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Effect of High Pressure on the Calpain–Calpastatin System in Fish Muscle

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Abstract:

Calpains (calcium-activated neutral proteases) of sea bass (Dicentrarchus labrax L.) muscle may participate in the degradation of muscle tissue during postmortem storage. These enzymes are regulated by calpastatin, their endogenous specific inhibitor. The objective of this study was to evaluate the changes encountered by the calpain system during the postmortem storage of fish muscle after high-pressure treatment. From 100 MPa, high-pressure treatment of purified calpains results in a loss of their activity as well as in the dissociation of the heterodimeric form. In muscle, the high-pressure processing decreases the initial activity of calpain. This loss in activity may be due to an inactivation by a change of structure. Initial calpastatin activity is not modified by the high-pressure treatment, but it decreases during the storage from the beginning for a treatment at 300 MPa after which calpastatin is stable during 2 d. Therefore, this study also suggests that high-pressure treatment could be a useful way to improve fish flesh quality.

Keywords: high pressure; postmortem denaturation; calpain system

Introduction

During *postmortem* storage, fish muscle undergoes degradation and the flesh quality decreases rapidly depending on the fish species. Loss of freshness, one of the most important aspects of raw fish, is often caused by a combination of physical, biochemical (enzymatic degradation), and microbiological reactions. Calpains are the enzymes often described to be involved in *postmortem* tenderization (Jiang 2000). The calpains (EC 3.4.22.17) are intracellular endopeptidases requiring calcium for their enzymatic activity. These proteases are ubiquitously distributed in cells of various organisms (insects, plants,etc), but some isoforms are tissue-specific. Two isoforms are widely present in muscle: µ-calpain (requiring 5-50 µM Ca^{2+} to be activated) and m-calpain (150-1000 µM Ca^{2+}). In sea bass muscle (*Dicentrarchus labrax* L.), 3 different isoforms have been identified and are differently expressed according to the season (Ladrat and others 2000). The calpains are heterodimers composed of a large subunit and a small subunit with a molecular weight of about 80 kDa and 28 kDa, respectively. The large subunit has a catalytic role and the small subunit a regulatory role. In the presence of calcium ions, the heterodimer is dissociated and also autolyzed (self-digested). Calpain is active as the dissociated form (Saido and others 1994; Goll and others 2003), although this is a controversial subject (Saido and others 1994; Bessière and others 1999b; Goll and others 2003). Moreover, these enzymes are regulated by an endogenous specific inhibitor, calpastatin.

High-pressure treatment is an innovative food preservation technology. High-pressure is an alternative to thermal or chemical treatment. This new technology causes inactivation of pathogenic and spoilage micro-organisms in foods (Yuste and others 2001). It also exhibits other numerous advantages; among them are no vitamin C loss, fresher taste, and better appearance, as well as better textural and nutritional qualities compared to other classical processing. High-pressure processing responds to the consumer demands for safer products. On

top of that, consumers do not have negative feeling about this process compared to irradiation treatment (Deliza and others 2005). Generally, the products are treated in the range of 100 to 1000 MPa, for 5 to 20 min. High-pressure processing affects chemical bonds of molecules, and therefore may also induce modifications in water, proteins, polysaccharides and lipids. This treatment can modify the hydrostatic and electrostatic molecular interactions with important consequences for the secondary, tertiary, and quaternary structures of proteins. In fish muscle, this processing affects enzymatic activities (Angsupanich and Ledward 1998; Ashie and Simpson 1996), as well as structural proteins (Ohshima and others 1993).

The objectives of this study were to identify the effects of the pressure treatment on the calpain system of sea bass (*Dicentrarchus labrax* L.) muscle upon refrigerated *postmortem* storage, as well as to gain an insight into the behavior of the calpain system in fish flesh treated by high pressure.

Materials and Methods

Materials

Unless specified, chemicals were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). The chromatographic gels were from Amersham Biosciences (Uppsala, Sweden).

Preparation of the fish samples

Alive sea bass (*Dicentrarchus labrax* L.), 4 years old, with an average weight of 325 g and total length of 30 cm were collected from a local aquaculture farm (“Les Viviers du Gois”, Beauvoir-sur-Mer, France) and brought back alive to the laboratory. Fish were killed by decapitation. Dorsal white muscle was excised and skinned in *prerigor* conditions.

Preparation of sarcoplasmic proteins from sea bass muscle

Frozen minced muscle (25 g) was homogenized twice for 30 s with an Ultra Turrax T25 (IKA Labortechnik, Staufen, Germany) equipped with an 18-mm-diameter head (S 25- 18 G) in 75 mL of buffer A containing 50 mM Tris–HCl (pH 7.5), 10 mM β-mercaptoethanol, and 1 mM ethylenediaminetetraacetic acid (EDTA). After centrifugation at 10000 x g (Model GR 20.22; Jouan, France) at 10000xg for 40 min at 10 °C, the supernatant was filtered through a 0.45-µm filter (Sartorius AG, Goettingen, Germany). Three sarcoplasmic extracts were realized for each pressure treatment and for each *postmortem* storage day.

Purification of calpains from muscle

The entire procedure following a method previously decribed (Ladrat and others, 2000) was carried out at 4 °C and with an Akta basic (Amersham Biosciences, Uppsala, Sweden) chromatographic equipment. The first chromatographic column (Phenyl Sepharose, Ø 26 mm, L 10.5 cm) was balanced with equilibration buffer composed of 50% buffer A and 50% buffer B (buffer A with 1 M NaCl).

Sarcoplasmic extract (50-mL) with 0.5 M NaCl (final concentration) was directly applied to the chromatographic column. The nonabsorbed proteins, including calpastatin, the endogenous inhibitor of calpains, were washed with the equilibration buffer. The calpain active fraction was then eluted in batch with 60 ml buffer A.

The calpain active fraction eluted during the purification by hydrophobic interaction chromatography was run to the DEAE Sepharose (16 mm diameter, 16 cm long, 1.5 mL/min) in buffer A. The elution was carried out with a 500-mL 0-400 mM NaCl gradient in buffer A. Only one peak containing active calpain was observed. The calpain active fractions recoved at 300 mM NaCl elution were collected (5 ml).

High-Pressure Processing

Two sample types were submitted to high-pressure treatment: muscle fillets at fish death before *postmortem* storage at 4 °C and calpain extract. To reduce the effect of individual fish variation, both dorsal fillets of each fish (a number of 12 fish in total) were divided into portions and mixed to finally obtain 12 homogenous samples, each about 100g. Prior to pressure processing, muscle portions and calpain extracts were individually packed under vacuum in polyethylene bags (La Bovida, Le Subdray, France). High-pressure processing was carried out in a 3.5-L reactor unit (ACB Pressure Systems, Nantes, France) equipped with temperature and pressure regulatory devices. The samples were subsequently subjected to high-pressure treatment: the levels of pressurization ranged from 0.1 to 500 MPa (± 7 MPa) for 5 min. The expected high-pressure was reached at 3 $MPa.s^{-1}$, and after 5 min of treatment it was then released at 250 $MPa.s^{-1}$. Temperature of transmitting medium in the vessel was settled at 10 °C (± 5 °C). Temperature of the cooling jacket which surrounded the pressure vessel was also maintained at 10 °C during the pressure treatment. One thermocouple, K-type (0.3 mm diameter; Omega, Stamford, Connecticut, USA), positioned close to the sample allowed to follow temperature variation during treatment.

Storage of fillet

In our experiment, samples (portion of 100g) treated at 0.1, 100 and 300 MPa were stored at 4 °C from 0 to 7 days. At the end of the appropriate storage period, triplicates of 30 g each were minced, vacuum-packed and frozen at -80 °C until use for enzymatic analysis.

Determination of protein amounts

The amount of proteins were evaluated by the biuret method according to Gornall and others (1949) with bovine serum albumin solution as the standard. The values were the means of 3 measurements for each sample.

Calpain activity measurement

Calpain activity was determined at 30 °C in a 303-µL reaction mixture containing 3 µL of 0.5 M $CaCl_2$, 6 µL of 5 % CHAPS {3-[3-(cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate} and 5 µl of 20 mM synthetic fluorogenic substrate SucLT (N-succinyl-Leu-Tyr-7-amido-4-methylcoumarin) prepared in methanol. The reaction was initiated by adding 255 µL of calpain active fraction. During a 40-min reaction, fluorescence was monitored in microplate wells using the spectrophotofluorometer Fluostar Optima reader (BMG Labtech, Champigny sur Marne, France) with an excitation wavelength set at 355 nm and emission wavelength set at 460 nm. A control in which 3 µL of 0.5 M $CaCl_2$ was replaced by 3 µL of 0.5 M EDTA was also performed. Enzymatic assays were performed in triplicates and activity was expressed as mean ± standard deviation. Calpain activity was expressed as fluorescence units per min per g of muscle (Cheret and others, 2005).

Quantification of calpastatin inhibitory activity

Calpastatin inhibitory activity was measured with a calpain-active sample produced separately from a whole fish white muscle (the nonabsorbed proteins, including calpastatin), as described above. Exactly 55 µL of calpastatin sample (or buffer for the control) was mixed with 200 µL of a calpain solution purified separately from a whole fish (*Dicentrarchus labrax* L.) muscle as described above and the resulting calpain activity was measured on SucLT fluorogenic substrate as previously described. One unit of calpastatin activity was defined as the amount

which inhibits one unit of calpain activity. The values were the means of 3 measurements for each sample (Cheret and others, 2007).

Electrophoresis

Denaturating gel electrophoresis was performed according to method of Laemmli (1970) using Mini-Protean dual slab cell (Bio-Rad, Richmond, CA, USA) using 10 % (w/v) polyacrylamide as the separating gel and 5% (w/v) polyacrylamide as the stacking gel. Sample was treated at 100°C for 5 min in ¼ volume of denatured buffer (10 mM Tris-HCl pH 8, 2% SDS, 0.1 M DTT, 0.01% bromophenol blue and 1 mM EDTA).

Gel electrophoresis under native conditions (PAGE) was carried out on calpain extracts. Samples (30 µL) were prepared with 10 µL buffer composed of 10 mM Tris-HCl pH 8, 0.1 M DTT, 0.01% bromophenol blue, and 1 mM EDTA, before putting them on the gel.

Both denaturated and native electrophoresis gels were silver-stained according to Blum and others (1987).

Results and Discussion

Effect of high-pressure treatment on the calpain activity

Calpain and its specific inhibitor, the calpastatin, were separated by hydrophobic interaction chromatography before measuring calpain activity. Regarding the study on the whole muscle, the purification was performed after the pressurization of the muscle. For the extracted calpain study, the pressurization was done after its purification. In both cases, the activity of calpains decreased significantly when the level of pressure increased (Figure 1). The activity of calpain decrease for pressures higher than 100 MPa; and above 350 MPa, the high-pressure treatment induced a total loss of activity. Bessière and others (1999a, 1999b) reported that the

high-pressure treatment led to a dissociation of the two subunits of calpains. The corresponding dissociation curve according to the pressure level was similar to our experimental curves. The decrease in activity could therefore be due to the dissociation that is responsible for the instability of the dissociated form (Saido and others 1994). A slight alteration of the calpain structure could also lead to the loss of activity.

Effect of high pressure on the structure of calpain

The calpain was purified by hydrophobic interaction chromatography followed by ion exchange-chromatography. The calpain active fractions were recovered, mixed, and subsequently pressurized at different intensities between 0.1 and 400 MPa. The treated samples were studied by native and denatured electrophoresis. Figure 2 shows the effect of high-pressure treatment on the calpain native structure. For the control (0.1 MPa), only one band was observed, must likely the heterodimeric form. In the pressurized samples, the presence of 2 bands were observed, probably corresponding to the catalytic and regulatory subunits. This indicates that the calpains were dissociated above 100 MPa. The SDS-PAGE analysis (Figure 3), showed that the 2 observed bands have a molecular weight similar to each calpain subunit, confirming the proposed identification of bands.

Effect of high-pressure treatment on the calpain system during the storage

Calpain activities after high-pressure treatment were followed during the *postmortem* storage (the pressures were 0.1, 100, and 300 MPa and the durations of storage were 0, 2, 4, and 7 days) (Figure 4). For the control (0.1 MPa), the calpain activity did not show any evolution during the 7 days of *postmortem* storage. After pressurization at 100 MPa, the initial calpain activity is not affected, but the activity increased between the day 0 and the day 4 and then

195 decreased at the end of storage. After a pressure treatment at 300 MPa, the initial calpain activity
196 was very low and continued to be stable during storage. This latter treatment was apparently too
197 drastic for the calpain molecules and might lead to degradation of the ternary structure.
198 The evolution of calpastatin activity was studied during refrigerated storage after high-pressure
199 treatment of the whole muscle (Figure 5). Initial activity was not affected by pressure treatment
200 but other previous studies have also established that this protein is stable and is resistant to
201 denaturing agents such as urea, SDS, and trichloroacetic acid (Goll and others 2003). Our data
202 confirmed that the protein could also be stable after a high-pressure treatment. The calpastatin
203 activity decreased during *postmortem* storage. The observed decreased could be due to a
204 degradation of calpastatin (Mellgren and others 1986); therefore calpains could be responsible
205 for the loss of calpastatin activity since the high-pressure treatment has not effect on calpastatin
206 activity.
207
208 **Conclusion**
209 In summary, we have shown that a high-pressure treatment at 100 MPa is able to
210 dissociate the calpain molecule and to activate it in the first days of storage following the
211 pressurization. After a 300-MPa treatment, calpain is denaturated and in no longer active.
212 Calpastatin initial activity is not affected whatever the intensity of pressure applied and its
213 subsequent decrease upon storage does not depend on the pressure level either. The decrease in
214 activity during storage may be explained by a proteolytic action of the calpains on their own
215 inhibitor. If the calpain system is responsible for fish flesh *postmortem* degradation as proposed
216 by several authors, such as Jiang (2000), high-pressure treatment at 300 MPa could therefore be a
217 useful way to keep fish flesh at high quality. From a practical and economical point of view, this

technique would be preferentially possible for high value fish fillets. It may be also a good technique for the conservation of a ready to eat.

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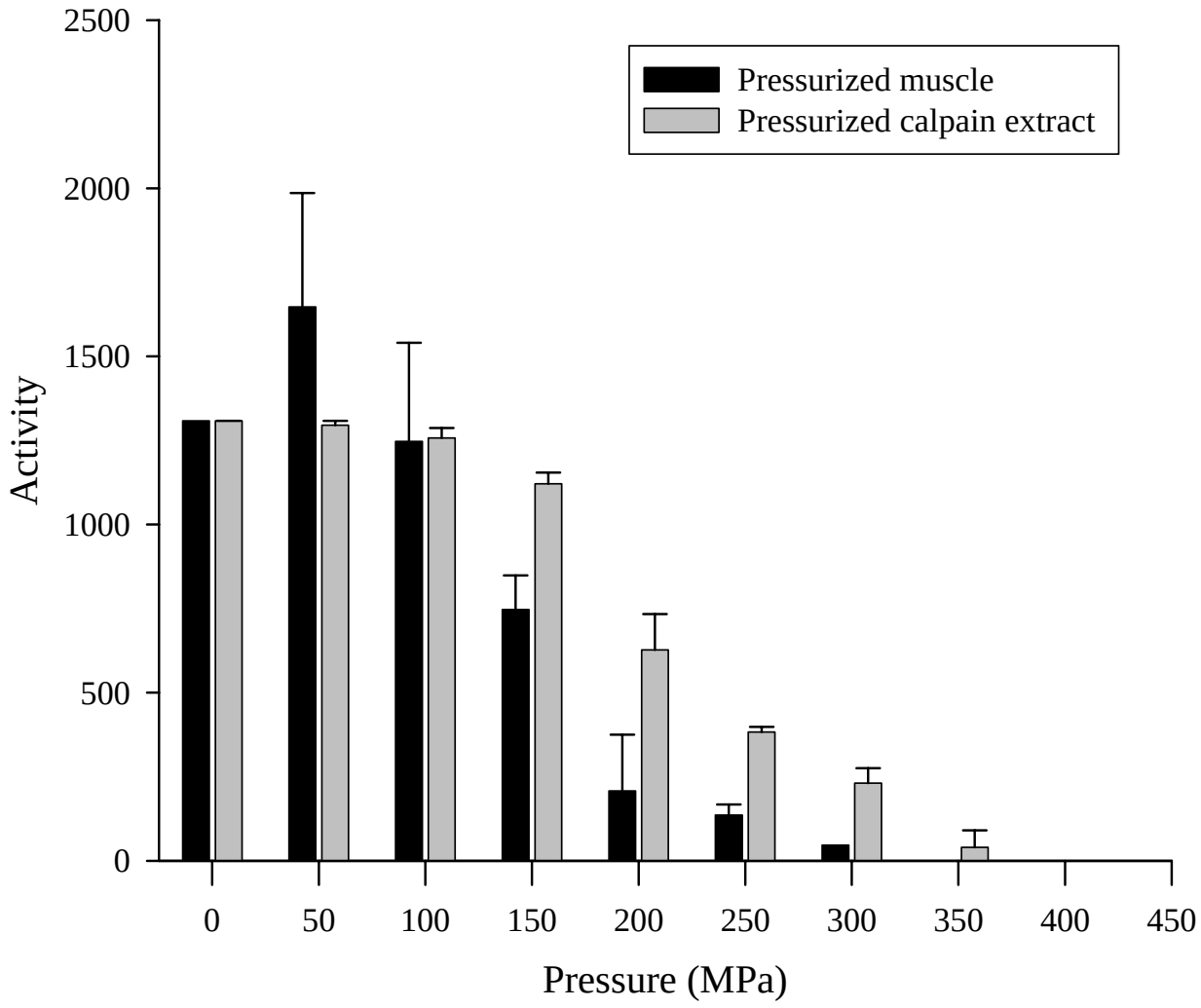
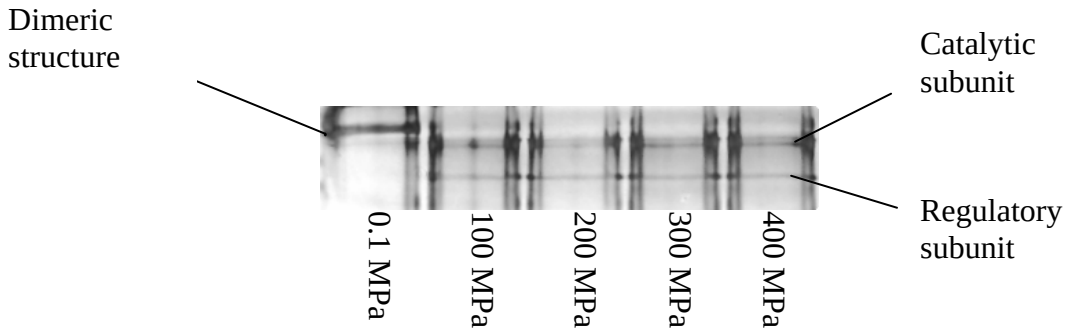


Figure.1. Evolution of calpain activity according to the pressure applied on calpain extract and on fish white muscle. Results are means of 3 samples; the vertical bars represent the standard deviation. The activities are expressed in fluorescence units per minute per g of muscle.



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270 Figure. 2. Native PAGE of calpains from fish (*Dicentrarchus labrax* L.) white muscle after high-

271 pressure treatment (0.1, 100, 300 and 400 MPa).

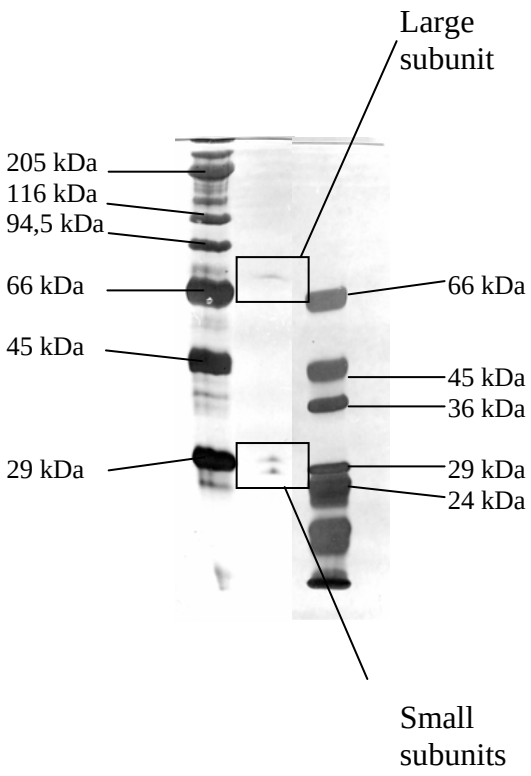
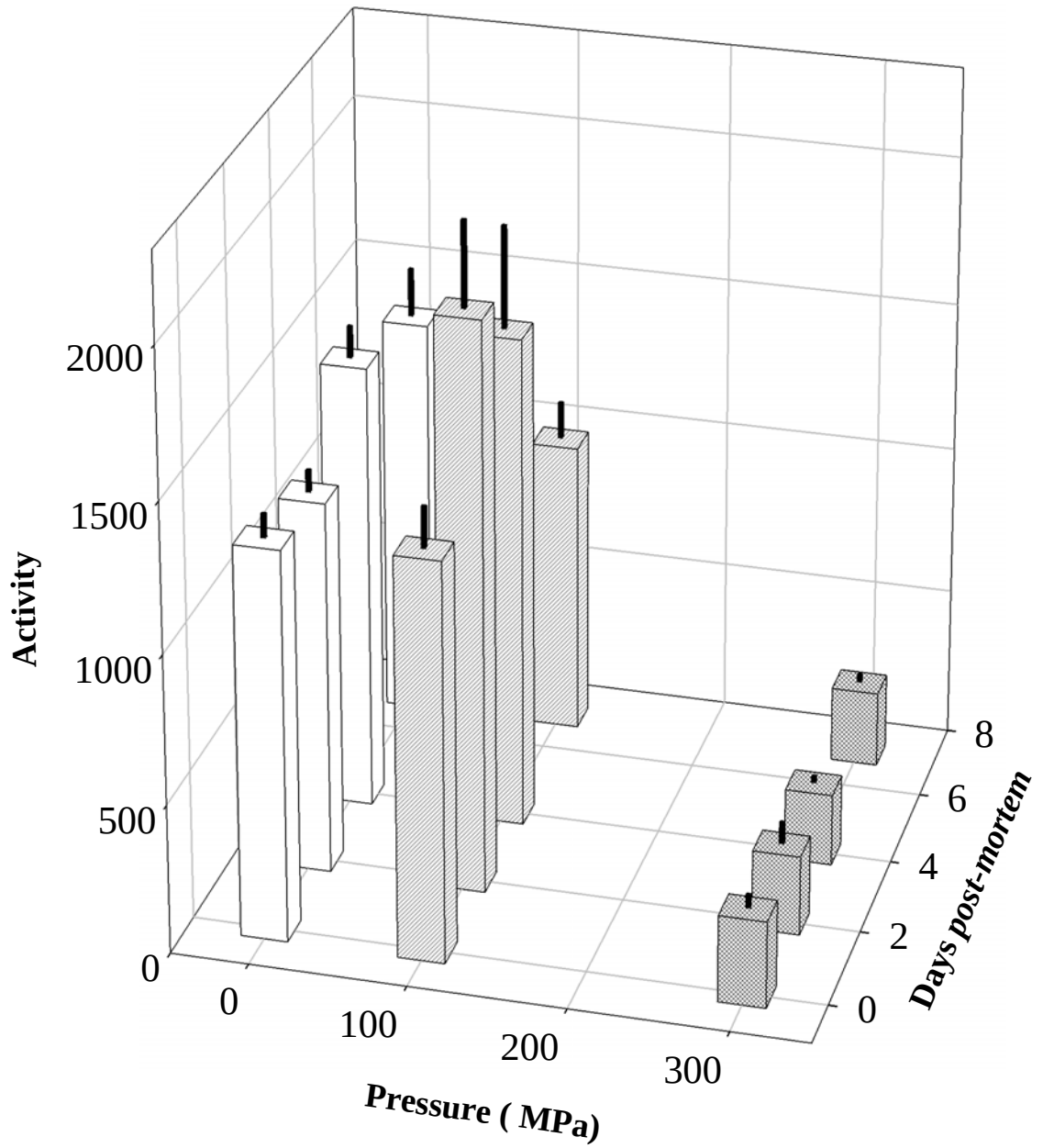


Figure. 3. Analysis of calpains by SDS PAGE from fish (*Dicentrarchus labrax* L.) white muscle after purification.



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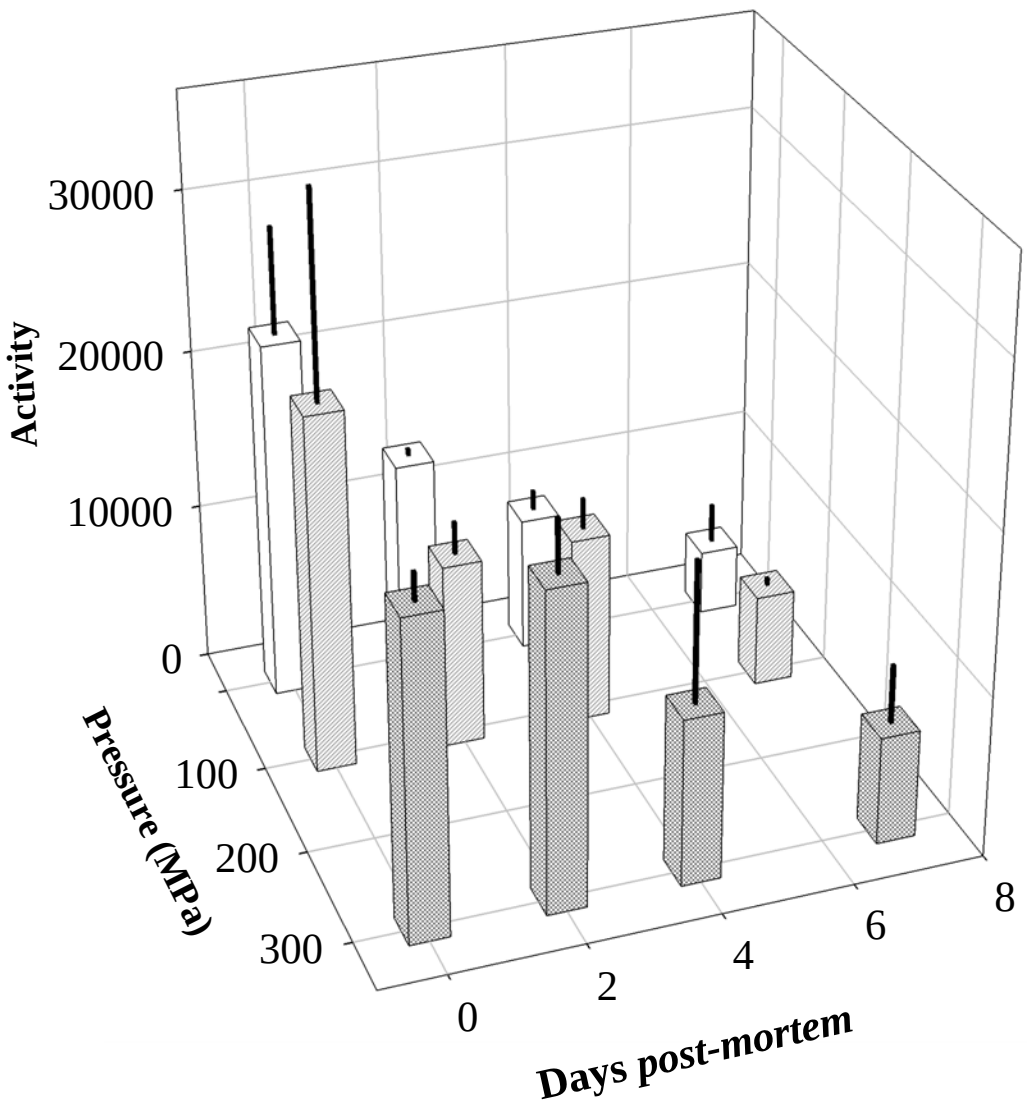
286 Figure. 4. Changes of calpain activities after 0, 2, 4, and 7 days *post-mortem* for the different
287 pressurized muscle samples: 0.1 MPa (control sample), 100 MPa, and 300 MPa. Results are
288 means (± S.D.) of 3 measurements; the vertical bars represent the standard deviation. The
289 activities are expressed in fluorescence units per minute per g of muscle.



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291 Figure. 5. Changes of the calpastatin activities after 0, 2, 4 and 7 days *post-mortem* after
292 pressurization of muscle samples at 0.1 MPa (control), 100 MPa, and 300 MPa. Results are
293 means (± S.D.) of 3 measurements; the vertical bars represent the standard deviation. One unit is
294 able to inhibit one unit (fluorescence unit per minute per g of muscle) of calpain.