pH 7 in the blue whiting) and at the peak activity pH, (b) pH 3 in the sardine and (c) pH 8 in the blue whiting. For purposes of calculating the residual activity, the results obtained for a control (no inhibitor, incubation at the assay temperature) were taken as 100% proteolytic activity. EDTA, ethylenediaminetetraacetic acid; IA, iodoacetic acid; PMSF, phenylmethyl sulfonyl fluoride.

Fig. 3 Sodium dodecylsulfate–polyacrylamide gel electrophoresis results for the non-pressurized (NP) and pressurized (P) (a) sardine and (b) blue whiting muscle held at 7°C for 0, 8 and 24 h. Ac, actin; H, high-molecular-weight standard; L, low-molecular-weight standard; MHC, myosin heavy chain.

Fig. 4 Sodium dodecylsulfate–polyacrylamide gel electrophoresis results for the non-pressurized (NP) and pressurized (P) sardine muscle incubated at (a) 40°C or (b) 55°C for 0, 4 and 8 h. Ac, actin; MHC, myosin heavy chain.

Fig. 5 Sodium dodecylsulfate–polyacrylamide gel electrophoresis results for the non-pressurized (NP) and pressurized (P) blue whiting muscle incubated at (a) 40°C or (b) 55°C for 0, 4 and 8 h. Ac, actin; MHC, myosin heavy chain.

Fig. 6 Sodium dodecylsulfate–polyacrylamide gel electrophoresis results for non-pressurized (NP) and pressurized (P) homogenates

prepared from the sardine and blue whiting muscle at natural post-mortem pH. (a) pH 6 in the sardine, (c) pH 7 in the blue whiting; and at the peak activity pH (b) pH 3 in the sardine, (d) pH 8 in the blue whiting. n.i., no incubation; i., incubation at 55°C for 1 h.

Fig. 7 Sodium dodecylsulfate–polyacrylamide gel results for non-pressurized (NP) and pressurized (P) enzyme extracts from the (a) sardine and (b) blue whiting. H, high-molecular-weight standard; L, low-molecular-weight standard.

Table 1 Protein content (µg) per lane during sodium dodecylsulfate–polyacrylamide gel electrophoresis performed on the homogenates

Sample		Treatment			
pH		Non-pressurized		Pressurized	
		n.i.	55°C/1 h	n.i.	55°C/1 h
Sardine	Muscle pH	30	30	30	30
	Peak activity pH	40	5	40	40
Blue whiting	Muscle pH	30	30	30	30
	peak activity pH		78	18	18
					9

Homogenates from each species were prepared at post-mortem muscle pH (pH 6 for the sardine, pH 7 for the blue whiting) and at the peak activity pH (pH 3 for the sardine, pH 8 for the blue whiting).

n.i., no incubation and samples incubated at 55°C for 1 h.

TCA-soluble peptides in the supernatant (after adding TCA to the homogenates and a centrifugation at 6.100 g at 7°C for 15 min) were analyzed by the method of Bradford.

Table 2 Proteolytic activity of the pressurized and non-pressurized homogenates and crude enzyme extracts from both species at the natural post-mortem muscle pH (pH 6 in the sardine, pH 7 in the blue whiting) and at the peak activity pH (pH 3 in the sardine, pH 8 in the blue whiting) at 55°C

Sample		Treatment			
		Sardine		Blue whiting	
		Non-pressurized	Pressurized	Non-pressurized	Pressurized
Homogenates	Muscle pH	1.74 ± 0.13 ^{a,x}	12.97 ± 0.67 ^{b,x}	7.89 ± 0.77 ^{b,x}	2.55 ± 0.16 ^{a,x}
	Peak activity pH	4.28 ± 0.45 ^{a,y}	14.14 ± 1.15 ^{b,x}	6.84 ± 0.54 ^{b,x}	4.38 ± 0.28 ^{a,y}
Enzyme extracts	Muscle pH	1.06 ± 0.20 ^{a,x}	0.94 ± 0.18 ^{a,x}	1.40 ± 0.17 ^{a,x}	1.97 ± 0.35 ^{b,x}
	Peak activity pH	3.92 ± 0.31 ^{b,y}	3.26 ± 0.036 ^{a,y}	1.91 ± 0.19 ^{b,y}	1.54 ± 0.15 ^{a,y}

For each specie, different letters (a, b) in the same file and in the same column (x, y), indicate significant differences ($P \leq 0.05$).

Following the incubation, trichloroacetic acid was added to stop proteolysis, and then, the sample was centrifuged at 6100 ×g for 15 min at 4°C. The content of soluble oligopeptides in the supernatant was analyzed by the method of Lowry *et al.* (28). Activity was expressed as μmoles of tyrosine released per gram of muscle per hour of incubation (μmol Tyr/g/h).

Table 3 Protein concentration (mg/mL of trichloroacetic acid-soluble peptides) in the supernatants from the sardine and blue whiting muscle homogenates prepared at the post-mortem muscle pH (pH 6 in the sardine, pH 7 in the blue whiting) and at the peak activity pH (pH 3 in the sardine, pH 8 in the blue whiting)

		Non-pressurized		Pressurized	
		n.i.	55°C/1 h	n.i.	55°C/1 h
Sardine	Muscle pH	9.95	4.24	10.54	8.00
	Peak activity pH	6.15	0.29	12.69	6.21
Blue whiting	Muscle pH	9.31	9.20	5.73	4.30
	Peak activity pH	8.69	7.09	2.17	0.53

n.i. no incubation and samples incubated at 55°C for 1 h.

Trichloroacetic acid (TCA)-soluble peptides in the supernatant (after adding TCA to the homogenates and a centrifugation at 6100 ×g at 7°C for 15 min) were analyzed by the method of Bradford.