JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Changes of Enzymes Activity and Protein Profiles Caused by High-Pressure Processing in Sea Bass (*Dicentrarchus labrax*) Fillets

Bárbara Teixeira,*[,†,‡] Liliana Fidalgo,[‡] Rogério Mendes,[†] Gonçalo Costa,[§] Carlos Cordeiro,[§] António Marques,[†] Jorge Alexandre Saraiva,[‡] and Maria Leonor Nunes[†]

[†]Division of Aquaculture and Upgrading, Portuguese Institute for the Sea and Atmosphere, I.P. (IPMA, I.P.), Avenida de Brasília, 1449-006 Lisbon, Portugal

[‡]Research Unit of Organic Chemistry, Natural, and Agro-food Products (QOPNA), Chemistry Department, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

[§]Chemistry and Biochemistry Department, Faculty of Sciences, University of Lisbon, Campo Grande C8, 1749-016 Lisbon, Portugal

ABSTRACT: High-pressure processing (HPP) is a technology of growing interest for food preservation, due to its ability to control the activity of degradative enzymes. The effect of three variables (pressure levels of 100, 250, and 400 MPa; pressure holding times of 0, 5, 15, and 30 min; and pressurization rates of 8 and 14 MPa/s) on the activity of the enzymes acid phosphatase, cathepsins (B and D), lipase, and calpains was studied using sea bass fillets as a case study model. Additionally, the effect of HPP on sarcoplasmic proteins was studied by SDS-PAGE and isoelectric focusing electrophoreses. The increase in pressure level and holding time decreased the protein concentration in sarcoplasmic extracts, and also the activity of calpains. As compared to nontreated samples, acid phosphatase activity was lower at 400 MPa, and for cathepsin D lower activities were observed at 100 and 400 MPa. The increase in pressurization rate increased the activity of cathepsin D, lipase, and calpains, although it was not always significant. In contrast, cathepsin B and lipase activities were less affected by HPP treatments. Electrophoresis separation of sarcoplasmic proteins showed that the intensity of many protein bands changed mainly due to pressure level and holding time. The results of this study suggest that HPP causes lysosomes disruption and also denaturation, aggregation, and fragmentation of sarcoplasmic proteins, and this evidence might be related to the decrease in enzymes activity especially at 400 MPa. In conclusion, HPP can be a potential tool to control the activity of degradative enzymes, which might prevent the softening of sea bass muscle due to autolytic reactions.

KEYWORDS: *high-pressure processing, sea bass, enzymes, water-soluble proteins, cathepsins, calpains, acid phosphatase, lipases, SDS-PAGE, IEF*

INTRODUCTION

The increase of consumer's preference toward fresh and minimally processed food products, rather than processed and frozen ones, demands for research on new processing and preservation methods, especially for products with short shelf life. High-pressure processing (HPP) is of growing interest for the processing and preservation of food. This technology has the potential to better retain food's nutritional and organoleptic characteristics, when compared to traditional thermal processing.[1] It is also a potential tool for the creation of new product textures by the food industry because of its ability to induce modifications on food functional properties.[2] HPP treatments have been used to inactivate microorganisms to extend food shelf life.[1] HPP is known to affect noncovalent chemical bonds of molecules, thus inducing modifications in water, proteins, polysaccharides, and lipids.[3]

Freshness of seafood is rapidly lost in the initial stages of post-mortem degradation due to autolytic degradation by endogenous enzymes, leading to an initial loss in prime quality.[4] Post-mortem changes, preprocessing, and processing methods can lead to tissue damage and disruption of cellular organelles, like mitochondria and lysosomes, releasing several enzymes into the cellular fluid.[5] The impact of autolytic enzymes on textural quality will limit the shelf life and cause an early downgrade of food products.[6] Therefore, enzymatic activity of fish has been used as an indicator of quality changes,[5] and calpains and cathepsins (B and D) have been reported as the most important enzymes involved in the softening of fish muscle tissues.[4,7,8]

Sea bass *Dicentrarchus labrax* (Linnaeus, 1758) is a teleost species widely distributed in the Mediterranean Sea and Atlantic Ocean and intensively farmed in several Mediterranean countries, for example, Greece, Spain, and Italy.[9] This species is highly appreciated because of its excellent organoleptical properties and reasonable price.[9]

The effect of pressure level in the activity of degradative enzymes has been studied in fish muscle, and results showed that the activity of degradative enzymes such as calpains and cathepsin D is reduced.[6,7] However, other pressure variables like pressure holding time and pressurization rate have not been a focus of attention. It is important to further investigate the effect of HPP in fresh fish to better understand the resultant effects on the quality and protein characteristics.

This study aims to understand the effect of HPP on the activity of several degradative enzymes of fresh sea bass fillets. In particularly, pressure level (0.1−400 MPa), pressure holding

Received: November 21, 2012
Revised: February 18, 2013
Accepted: February 21, 2013
Published: February 21, 2013

Table 1. Treatment Conditions Tested[a]

	Pressurization rate	Pressure level	Pressure holding time
Non-treated samples not subjected to high pressure processing (0.1 MPa)			
Treated samples subjected to high pressure processing	8 MPa/s	100 MPa	0 min
			5 min
			15 min
			30 min
		250 MPa	0 min
			5 min
			15 min
			30 min
		400 MPa	0 min
			5 min
			15 min
			30 min
	14 MPa/s	100 MPa	0 min
			5 min
		250 MPa	0 min
			5 min
		400 MPa	0 min
			5 min

[a]The time indicated in each treatment does not include the come up time or the depressurization time.

time (0−30 min), and pressurization rate (8 and 14 MPa/s) were tested to evaluate their effect on enzymatic activity of acid phosphatase, cathepsins (B and D), lipases, and calpains. Additionally, sarcoplasmic proteins of HPP sea bass were separated according to sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and isoelectric focusing (IEF) electrophoreses, to identify changes on protein profiles.

MATERIALS AND METHODS

Chemicals. The chemicals sodium dodecyl sulfate (SDS), trichloroacetic acid, and acetic acid were obtained from Fluka (Buchs, Germany); bromophenol blue was from Merck (Darmstadt, Germany); potassium hydroxide was from Panreac Quimica S.A.U. (Barcelona, Spain); Commassie Brilliant Blue R-250 was from Bio-Rad (Philadelphia, PA); Coomassie Phastgel blue R was from Amersham Pharmacia Biotech (Uppsala, Sweden); *p*-nitrophenylphosphate, L-methionine-7-amido-4-methylcoumarin triflouroacetic salt, *Z*-arginine-arginine-7-amido-4-methylcoumarin hydrochloride, hemoglobin from bovine blood, olive oil, trizma hydrochloride (Tris-HCl), dithiothreitol (DTT), 2-bis-(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol (Bis-Tris), ethylene diamine tetracetic acid (EDTA), ethylene glycol-bis-(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), glycine, potassium chloride, glycerol, magnesium chloride, sodium acetate, calcium chloride, thymolphtalein, potassium phosphate monobasic, sodium hydroxide, monochloroacetic acid, *β*-mercaptoethanol, citric acid, trisodium citrate, and bovine serum albumin were from Sigma-Aldrich (Steinheim, Germany); ethanol had a purity grade of 99%; and the water used was Milli-Q purified and distilled.

Preparation of Samples. Fresh farmed sea bass *Dicentrarchus labrax* (Linnaeus, 1758) specimens, with commercial quality, were acquired from a local market. The average weight of each fish was 465 ± 57 g, and the total length was 35 ± 1 cm. Fish samples (n = 16) were filleted, and skin was removed. Fillets were divided in portions of ca. 15 g, and eight portions randomly selected were used for each treatment to reduce the effect of specimen variability. Fillet portions were vacuum packed in low-oxygen permeable barrier bags (Colamin XX 100e, Obermühle, Pössneck, Germany) with a vacuum packager (Packman, Albipack, Águeda, Portugal) before pressurization treatments.

High-Pressure Treatments. HPP treatments were carried out in a hydrostatic press (high-pressure system U33, Unipress Equipment, Poland). This equipment has a pressure vessel of 35 mm diameter and 100 mm height surrounded by an external jacket, connected to a thermostatic bath to control the temperature (6 °C), using a mixture of propylene glycol and water (1:1) as pressurizing fluid. Samples (8 fillet portions per treatment) were processed at pressure levels of 100, 250, and 400 MPa for 5, 15, and 30 min using a pressurization rate of ca. 8 MPa/s. A higher pressure rate increment, ca. 14 MPa/s, was also tested to process samples at the same pressure levels (100, 250, and 400 MPa) for 5 min. Control treatments of 0 min pressure holding time were also performed subjecting the fillets to pressurization and immediate depressurization. Nineteen treatments were studied (Table 1) including nontreated samples (0.1 MPa). After HPP treatments, samples were immediately frozen in liquid nitrogen and stored at −20 °C until further analysis.

Enzymatic Activity. *Preparation of Extracts.* The extraction of enzymes was performed following the methodology previously described by Lakshmanan and coauthors,[6] with minor modifications. Briefly, minced sea bass (20 g) was homogenized with cold water (40 mL; 4 °C) using an Ultra Turrax homogenizer (2 min, 8000 rpm; Ultraturrax T25, Janke & Kunkel IKA-Labortechnik). The homogenate was kept on ice for 30 min with occasional stirring and centrifuged (14 600*g*, 4 °C, 20 min; centrifuge 3K30, Sigma, Osterode, Germany). The supernatant was filtered (Macherey-Nagel MN 640 W), frozen in liquid nitrogen, and stored at −20 °C until further analysis (enzymatic analysis and protein concentration).

Acid Phosphatase. Acid phosphatase activity was assayed with *p*-nitrophenylphosphate as a substrate following the methodology of Ohmori and coauthors.[10] Extracts (1 mL) were mixed with substrate solution (0.9 mL, 4 mmol/L *p*-nitrophenylphosphate in 0.1 mmol/L sodium acetate buffer and 1 mmol/L EDTA, pH 5.5) and incubated at 37 °C during 15 min. The reaction was stopped by the addition of potassium hydroxide (4 mL, 0.1 mol/L), and the *p*-nitrophenol released was measured spectrophotometrically (Perkin-Elmer Instruments Lambda 35 UV/vis spectrometer) at 400 nm. Acid phosphatase activity was expressed as absorbance units (AU) change per minute per gram of muscle. Four replicates were performed per treatment.

Cathepsin B. The activity of cathepsin B was assayed by the Barrett and Kirschke[11] method. Enzyme extract (0.1 mL) was mixed with substrate solution (0.1 mL, 0.0625 mmol/L of *Z*-arginine-arginine-7-amido-4-methylcoumarin hydrochloride in 100 mmol/L Bis-Tris, 20 mmol/L EDTA, 4 mmol/L DTT, pH 6.5) and incubated at 37 °C during 5 min. The reaction was stopped by the addition of SDS (1 mL, 30 mg/mL in 50 mmol/L Bis-Tris, pH 7.0), and the fluorescence of 7-amino-4-methylcoumarin liberated was measured (Ex = 360 nm; Em = 460 nm) using a spectrofluorometer (FluoroMax 3 spectrofluorometer, Horiba Jovin Yvon, NJ). Cathepsin B activity was expressed as fluorescence units (FU) change per minute per gram of muscle. Four replicates were performed per treatment.

Cathepsin D. The cathepsin D assay used was based on the procedure described by Anson,[12] with small modifications. Enzyme extract (0.5 mL) was mixed with substrate solution (1.5 mL of 20 mg/mL denatured hemoglobin from bovine blood in 0.2 mol/L citrate buffer, pH 3.7) and incubated at 37 °C during 3 h. The reaction was stopped by the addition of trichloroacetic acid (1.5 mL, 0.1 g/mL). After being

vigorously stirred, the precipitate was removed by centrifugation (18 000*g*, 15 min), and soluble peptides were measured spectrophotometrically at 280 nm. Cathepsin D activity was expressed as AU change per hour per gram of muscle. Four replicates were performed per treatment.

Lipase. The activity of lipase was assayed with olive oil as substrate following the titrimetric enzymatic assay described by Sigma-Aldrich.[13] Enzyme extract (1 mL) was mixed with substrate solution (1.5 mL of olive oil, 1.25 mL of water, and 0.5 mL of 200 mmol/L Tris-HCl buffer pH 7.7) and incubated at 37 °C during 24 h. The reaction was stopped by adding ethanol (2 mL, 95% v/v), and the liberated free fatty acid was titrated against sodium hydroxide (25 mmol/L) using thymolphtalein as indicator. Lipase activity was expressed in units per gram of muscle, where one unit corresponds to the amount of enzyme that hydrolyzes one microequivalent of fatty acids from triglycerides in 1 h. Four replicates were performed per treatment.

Calpains. Activity of calpains was measured using the method of Sasaki and coauthors.[14] Enzyme extract (0.1 mL) was mixed with substrate solution (0.1 mL, 0.125 mmol/L L-methionine-7-amido-4-methylcoumarin trifluoroacetic salt in 100 mmol/L Bis-Tris, 5 mmol/L calcium chloride, pH 6.5) and incubated at 37 °C during 2 min. The reaction was stopped by adding 30 mmol/L monochloroacetic acid, 21 mmol/L acetic acid, and 9 mmol/L sodium acetate (3 mL, pH 4.3). The fluorescence of 7-amino-4-methylcoumarin liberated was measured (Ex = 360 nm; Em = 460 nm) using a spectrofluorometer (FluoroMax 4 spectrofluorometer, Horiba Jobin Yvon, NJ). Calpains activity was expressed as FU change per minute per gram of muscle. Four replicates were performed per treatment.

Electrophoresis. *Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).* Muscle samples were added to cold buffer (75 mmol/L potassium chloride, 10 mmol/L potassium phosphate monobasic, 2 mmol/L magnesium chloride, 2 mmol/L EGTA, pH 7.0; 4 °C) and homogenized using an Ultra Turrax homogenizer. The homogenate was centrifuged (10 000*g*, 4 °C, 10 min), and the supernatant containing the sarcoplasmic proteins was frozen until further use. Sarcoplasmic protein solutions (with the same adjusted protein concentration) were diluted 1:1 in loading buffer (62.5 mmol/L Tris-HCl, 0.2 g/mL glycerol, 20 mg/mL SDS, 5% v/v β-mercaptoethanol, and 0.1 mg/mL bromophenol blue at pH 6.8) and heated (100 °C, 3 min) prior to gel running.

For SDS-PAGE, gels with different resolving power were used (10%, 12%, and 15% polyacrylamide; 18 × 16 cm) with stacking gels of 4%. The amount of sample loaded per lane was 100 μg of protein in gels with 10% and 12% polyacrylamide, and 300 μg of protein in gels with 15% polyacrylamide. Molecular weight standards (Precision Plus Protein Standards All Blue, Bio-Rad, Philadelphia, PA) were used in every gel. Electrophoresis was performed in a vertical unit (Hoefer SE 600 series, Amersham Pharmacia Biotech, Uppsala, Sweden) at 10 °C (LAUDA Ecoline Staredition RE 104, Lauda-Konigshofen, Germany) with running buffer (25 mmol/L Tris-HCl, 192 mmol/L glycine, and 1 mg/mL SDS, pH 9), and the running conditions limited to 25 mA (EPS 601, Amersham Pharmacia Biotech, Uppsala, Sweden). Protein bands were fixed and stained with Coomassie Brilliant Blue R-250. Determinations were performed in triplicate.

Isoelectric Focusing (IEF) with CleanGel. For IEF of sarcoplasmic extracts, the homogenization of muscle was performed with cold water (4 °C) to avoid band disturbances due to the presence of salts. The homogenates were centrifuged (40 000*g*, 4 °C, 20 min), and the supernatants were used as sarcoplasmic proteins.

Sarcoplasmic protein solutions (1 mg/mL) were analyzed by IEF using a CleanGel (25 × 11 cm; Amersham Pharmacia Biotech, Uppsala, Sweden) according to Silva and coauthors [15] with modification on gel rehydration. For a full-size CleanGel, 21 mL of a solution containing ampholine preblended (0.26 mL, pH 3.5−9.5; Pharmacia Biotech, Uppsala, Sweden) and ampholine (1.31 mL, pH 3.5−5.0; Pharmacia Biotech, Uppsala, Sweden) was used to rehydrate. Electrode wicks (Serva 42972, Heidelberg, Germany) were soaked with anode fluid (Serva 42984, Heidelberg, Germany) and cathode fluid (Serva 42986, Heidelberg, Germany). The electrophoresis running conditions were: prefocusing (30 min) − 500 V, 8 mA, 8 W; sample entrance (20 min) − 500 V, 8 mA, 8 W; and isoelectric focusing (5000 Vh) − 2000 V, 14 mA, 14 W (MultiDrive XL, Amersham Pharmacia Biotech, Uppsala, Sweden) at 10 °C (Multitemp II thermostatic circulator model 2219, Amersham, Sweden). After prefocusing, the sample application pieces (Amersham Pharmacia Biotech, Uppsala, Sweden) were placed 2 cm in front of the cathodic wick, and 30 μL of samples and 7.5 μL of isoelectric point (pI) marker solutions (isoelectric focusing calibration kit pH 3−10 and pH 5−10.5, Amersham Pharmacia Biotech, Uppsala, Sweden) were applied. After the IEF run, proteins were fixed and stained with Coomassie Phastgel blue R. Determinations were performed in triplicate.

Identification and Quantification of Protein Bands. Gels were scanned using a densitometer (model GS-800, Bio-Rad, Philadelphia, PA), and the images were analyzed with PDI Discovery Series software (module Quantity One version 2.4; Bio-Rad, PA). Molecular weights, pI values, and changes in integrated optical density (IOD) of protein bands were calculated.

Protein Concentration in Extracts. Concentration of proteins in extracts, for enzymatic activity and electrophoreses analyses, was determined with the Bradford method[16] using bovine serum albumin as standard. Four replicates were performed per treatment.

Statistical Analysis. Differences between treatments were tested at a 0.05 level of probability with the software STATISTICA 6.1 (Statsoft, Inc., Tulsa, OK). The Dunnett test was used to identify differences between HPP and nontreated samples. The effects of pressure level and pressure holding time were tested with a two-way analysis of variance, followed by a multiple comparisons test (Tukey HSD) to identify the differences between treatments. To study the effect of pressurization rate, a *t* test for independent samples was used.

RESULTS AND DISCUSSION

Protein Concentration. Protein concentration in water extracts was ca. 90 mg/mL in nontreated samples. The solubility of proteins of sea bass fillets treated with HPP was found to be dependent on pressure level and holding time (Figure 1).

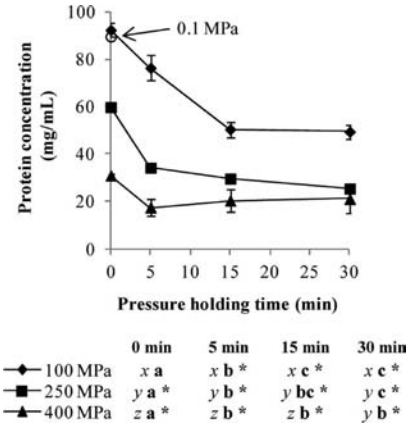

Figure 1. Protein concentration in sarcoplasmic extracts of sea bass fillets treated with high pressure. Vertical error bars represent the standard deviation. Different letters denote significant differences ($p < 0.05$) between pressure levels (x−z) or between pressure holding times (a−c), and the symbol "*" denotes significant differences with the nontreated sample (0.1 MPa).

In fact, apart from the treatment at 100 MPa during 0 min, HPP induced a significant reduction in protein solubility with pressure level and holding time. Water-soluble proteins reduced 4.5 times in samples treated at 400 MPa (5−30 min) when compared to nontreated samples. In contrast, pressurization rate did not influence sarcoplasmic protein solubility for the short-time treatments (0 and 5 min; Table 2).

The decrease of protein solubility with the increase of pressure level is in accordance with the results obtained with bovine

Table 2. Protein Concentration in Sarcoplasmic Extracts and Enzymatic Activities of Sea Bass Fillets Treated with High Pressure at 8 and 14 MPa/s Pressurization Rates[a]

		0 min		5 min	
	pressure level (MPa)	8 MPa/s	14 MPa/s	8 MPa/s	14 MPa/s
protein concentration in sarcoplasmic extracts (mg/mL)	100	92.4 ± 3.1 a	91.0 ± 5.9 a	76.4 ± 5.2 A	76.7 ± 2.3 A
	250	59.8 ± 1.4 a	71.4 ± 14.0 a	34.2 ± 1.0 A	33.6 ± 7.5 A
	400	30.9 ± 0.8 a	31.6 ± 0.8 a	17.5 ± 3.7 A	19.8 ± 0.2 A
acid phosphatase activity (AU/min/g)	100	5.4 ± 0.4 a	5.3 ± 0.7 a	5.6 ± 0.3 A	6.0 ± 0.3 A
	250	4.6 ± 0.1 a	5.9 ± 0.1 a	5.3 ± 0.6 A	5.1 ± 0.5 A
	400	4.5 ± 0.4 a	4.4 ± 0.4 a	4.4 ± 1.0 A	4.3 ± 0.3 A
cathepsin B activity ($\times 10^3$ FU/min/g)	100	3.7 ± 0.2 a	3.6 ± 1.4 a	4.4 ± 0.4 A	4.9 ± 1.5 A
	250	2.8 ± 0.2 a	3.8 ± 1.7 a	5.3 ± 1.5 B	7.1 ± 1.3 A
	400	6.3 ± 1.7 a	7.7 ± 1.1 a	6.9 ± 1.2 A	5.5 ± 0.6 B
cathepsin D activity (AU/h/g)	100	0.15 ± 0.04 b	0.22 ± 0.02 a	0.20 ± 0.05 A	0.27 ± 0.05 A
	250	0.25 ± 0.05 a	0.25 ± 0.04 a	0.21 ± 0.01 B	0.28 ± 0.06 A
	400	0.31 ± 0.02 b	0.42 ± 0.07 a	0.10 ± 0.04 A	0.15 ± 0.09 A
lipase activity ($\times 10^{-2}$ units/g)	100	3.64 ± 0.86 a	4.47 ± 0.52 a	1.11 ± 0.48 B	3.92 ± 1.20 A
	250	3.33 ± 0.77 a	4.36 ± 0.55 a	5.20 ± 2.22 A	5.10 ± 1.65 A
	400	4.89 ± 2.10 a	5.82 ± 0.76 a	3.95 ± 1.73 A	6.01 ± 1.61 A
calpains activity ($\times 10^4$ FU/min/g)	100	13.7 ± 1.9 a	14.9 ± 1.5 a	12.2 ± 1.0 B	15.3 ± 1.5 A
	250	11.0 ± 0.6 b	13.5 ± 1.3 a	5.3 ± 0.5 A	4.7 ± 0.8 A
	400	5.3 ± 1.1 b	7.2 ± 1.4 a	1.4 ± 0.1 B	1.6 ± 0.1 A

[a]Values are presented as average ± standard deviation. Different letters denote significant differences ($p < 0.05$) between pressurization rates at the same pressure level for 0 min (a,b) or 5 min (A,B). Abbreviations: AU, absorbance units; FU, fluorescence units.

muscle by Marcos and coauthors.[17] The protein solubility represents a measure of protein denaturation, and the decrease observed with the raise of pressure level could be due to the formation of protein aggregates that can no longer be solubilized in water,[17] which were pronounced with the increase in pressure holding time. These results also indicate that the increase in pressure level and holding time might decrease the amount of enzymes available, as soluble proteins in these extracts include mainly enzymes.[18]

Enzymatic Activity. *Acid Phosphatase.* Phosphatase activities in fish muscles, particularly ATP-, ADP-, and IMP-degrading enzymatic activities, have been reported to be related to the freshness index, *K*-value.[19]

The activity of acid phosphatase in nontreated samples (5.6 AU/min/g) was not significantly different from that found in 100 MPa (0−30 min) and 250 MPa (5−15 min) samples, and these values were significantly higher than in the remaining treatments (Figure 2). In general, pressure holding time and pressurization rate did not affect the activity of acid phosphatase (Figure 2; Table 2), but still the activity of acid phosphatase decreased to a minimum of 3.7 AU/min/g in samples treated at 400 MPa (15 min). HPP treatments that cause inactivation of acid phosphatase might be suitable for the preservation of fish muscle, as a decrease in its activity indicates less degradation of IMP, and thus pleasant flavors might be kept for longer.

Ohmori and coauthors[10] obtained similar results in bovine liver pressurized during 10 min at levels up to 500 MPa. At lower pressure levels (up to 300 MPa), it was observed a gradual increase in acid phosphatase activity in the cytosolic fraction and a decrease in the lysosomal fraction, but the total activity remained almost the same.[10] This effect was explained by the enzyme being released from lysosomes due to their disruption, as approximately 40−60% of this enzyme is bound to lysosomes membranes.[10] The decrease in the phosphatase activity with the increase in pressure level can be a result of enzyme inactivation caused by HPP treatments. HPP affects proteins differently in minced and whole fish muscle, as in HPP minced sea bass values increased ca. 2.5 times in samples treated at 500 MPa (5 min) as compared to nontreated samples.[7]

Cathepsin B. Cathepsins are a group of proteases responsible for the softening of the muscle tissue.[4] This group of enzymes is found in lysosomes and can be released into the cytoplasm and intracellular spaces as a consequence of lysosomal disruption, mainly during post-mortem storage.[7] In particular, cathepsin B is a cysteine carboxypeptidase that is regulated in vivo by a inhibitor named cystatin.[7]

The effect of HPP on the activity of cathepsin B is shown in Figure 2 and Table 2. No significant differences were determined between nontreated samples (5.2×10^3 FU/min/g) and samples treated at 100 MPa (0−30 min). In what concerns pressurization rate, the activity of this enzyme at 250 MPa (5 min) increased with the increase in pressurization rate, while at 400 MPa (5 min) the opposite was observed (Table 2).

In general, pressure level did not affect significantly the activity of cathepsin B. In previous studies, the activity of cathepsin B increased almost 2 times with the increase in pressure level from 0.1 to 300 MPa (5 min).[20]

In the current study, the highest values were observed in treatments at 250 MPa (30 min) and 400 MPa (5−15 min). At 250 MPa, the increase in pressure holding time from 0 to 30 min increased cathepsin B activity in 2.8 times. The cathepsin B activity increase might be due to disruption of lysosomes and consequent release of enzymes that became available to interact with substrate, and this can promote the softening of sea bass muscle tissues.

Cathepsin D. Cathepsin D is considered to be the most important enzyme in post-mortem degradation due to the absence of a specific inhibitor in the fish muscle.[7] This enzyme is an aspartic acid protease that occurs in the lysosomal system.[21]

The activity of cathepsin D in nontreated samples was 0.28 AU/h/g, and the variations caused by HPP treatments did not exceed the activity values observed in nontreated samples (Figure 2). With the exception of the 0 min treatments for which the increase in pressure level increased cathepsin D

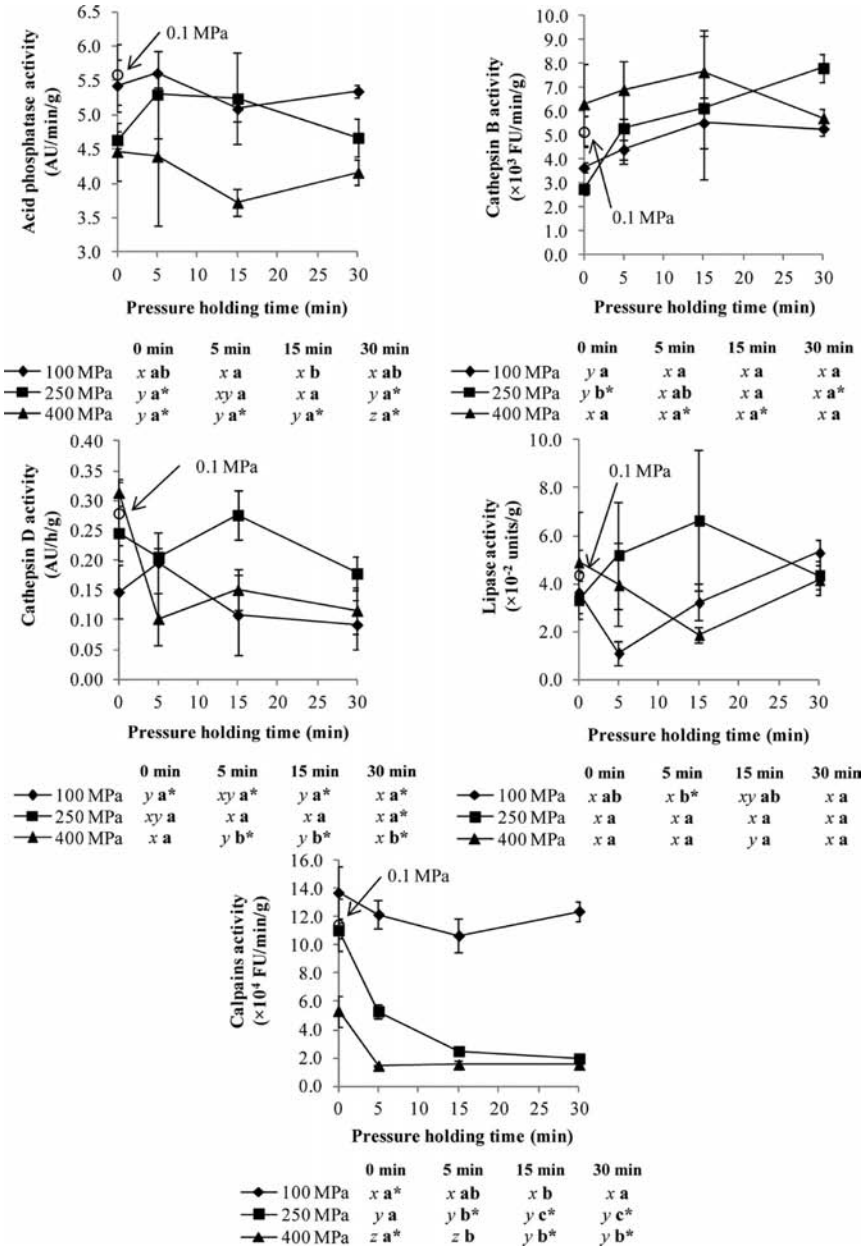

Figure 2. Enzymes activity (acid phosphatase, cathepsin B, cathepsin D, lipase, and calpains) in sea bass fillets treated with high pressure. Vertical error bars represent the standard deviation. Different letters denote significant differences ($p < 0.05$) between pressure levels (x–z) or between pressure holding times (a–c), and the symbol "*" denotes significant differences with the nontreated sample (0.1 MPa). Abbreviations: AU, absorbance units; FU, fluorescence units.

activity, the treatments at 100 and 400 MPa showed lower activity values. Pressure holding time only affected the activity of cathepsin D in the 400 MPa treatments, decreasing the activity with the increase in pressure holding time. In relation to pressurization rate, higher values of cathepsin D activity were, in general, observed in samples treated with the fastest pressurization rate treatment, but not always significant (Table 2).

In what concerns the effect of pressure level, the activity of cathepsin D followed a similar trend in previous studies with minced and whole muscle of sea bass, but the activity in nontreated samples was lower than in treatments at higher pressure levels.[7,20] At lower pressure levels (100 MPa), the variations in cathepsin D activity seem to be due principally to inactivation of this enzyme. With the increase in pressure level to 250 MPa, the rupture of lysosomes and consequent release of enzymes might caused an increase of cathepsin D activity. It is not set aside the hypothesis that HPP continues to inactivate the enzyme, but proportionally the release of enzymes from lysosomes might be more important at this pressure level. At 400 MPa, inactivation predominates again the effects of HPP on the activity of this enzyme, and these treatments (pressure holding time of at least 5 min) might be suitable for the preservation of fish muscle.

Lipase. Lipases are triacylglycerol acylhydrolases that degrade phospholipids and catalyze the hydrolysis of fatty acid ester bonds in triacylglycerols to free fatty acids and glycerol.[22,23] Accumulation of free fatty acids has been associated with quality deterioration due to changes on textural properties by stimulation of protein denaturation and production of off flavors by promotion of lipid oxidation.[23]

dx.doi.org/10.1021/jf3049643 | J. Agric. Food Chem. 2013, 61, 2851–2860

Lipase activity (Figure 2) in nontreated samples was 4.4 × 10^{-2} units/g, and it was not significantly affected in most of the HPP treatments. In what concerns pressurization rate, higher values of lipase activity were only observed in samples treated at 100 MPa during 5 min with the fastest pressurization rate treatment (Table 2).

Published data show that the degradation of lipids is enhanced by HPP treatments in salmon and tuna,[24] but lipid degradation inhibition has also been reported in tuna.[25] Differences between species as well on product processing conditions (e.g., fresh, smoked, fillet, *carpaccio*, minced fish) might explain these differences. Nayak and coauthors[26] suggested that differences in lipase activity can also be attributed to feeding habits, food composition, and physiological status of fish.

Nevertheless, the increase in lipids degradation with HPP treatments was not associated with a reduction in texture quality due to changes in structural proteins of fish muscle.[24] Particularly, the reactions related to lipid degradation are of less importance in fish with low fat content, where storage conditions are the determinant factors in the evolution of lipid degradation.[24]

Calpains. Calpains are one of the main proteinase groups that hydrolyze myofibrillar proteins, affecting the texture of post-mortem fish muscle.[8] Calpains are intracellular cysteine proteases, free in the cells cytoplasm, active at neutral pH, dependent upon calcium, and with their activity regulated by the endogenous inhibitor calpastatin.[27]

The overall activity of calpains was high (11.4 × 10^4 FU/min/g in nontreated samples). In general, samples treated at 100 MPa showed calpain activities similar to nontreated samples (Figure 2). The increase in pressure level and holding time inactivated this enzyme to levels of 1.5 × 10^4 FU/min/g in samples treated at 250 (15−30 min) and 400 MPa (5−30 min). The increase in pressurization rate caused a significant increase in calpains activity in samples treated at 100 MPa (5 min), 250 MPa (0 min), and 400 MPa (0−5 min) (Table 2).

The decrease in calpains activity at higher pressure levels and longer holding times was probably caused by the dissociation of both subunits of calpains.[28] A similar decrease of calpains activity with pressure level was observed in previous studies with minced sea bass,[28] which may delay post-mortem degradation due to the higher hardness of HPP samples during refrigerated storage.[29]

Electrophoresis. *SDS-PAGE.* Molecular weights of major sarcoplasmic proteins in sea bass fillets were 84.3, 52.6−54.0, 41.0, 20.9−27.2, 14.1−15.6, 10.8, 3.9, and 1.6−2.2 kDa (Figure 3).

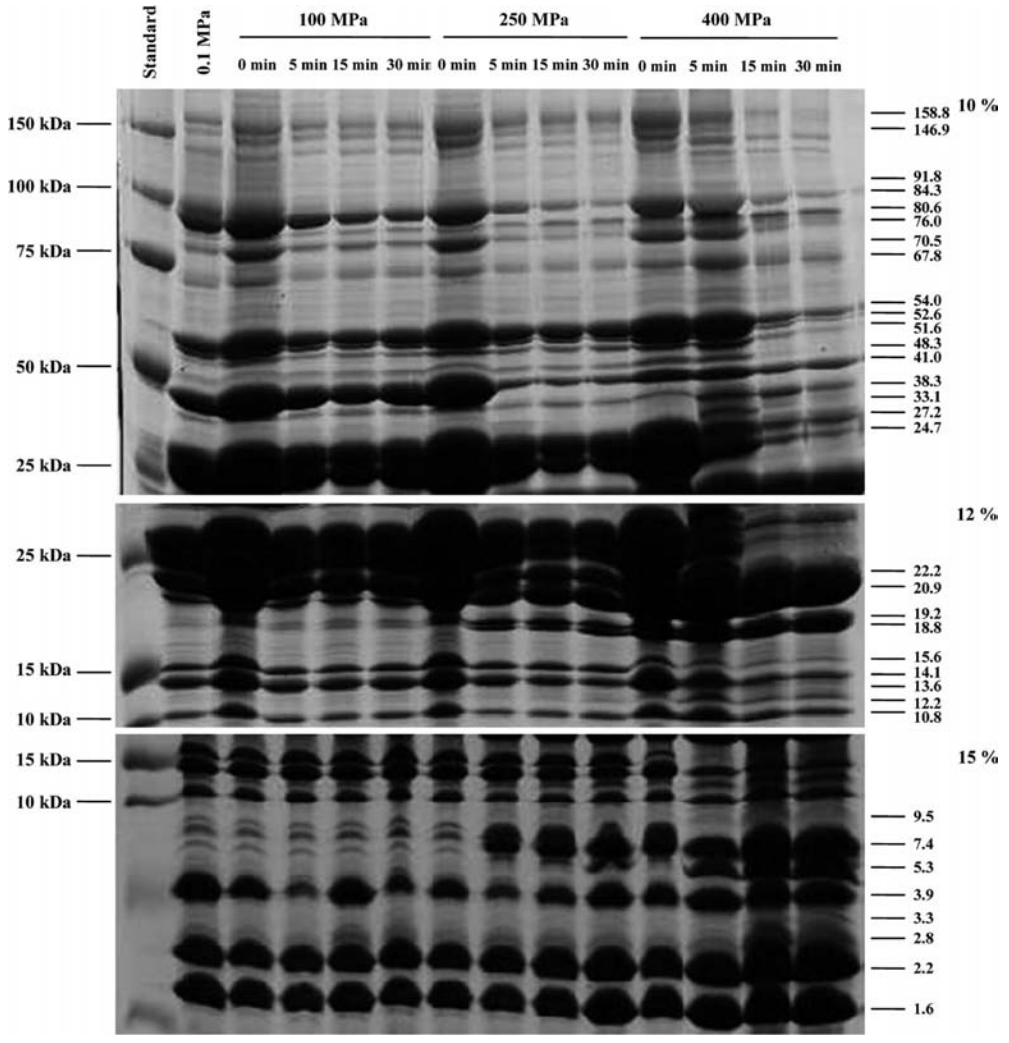

Figure 3. SDS-PAGE protein profiles of sarcoplasmic proteins extracted from sea bass fillets treated with high pressure. Proteins separation was achieved in gels containing 10%, 12%, and 15% of polyacrilamide (identified in the right side of the figure). Molecular weights (kDa) of selected protein bands are indicated in the right side of the figure, aligned with protein bands. Molecular weight of standard proteins is indicated on the left side of the figure.

Table 3. Integrated Optical Densities (IOD) of Protein Bands Obtained by SDS-PAGE of Sarcoplasmic Proteins Extracted from Sea Bass Fillets Treated with High Pressure (Electropherograms Shown in Figure 3)[a]

	100 MPa				250 MPa				400 MPa			
molecular weight (kDa)	0 min	5 min	15 min	30 min	0 min	5 min	15 min	30 min	0 min	5 min	15 min	30 min
158.8 ± 10.2	1.48 ± 0.25 ya*	0.52 ± 0.16 yb	0.43 ± 0.07 xb	0.70 ± 0.19 xb	2.35 ± 0.12 xa*	0.75 ± 0.04 yb	0.65 ± 0.01 xb	0.65 ± 0.07 xb	2.12 ± 0.29 xya*	1.61 ± 0.34 xa*	0.31 ± 0.15 xb	0.22 ± 0.04 xb
146.9 ± 10.8	1.08 ± 0.28 xa*	0.43 ± 0.07 xb	0.43 ± 0.05 xb	0.50 ± 0.13 xb	1.48 ± 0.22 xa*	0.43 ± 0.10 xb	0.36 ± 0.14 xb	0.28 ± 0.11 xb	1.08 ± 0.02 xa*	0.57 ± 0.13 xab	0.17 ± 0.06 xb	n.d.*
91.8 ± 9.9	1.55 ± 0.15 a*	0.29 ± 0.13 b*	0.25 ± 0.01 b*	0.23 ± 0.01 b*	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*
84.3 ± 7.7	5.88 ± 0.48 xa	2.77 ± 0.21 xb*	1.93 ± 0.18 xb	1.97 ± 0.31 xb	4.71 ± 0.23 ya	1.19 ± 0.10 yb	0.75 ± 0.09 yb	0.47 ± 0.07 yb	3.13 ± 0.43 za*	2.40 ± 0.28 xa	0.69 ± 0.06 yb	0.38 ± 0.05 yb
80.6 ± 7.2	0.51 ± 0.00 xa	0.27 ± 0.05 ya	0.41 ± 0.11 ya	0.45 ± 0.12 xa	0.51 ± 0.04 xa	0.49 ± 0.11 ya	0.68 ± 0.17 ya*	0.60 ± 0.13 xa	0.92 ± 0.08 xb*	1.62 ± 0.07 xa*	1.16 ± 0.16 xb*	0.85 ± 0.13 xb*
76.0 ± 6.7	2.85 ± 0.03 xa*	0.60 ± 0.11 yb	0.84 ± 0.08 xb	0.70 ± 0.13 xb	2.95 ± 0.22 xa*	0.53 ± 0.07 yb	0.43 ± 0.07 xb	0.35 ± 0.06 xb*	2.10 ± 0.14 ya*	1.66 ± 0.17 xb*	0.49 ± 0.04 xc	0.26 ± 0.04 xc*
70.5 ± 4.5	0.89 ± 0.01 xa	0.25 ± 0.11 ya	0.47 ± 0.34 xa	0.48 ± 0.31 xa	0.83 ± 0.23 xa	0.41 ± 0.20 ya	0.41 ± 0.21 xa	0.53 ± 0.10 xa	0.51 ± 0.17 xb	1.29 ± 0.10 xa*	0.29 ± 0.07 xb	0.25 ± 0.03 xb
67.8 ± 4.8	1.22 ± 0.13 xa*	0.39 ± 0.21 yb	0.46 ± 0.20 xb	0.43 ± 0.18 xb	0.88 ± 0.09 xa	0.43 ± 0.21 ya	0.36 ± 0.15 xa	0.25 ± 0.01 xa	0.77 ± 0.24 xab	1.32 ± 0.20 xa*	0.69 ± 0.08 xb	0.59 ± 0.03 xb
54.0 ± 3.4	6.24 ± 0.47 xa*	3.33 ± 0.44 yb	3.08 ± 0.39 xb	3.86 ± 0.47 xb	6.34 ± 0.78 xa*	3.47 ± 0.39 yb	3.48 ± 0.54 xb	3.55 ± 0.47 xb	6.14 ± 0.32 xa*	6.65 ± 0.47 xa*	1.70 ± 0.22 xb*	0.95 ± 0.04 yb*
52.6 ± 3.1	1.59 ± 0.03 ya*	0.72 ± 0.03 yb	0.81 ± 0.02 xb	0.90 ± 0.01 xb	1.60 ± 0.15 ya*	0.86 ± 0.05 yb	0.65 ± 0.04 xybc	0.53 ± 0.02 yc*	1.96 ± 0.03 xa*	1.35 ± 0.14 xb*	0.50 ± 0.08 yc*	0.26 ± 0.02 yc*
51.6 ± 2.9	1.06 ± 0.06 xa*	0.44 ± 0.09 yb	0.55 ± 0.06 xb	0.55 ± 0.09 xyb	1.25 ± 0.01 xa*	0.66 ± 0.11 yb	0.73 ± 0.13 xb*	0.90 ± 0.10 xab*	1.38 ± 0.10 xb*	2.25 ± 0.23 xa*	0.57 ± 0.12 xc	0.29 ± 0.00 yc
48.3 ± 4.0	0.56 ± 0.01 xa	0.31 ± 0.10 ya	0.48 ± 0.09 xa	0.56 ± 0.10 xa	0.73 ± 0.02 xa*	0.56 ± 0.03 ya	0.55 ± 0.03 xa	0.51 ± 0.04 xa	1.08 ± 0.09 xa*	1.23 ± 0.07 xa*	0.28 ± 0.12 xb	0.13 ± 0.02 yb*
41.0 ± 2.6	10.96 ± 0.14 xa*	6.15 ± 0.27 xbc	5.31 ± 0.35 xc	6.45 ± 0.45 xb*	10.54 ± 0.12 xa*	1.42 ± 0.07 zb*	1.23 ± 0.15 yb*	1.33 ± 0.17 yb*	3.19 ± 0.19 ya*	3.65 ± 0.22 ya*	1.61 ± 0.17 yb*	1.32 ± 0.03 yb*
38.3 ± 2.5	1.29 ± 0.15 xa*	0.46 ± 0.02 yb	0.66 ± 0.02 yb	0.49 ± 0.23 yb	1.10 ± 0.08 xa*	0.62 ± 0.10 ya	0.82 ± 0.16 ya	0.66 ± 0.07 ya	1.16 ± 0.18 xc*	3.30 ± 0.06 xa*	2.09 ± 0.31 xb*	1.05 ± 0.18 xc*
33.1 ± 2.3	0.62 ± 0.17 xa	0.24 ± 0.19 ya	0.53 ± 0.01 xa	0.42 ± 0.07 xa	0.58 ± 0.12 xa	0.50 ± 0.33 ya	0.42 ± 0.23 xa	0.31 ± 0.17 xa	0.66 ± 0.01 xb	3.38 ± 0.32 xa*	0.57 ± 0.02 xb	0.50 ± 0.06 xb
27.2 ± 1.6	12.45 ± 1.25 xa*	9.04 ± 0.78 xa	8.97 ± 1.27 xa	9.58 ± 0.95 xa	12.52 ± 0.76 xa*	9.10 ± 1.08 xab	7.44 ± 0.77 xb	9.28 ± 0.65 xab	11.34 ± 2.25 xa	4.78 ± 0.39 yb*	2.54 ± 0.24 yb*	2.20 ± 0.07 yb*
24.7 ± 1.0	12.38 ± 0.62 xa*	6.48 ± 0.24 xb	5.91 ± 0.75 xb	7.84 ± 0.23 xb	12.47 ± 1.52 xa*	6.47 ± 0.33 xb	4.50 ± 0.37 xbc*	3.75 ± 0.30 yc*	10.62 ± 1.37 xa*	7.25 ± 0.01 xb	1.61 ± 0.11 yc*	1.03 ± 0.09 zc*
22.2 ± 0.2	11.83 ± 0.37 xa*	7.53 ± 0.23 yb	7.95 ± 0.49 xyb	8.30 ± 0.20 xb	12.46 ± 1.14 xa*	5.14 ± 0.06 yb	5.68 ± 0.05 yb	5.51 ± 0.19 yb	12.51 ± 0.68 xb*	15.85 ± 0.38 xa*	9.13 ± 0.75 xc	7.48 ± 1.69 xyc
20.9 ± 0.2	4.61 ± 0.15 ya	2.95 ± 0.59 za	3.02 ± 0.30 ya	3.09 ± 0.18 ya	4.80 ± 0.81 yb*	6.44 ± 0.24 yab*	7.63 ± 0.22 xa*	8.10 ± 0.01 xa*	9.08 ± 1.71 xa*	9.93 ± 0.07 xa*	9.27 ± 1.21 xa*	7.60 ± 0.65 xa*
19.2 ± 0.3	1.72 ± 0.49 ya*	0.60 ± 0.37 za	0.88 ± 0.11 za	0.85 ± 0.24 za	2.14 ± 0.60 ya*	2.52 ± 0.13 ya*	2.92 ± 0.19 ya*	2.59 ± 0.11 ya*	5.06 ± 0.89 xa*	5.48 ± 0.46 xa*	4.73 ± 0.52 xa*	4.45 ± 0.16 xa*
18.8 ± 0.3	1.57 ± 0.30 ya*	0.40 ± 0.01 zb	0.65 ± 0.14 yb	0.59 ± 0.21 yb	1.55 ± 0.20 yab*	1.39 ± 0.09 yab*	1.24 ± 0.07 xyb*	2.12 ± 0.06 xa*	4.35 ± 0.3 xa*9	4.47 ± 0.25 xa*	1.69 ± 0.10 xb*	2.31 ± 0.02 xb*
15.6 ± 0.3	4.54 ± 0.21 xa*	1.75 ± 0.00 xb	1.71 ± 0.01 xb	1.91 ± 0.03 xb	4.86 ± 0.40 xa*	1.79 ± 0.05 xb	1.85 ± 0.02 xb	1.88 ± 0.09 xb	2.12 ± 0.06 ya*	1.60 ± 0.06 xab	1.02 ± 0.19 ybc*	0.76 ± 0.10 yc*
14.1 ± 0.5	6.86 ± 0.89 xya*	2.87 ± 0.13 yb	2.85 ± 0.09 xb	3.21 ± 0.04 xb	6.54 ± 0.19 ya*	2.70 ± 0.02 yb	2.18 ± 0.01 xb	1.83 ± 0.04 yb*	7.92 ± 0.08 xa*	4.23 ± 0.28 xb*	1.84 ± 0.06 xc*	1.67 ± 0.13 yc*
13.6 ± 0.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.05 ± 0.03 a*	0.66 ± 0.01 b*	0.44 ± 0.03 c*
12.2 ± 0.4	0.69 ± 0.06 za*	0.42 ± 0.02 yb	0.52 ± 0.03 yab	0.51 ± 0.01 yab	0.96 ± 0.05 ya*	0.52 ± 0.03 yb	0.54 ± 0.02 yb	0.68 ± 0.02 yb*	1.32 ± 0.00 xb*	2.60 ± 0.05 xa*	1.13 ± 0.13 xbc*	1.00 ± 0.06 xc*
10.8 ± 0.4	3.63 ± 0.30 xa*	1.26 ± 0.16 yb	1.24 ± 0.13 xb	1.28 ± 0.11 xb	3.77 ± 0.19 xa*	1.01 ± 0.08 yb	1.14 ± 0.05 xb	1.26 ± 0.01 xb	3.79 ± 0.13 xa*	4.15 ± 0.34 xa*	1.81 ± 0.27 xb*	1.65 ± 0.19 xb
9.5 ± 0.4	0.32 ± 0.04 xa	0.29 ± 0.07 ya	0.38 ± 0.07 ya	0.50 ± 0.07 ya	0.34 ± 0.08 xa	0.57 ± 0.10 xya	0.55 ± 0.09 ya	0.54 ± 0.22 ya	0.43 ± 0.09 xc	0.69 ± 0.01 xbc	1.87 ± 0.08 xa*	1.02 ± 0.02 xb*
7.4 ± 0.3	1.24 ± 0.05 ya	1.28 ± 0.03 ya	1.62 ± 0.09 za	2.43 ± 0.17 za*	1.66 ± 0.04 yb	7.12 ± 0.23 xa*	7.58 ± 0.50 ya*	6.84 ± 0.29 ya*	8.05 ± 0.74 xb*	5.99 ± 0.23 xc*	10.21 ± 0.10 xa*	9.25 ± 0.26 xab*
5.3 ± 0.2	0.24 ± 0.03 xa	0.20 ± 0.07 ya	0.22 ± 0.08 ya	0.25 ± 0.06 za	0.21 ± 0.00 xb	0.19 ± 0.00 yb	n.d.	3.86 ± 1.28 ya*	n.d.	4.48 ± 0.19 xb*	9.45 ± 0.32 xa*	8.04 ± 0.56 xa*
3.9 ± 0.1	5.28 ± 0.02 xb*	1.98 ± 0.01 yc*	7.21 ± 0.44 xa	4.05 ± 0.29 yb*	4.62 ± 0.11 xb*	2.57 ± 0.22 yc*	3.98 ± 0.09 ybc*	6.60 ± 0.23 xa*	5.24 ± 0.21 xb	7.00 ± 0.12 xa	7.90 ± 0.99 xa	7.13 ± 0.5 xa*0
3.3 ± 0.0	0.22 ± 0.09 xa	0.27 ± 0.12 xa	0.27 ± 0.11 ya	0.34 ± 0.14 ya	0.28 ± 0.05 xa	0.21 ± 0.01 xa	0.31 ± 0.04 ya	0.32 ± 0.14 ya	0.23 ± 0.12 xb	0.37 ± 0.07 xb	1.09 ± 0.19 xa*	0.90 ± 0.18 xa*
2.8 ± 0.1	0.46 ± 0.05 xa	0.48 ± 0.19 xa	1.04 ± 0.16 ya	0.73 ± 0.11 ya	0.54 ± 0.13 xa	0.84 ± 0.22 xa	0.82 ± 0.18 ya	0.69 ± 0.06 ya	0.50 ± 0.08 xb	0.97 ± 0.35 xb	3.61 ± 0.28 xa*	3.14 ± 0.22 xa*
2.2 ± 0.1	6.80 ± 0.59 xa	6.26 ± 0.50 ya	7.34 ± 0.91 ya	8.72 ± 0.84 ya	6.85 ± 0.48 xa	7.24 ± 0.36 xya	7.90 ± 0.85 ya	9.69 ± 0.84 xya	7.69 ± 0.74 xc	10.40 ± 0.60 xbc	15.32 ± 1.94 xa*	13.18 ± 2.04 xab*
1.6 ± 0.2	7.00 ± 0.59 xa	5.92 ± 0.17 ya*	7.40 ± 0.49 ya	7.11 ± 0.42 ya	7.42 ± 0.26 xb	6.01 ± 0.01 yb	7.11 ± 0.50 yb	10.53 ± 0.91 xa	8.60 ± 0.82 xb	10.70 ± 0.50 xab*	11.62 ± 1.60 xa*	12.01 ± 0.75 xa*

[a]Only bands with IOD of 1 or higher in at least one treatment are shown. Values are presented as average ± standard deviation. Different letters denote significant differences ($p < 0.05$) between pressure levels (x–z) or between pressure holding times (a–c), and the symbol "*" denotes significant differences with the nontreated sample (0.1 MPa). Abbreviations: n.d., band not detected.

dx.doi.org/10.1021/jf3049643 | J. Agric. Food Chem. 2013, 61, 2851−2860

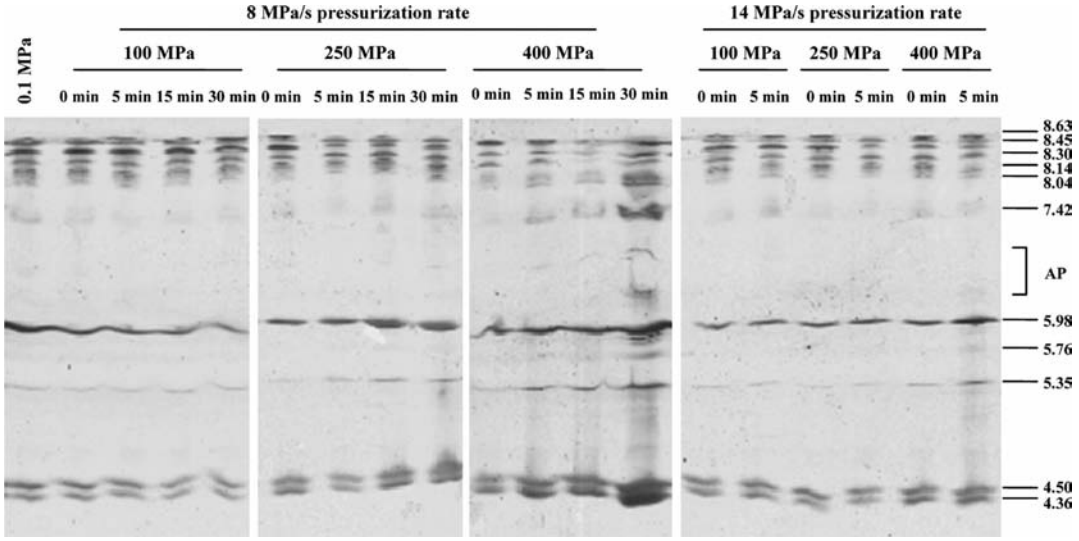

Figure 4. IEF protein profiles of sarcoplasmic proteins extracted from sea bass fillets treated with high pressure. Isoelectric point of proteins bands is indicated on the right side of the figure. Abbreviations: AP, sample application point.

The protein bands with 48.3−41.0 kDa, those with 27.2−24.7 kDa, and those with 80.6 and 27.2 kDa showed molecular weights similar to those of cathepsin D, cathepsin B, and calpains, respectively.[27,30] Still, different proteins might be included in the same protein bands.

Changes in sarcoplasmic proteins profile from HPP sea bass fillets were followed by SDS-PAGE after protein adjustment to the same concentration (Figure 3 and Table 3). Electrophoretic profiles show that a total of 34 protein bands in the range 1.6−158.8 kDa presented significant differences among HPP treatments. Also, protein profiles of HPP control samples (0 min of pressure holding time at 100, 250, and 400 MPa) presented 16 protein bands with higher intensities than the same bands in 0.1 MPa samples (158.8, 146.9, 76.0, 54.0−51.6, 41.0−38.3, 24.7−22.2, 19.2−14.1, and 12.2−10.8 kDa). A similar pattern was found in HPP squid treated at 300 MPa.[31]

Concerning pressure level, 400 MPa treatments caused marked changes in the profile of sarcoplasmic proteins, as compared to 100 MPa (Figure 3). The intensity of protein bands with 20.9, 19.2, 18.8, 12.2, and 7.4 kDa increased with pressure level in all pressure holding times, while the opposite was observed with the protein band with 91.8 kDa, which was undetectable at 250 and 400 MPa. On the other hand, the protein band with 13.6 kDa appeared only at 400 MPa (5−30 min).

In what concerns pressure holding time, the decrease in intensity of protein bands with higher molecular weights was accompanied by an increase in intensities of protein bands with lower molecular weights. At 400 MPa, the intensity of 14 protein bands (158.8, 146.9, 84.3, 76.0, 54.0, 52.6, 48.3, 41.0, 24.7, 18.8, 15.6, 14.1, 13.6, and 10.8 kDa) gradually decreased with longer processing times. The same pattern was observed in the protein bands with 52.6 and 24.7 kDa at 250 MPa. In contrast, the intensity of the protein band with 20.9 kDa increased with pressure holding time at 250 MPa, and those with 5.3, 3.3, and 2.8 kDa increased at 400 MPa. These variations with pressure holding time do not seem to happen in squid, as it was not highlighted in the study of Gou and coauthors[31] where squid was treated at 300 MPa (0−20 min).

In terms of pressurization rate (data not shown), the intensity of most protein bands decreased with the increase in pressurization rate at 250 MPa, while the opposite was observed at 400 MPa. However, no clear pattern was observed at 100 MPa, as the fastest rate decreased the intensity of several protein bands in samples treated during 0 min, but not during 5 min.

The results obtained in the current study revealed a higher effect of pressure level in the protein profiles of sarcoplasmic proteins as compared to other food matrixes like turkey[32] and bovine.[17] The muscle of terrestrial animals and fish tenderizes at different rates, and fish tenderization diminishes firmness and acceptability by consumers.[33] The results suggest that fish proteins may be more susceptible to HPP treatments, as compared to terrestrial animals. Chéret and coauthors[20] also studied the effect of pressure level in sea bass fillets, but protein profiles of sarcoplasmic extracts revealed less differences, which might be due to the smaller amount of proteins loaded for the electrophoresis.

The decrease in the intensity of protein bands might be due to protein degradation or fragmentation, and to insolubilization of sarcoplasmic proteins as a result of the formation of proteins aggregates.[17] On the other hand, the increase in the intensity of some protein bands could be explained by an increase in the solubility of myofibrillar proteins as a result of modifications in the protein structure with the HPP treatment.[17]

IEF. The effect of HPP in the protein profiles of native sarcoplasmic proteins was followed by IEF (Figure 4). The pI values of major sarcoplasmic proteins in sea bass fillets were ca. 8.04−8.63, 5.98, and 4.36−4.50. Comparing the profiles of HPP samples obtained by IEF and SDS-PAGE, the results of IEF showed less changes, as only 12 protein bands were detected in the IEF profiles. Once again, the differences in protein bands intensities were mainly due to variations in pressure level and holding time, and not as much with pressurization rate.

The increase in pressure level and holding time led to the intensification of the protein bands with pI in the range from 4.36 to 7.42. Although this increase has been observed for most protein bands, samples treated at 400 MPa (15 min) showed lower intensities in protein bands with pI of 8.30−8.63 than 400 MPa (5 and 30 min). Additionally, the protein band with pI value of 5.76 appeared only in the treatments at 400 MPa (5−30 min). In relation to pressurization rate, most differences in IOD values were not evident by human visual inspection of electropherograms. The exceptions were for protein bands with pI of 8.45, 8.30, and 8.04, where higher intensities were observed in the fastest pressurization rate treatments at 400 MPa.

HPP treatment effects in IEF profiles were studied in minced salmon and saithe samples,[34] but sarcoplasmic proteins of these species behaved differently when compared to the current study. In fact, three protein bands with pI near 5.85 were not detected in samples treated at 350 MPa (15 min). Still, the IEF profile was differently affected with the HPP treatment according to fish species.

In conclusion, HPP induced significant changes in the activities of several enzymes of sea bass muscle with pressure level and holding time, but only minor changes were detected with pressurization rate. The increase in pressure level and holding time decreased the protein concentration in sarcoplasmic extracts, and also the activity of calpains. The highest reductions were observed at 400 MPa, for acid phosphatase, cathepsin D, and calpains activities. HPP also caused noticeable modifications in sarcoplasmic proteins profiles (SDS-PAGE and IEF) accompanying pressure level and holding time, which might be due to denaturation, fragmentation, and aggregation of proteins. This study brought new evidence about the effect of HPP in sarcoplasmic protein profiles and in the activity of degradative enzymes, which might be useful to control the softening of fish muscle tissues due to autolytic reactions. Nevertheless, further studies are still needed to understand the effect of HPP treatments in the quality attributes of sea bass fillets during refrigerated storage.

AUTHOR INFORMATION

Corresponding Author

*Tel.: +351 213 027 025. Fax: +351 213 015 948. E-mail: barbara.p.b.teixeira@gmail.com.

Funding

B.T. and A.M. acknowledge the Portuguese Foundation for Science and Technology (FCT) and the European Social Fund (FSE) for supporting a Ph.D. grant (ref SFRH/BD/44254/2008) and a Research contract (Programa Ciência 2008), respectively.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Patterson, M. F.; Linton, M.; Doona, C. J. Introduction to high pressure processing of foods. In *High Pressure Processing of Foods*; Doona, C. J., Feeherry, F. E., Eds.; Blackwell Publishing and the Institute of Food Technologists: Ames, IA, 2007; pp 1−14.

(2) Chapleau, N. J.; Lamballerie-Anton, M. I. d. Changes in myofibrillar proteins interactions and rheological properties induced by high-pressure processing. *Eur. Food Res. Technol.* **2003**, *216*, 470−476.

(3) Fellows, P. J. *Food Processing Technology: Principles and Practice*, 2nd ed.; Woodhead Publishing in Food Science and Technology, CRC Press: England, 2000; p 575.

(4) Nielsen, M. K.; Nielsen, H. H. Seafood enzymes. In *Food Biochemistry and Food Processing*; Hui, Y. H., Nip, W., Nollet, L. M. L., Paliyath, G., Simpson, B. K., Eds.; Blackwell Publishing: Ames, IA, 2006; pp 379−400.

(5) Gopakumar, K. Enzymes and enzyme products as quality indices. In *Seafood Enzymes − Utilization and Influence on Postharvest Seafood Quality*; Haard, N. F., Simpson, B. K., Eds.; Marcel Dekker Inc.: Basel, Switzerland, 2000; pp 337−363.

(6) Lakshmanan, R.; Patterson, M. F.; Piggott, J. R. Effects of high-pressure processing on proteolytic enzymes and proteins in cold-smoked salmon during refrigerated storage. *Food Chem.* **2005**, *90*, 541−548.

(7) Chéret, R.; Delbarre-Ladrat, C.; Lamballerie-Anton, M. d.; Verrez-Bagnis, V. High-pressure effects on the proteolytic enzymes of sea bass (*Dicentrarchus labrax* L.) fillets. *J. Agric. Food Chem.* **2005**, *53*, 3969−3973.

(8) Jiang, S.-T. Enzymes and their effects on seafood texture. In *Seafood Enzymes − Utilization and Influence on Postharvest Seafood Quality*; Haard, N. F., Simpson, B. K., Eds.; Marcel Dekker Inc.: Basel, Switzerland, 2000; pp 411−450.

(9) Ayala, M. D.; Albors, O. L.; Blanco, A.; Alcázar, A. G.; Abellán, E.; Zarzosa, G. R.; Gil, F. Structural and ultrastructural changes on muscle tissue of sea bass, *Dicentrarchus labrax* L., after cooking and freezing. *Aquaculture* **2005**, *250*, 215−231.

(10) Ohmori, T.; Shigehisa, T.; Tagi, S.; Hayashi, R. Biochemical effects of high hydrostatic pressure on the lysosome and proteases involved in it. *Biosci. Biotechnol. Biochem.* **1992**, *56*, 1285−1288.

(11) Barrett, A. J.; Kirschke, H. Cathepsin B, cathepsin H, and cathepsin L. In *Methods in Enzymology. Proteolytic enzymes, Part C*; Lorand, L., Ed.; Academic Press: New York, 1981; pp 535−561.

(12) Anson. The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *J. Gen. Physiol.* **1938**, *22*, 78−89.

(13) Sigma-Aldrich, Enzymatic assay of lipase (EC 3.1.1.3) (olive oil as substrate). Sigma Aldrich Chemie GmbH, http://www.sigmaaldrich.com, 1999.

(14) Sasaki, T.; Kikuchi, T.; Yumoto, N.; Yoshimura, N.; Murachi, T. Comparative specificity and kinetic studies on porcine calpain I and calpain II with naturally occurring peptides and synthetic fluorogenic substrates. *J. Biol. Chem.* **1984**, *259*, 12489−12494.

(15) Silva, H.; Mendes, R.; Nunes, M.; Empis, J. Protein changes after irradiation and ice storage of horse mackerel (*Trachurus trachurus*). *Eur. Food Res. Technol.* **2006**, *224*, 83−90.

(16) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(17) Marcos, B.; Kerry, J. P.; Mullen, A. M. High pressure induced changes on sarcoplasmic protein fraction and quality indicators. *Meat Sci.* **2010**, *85*, 115−120.

(18) Tahergorabi, R. Seafood proteins. In *Handbook of Food Proteins*; Phillips, G. O., Williams, P. A., Eds.; Woodhead Publishing Ltd.: Sawston, Cambridge, UK, 2011; pp 116−149.

(19) Gill, T. A. Chemical and biological indices in seafood quality. In *Quality Assurance in the Fish Industry*; Huss, H. H., Jacobsen, M., Liston, J., Eds.; Elsevier: Amsterdam, 1992; pp 337−387.

(20) Chéret, R.; Hernández-Andrés, A.; Delbarre-Ladrat, C.; Lamballerie, M. d.; Verrez-Bagnis, V. Proteins and proteolytic activity changes during refrigerated storage in sea bass (*Dicentrarchus labrax* L.) muscle after high-pressure treatment. *Eur. Food Res. Technol.* **2006**, *222*, 527−535.

(21) Mommsen, T. P. Salmon spawning migration and muscle protein metabolism: the August Krogh principle at work. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **2004**, *139*, 383−400.

(22) Hasan, F.; Shah, A. A.; Hameed, A. Methods for detection and characterization of lipases: A comprehensive review. *Biotechnol. Adv.* **2009**, *27*, 782−798.

(23) López-Amaya, C.; Marangoni, A. G. Lipases. In *Seafood Enzymes − Utilization and Influence on Postharvest Seafood Quality*; Haard, N. F., Simpson, B. K., Eds.; Marcel Dekker Inc.: Basel, Switzerland, 2000; pp 121−146.

(24) Gómez-Estaca, J.; López-Caballero, M. E.; Gómez-Guillén, M. C.; Lacey, A. L. d.; Montero, P. High pressure technology as a tool to obtain high quality *carpaccio* and *carpaccio*-like products from fish. *Innovative Food Sci. Emerging Technol.* **2009**, *10*, 148−154.

(25) Ramirez-Suarez, J. C.; Morrissey, M. T. Effect of high pressure processing (HPP) on shelf life of albacore tuna (*Thunnus alalunga*) minced muscle. *Innovative Food Sci. Emerging Technol.* **2006**, *7*, 19−27.

(26) Nayak, J.; Nair, P. G. V.; Ammu, K.; Mathew, S. Lipase activity in different tissues of four species of fish: rohu (*Labeo rohita* Hamilton), oil sardine (*Sardinella longiceps* Linnaeus), mullet (*Liza subviridis* Valenciennes) and Indian mackerel (*Rastrelliger kanagurta* Cuvier). *J. Sci. Food Agric.* **2003**, *83*, 1139−1142.

(27) Delbarre-Ladrat, C.; Chéret, R.; Taylor, R.; Verrez-Bagnis, V. Trends in *postmortem* aging in fish: understanding of proteolysis and disorganization of the myofibrillar structure. *Crit. Rev. Food Sci. Nutr.* **2006**, *46*, 409−421.

(28) Chéret, R.; Delbarre-Ladrat, C.; Verrez-Bagnis, V.; Lamballerie, M. d., Effect of high pressure on the calpain−calpastatin system in fish muscle. *J. Food Sci.* **2007**, *72*, C313−C316.
(29) Chéret, R.; Chapleau, N.; Delbarre-Ladrat, C.; Verrez-Bagnis, V.; Lamballerie, M. d., Effects of high pressure on texture and microstructure of sea bass (*Dicentrarchus labrax* L.) fillets. *J. Food Sci.* **2005**, *70*, e477−e483.
(30) Obinata, T.; Maruyama, K.; Sugita, H.; Kohama, K.; Ebashi, S. Dynamic aspects of structural proteins in vertebrate skeletal muscle. *Muscle Nerve* **1981**, *4*, 456−488.
(31) Gou, J.; Lee, H.-Y.; Ahn, J. Effect of high pressure processing on the quality of squid (*Todarodes pacificus*) during refrigerated storage. *Food Chem.* **2010**, *119*, 471−476.
(32) Chan, J. T. Y.; Omana, D. A.; Betti, M. Application of high pressure processing to improve the functional properties of pale, soft, and exudative (PSE)-like turkey meat. *Innovative Food Sci. Emerging Technol.* **2011**, *12*, 216−225.
(33) Hui, Y. H.; Cross, N.; Kristinsson, H. G.; Lim, M. H.; Nip, W. K.; Siow, L. F.; Stanfield, P. S., Biochemistry in seafood processing. In *Food Biochemistry and Food Processing*, 1st ed.; Hui, Y. H., Nip, W., Nollet, L. M. L., Paliyath, G., Simpson, B. K., Eds.; Blackwell Publishing: Ames, IA, 2006; pp 351−378.
(34) Etienne, M.; Jérôme, M.; Fleurence, J.; Rehbein, H.; Kündiger, R.; Mendes, R.; Costa, H.; Martínez, I. Species identification of formed fishery products and high pressure-treated fish by electrophoresis: a collaborative study. *Food Chem.* **2001**, *72*, 105−112.