European Food Research and Technology
March 2006; 222(5-6) : 527-535
http://dx.doi.org/10.1007/s00217-005-0158-z

Proteins and proteolytic activity changes during refrigerated storage in sea bass (Dicentrarchus labrax L.) muscle after high-pressure treatment

Romuald Chéret[1,2], Aránzazu Hernández-Andrés[3], Christine Delbarre-Ladrat[2], Marie de Lamballerie[1] and Véronique Verrez-Bagnis[2*]

[1]UMR CNRS 6144 GEnie des Procédés Environnement et Agroalimentaire, École Nationale d'Ingénieurs des Techniques des Industries Agricoles et Alimentaires, BP 82225, 44322 Nantes Cedex 3, France
[2]IFREMER, Rue de l'Ile d'Yeu, BP 21105, 44311 Nantes Cedex 3, France
[3]Instituto del Frío (CSIC), C/José Antonio Nováis, 10, 28040 Madrid, Spain

*: Corresponding author : veronique.verrez@ifremer.fr

Abstract: Contrary to other preservation methods like thermal treatments, high pressure can destroy microorganisms without affecting the nutritional quality, color, or food texture. The firm texture of fish flesh is an important quality parameter. During the refrigerated storage, the tissue becomes softer and the muscle is deteriorated by different proteases. The aim of this study was to study the modification of the fish muscle proteins after high-pressure treatment during the refrigerated storage and to evaluate the effect of high-pressure treatment level on the post-mortem protein changes and enzyme activities. The calpain activity decreased with the high-pressure treatment and evolved differently during the refrigerated storage, depending on the level of pressurisation. Its inhibitor, the calpastatin was not affected by high pressure, but its inhibiting potential decreased during the post-mortem storage. The activities of cathepsins were modified by the high-pressure treatment and the time of storage, but depended according to their class. The electrophoresis profiles showed that sarcoplasmic proteins were modified according both the high-pressure treatment and the period of storage. For the myofibrillar proteins, the only changes were due to the high-pressure treatment.

Keywords: Fish - High pressure - Post-mortem denaturation - Myofibrillar proteins - Sarcoplasmic proteins - Calpains - Cathepsins

1. Introduction

Freshness is one of the most important aspects of raw fish which contributes the best to define the quality of fish as food. The loss of freshness is often caused by a combination of physical, biochemical and microbiological reactions. Thus, the enzymatic degradations introduce the *post-mortem* softening of fish muscle and allow a proliferation of bacterial flora.

The contributions of proteolytic systems to the fish muscle degradation have been only little clarified. Calpains and cathepsins are the enzymes often cited as they seem to be involved in *post-mortem* tenderization [1, 2]. The calpains and the cathepsins are thought to act in a synergistic way [2].

The calpains (EC 3.4.22.17) are intracellular endopeptidases requiring calcium for their enzymatic activity. Two isoforms are widely present in muscle: μ-calpain (5-50 μM Ca^{2+} to be active) and m-calpain (150-1000 μM Ca^{2+}). 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. Moreover, these enzymes are regulated by an endogenous specific inhibitor, the calpastatin. In sea bass muscle (*Dicentrarchus labrax* L.), three different isoforms have been identified [3]. The calpains seem to be responsible for the early beginning of the proteolytic degradation of myofibrils [4].

The cathepsins are "acid" proteases, which are embedded in organelles called lysosomes [5]; they are widely distributed in muscles and organs. After the death and during the storage of the muscle, they may be released into both the cytoplasm and the intracellular spaces as a consequence of lysosomal breakdown. These enzymes contribute to the breakdown of cells and tissues. Some cathepsins are regulated by an inhibitor, such as a cystatin for the cathepsins B and L. Among all the lysosomal proteases, cathepsins B (EC 3.4.22.1), D (EC 3.4.23.5) and L (EC 3.4.22.15) are often described to be involved in the softening of the fish muscle [1, 2].

High-pressure treatment is an innovative food preservation technology. The high-pressure is an alternative to thermal treatment or chemical preservations. There are several high-pressure processed products on the market: ham, sausages, bacon (Spain), fruit jams, fruit jellies and rice (Japan), fruit juices (France, Italy, UK, USA), guacamole, and oysters (USA). This technology allows inactivation of pathogenic and spoilage micro-organisms in foods [6]. But it exhibits also other numerous advantages. Foods have fresher taste, no vitamin C loss and better appearance, textural and nutritional qualities compared to other classical processing. On the top of that, consumers do not have a negative feeling of the process as it is the case with irradiation treatment [7]. Generally, the products are treated in the range of 100 to 1000 MPa, for 5 to 20 minutes. The high-pressure processing affects chemical bonds of molecules, and that may induce modifications of 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 the fish muscle, this processing affects enzymatic activities [8, 9] as well as structural proteins [10]. Therefore, high-pressure processing changes structural matrix of muscle and consequently the texture [8].

The aim of the study was to understand how the proteolytic enzymes behave during the refrigerated *post-mortem* storage after different high pressure treatments of fish muscle and how the sarcoplasmic and myofibrillar proteins were modified

2. Materials and methods

2. 1. Materials

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

94

95 **2.2. Preparation of the fish samples**

96 Twelve farmed fresh sea bass (*Dicentrarchus labrax* L.), 4 years old, with an average
97 weight of 325 g and total length of 30 cm were collected from a local aquaculture farm ('Les
98 Viviers du Gois', Beauvoir-sur-Mer, France) and brought back alive to the laboratory. Fish
99 were killed by decapitation, dorsal white muscle was excised and skinned in *pre-rigor*
100 conditions. To reduce the effect of fish variation, both dorsal fillets of each fish were divided
101 into portions and mixed to finally obtain out twelve homogeneous samples each of about 100
102 g.

103

104 **2.3. High-Pressure Processing**

105 High-pressure processing was carried out in a 3.5 L vessel (ACB Pressure Systems,
106 Nantes, France) equipped with temperature and pressure regulator device. Prior to pressure
107 processing, sea bass muscle samples at the day of death prepared as described above were
108 individually packed under vacuum in polyethylene bags (La Bovida, France). The samples
109 were subsequently subjected to high-pressure treatment: 4 samples at 100 MPa and 4 samples
110 at 300 MPa (± 7 MPa) for 5 minutes. The remaining four samples were studied without
111 pressurization as control. The expected high-pressure was reached at 3 MPa/s, and after 5
112 minutes was then quickly released (250 MPa/s). Temperature of transmitting medium in the
113 vessel was settled at 10°C (± 5°C). Temperature of the cooling jacket which surrounded the
114 pressure vessel was also controlled at 10 °C during pressure treatment. One thermocouple K-
115 type (0.3 mm diameter, Omega, Stamford, Connecticut, USA) positioned close to sample
116 allowed to follow temperature variation during treatment.

117

118 **2.4. Storage of fillet**

The figure 1 summarizes the planning of the experimentation. After each pressurization, the different portions of muscles were stored at 4 °C from 0 to 7 days. At the end of the appropriate storage period, three portions of 30 g in triplicates were minced and vacuum packed (triplicate samples). And finally, the minced muscle samples were frozen at -80°C until use.

2.5. Preparation of sarcoplasmic proteins from sea bass muscle

25 g of frozen minced muscle was homogenized twice for 30 s with Ultra Turrax (T25, IKA, Labortechnik, Staufen, Germany) equipped with a 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 (GR 20.22, Jouan, France) 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 different post-mortem storage days.

2.6. Preparation of myofibrillar proteins

5 g of dorsal frozen fish muscle was homogenized with 4 volumes of buffer A with Ultra Turrax (T25, IKA, Labortechnik, Staufen, Germany) (22000 rpm) during 1 min. After centrifugation at 10000 x g (GR 20.22, Jouan, France) for 40 min at 10°C, the pellet was again homogenized with 4 volumes of buffer A with Ultra Turrax (T25, IKA, Labortechnik, Staufen, Germany) (22000 rpm) during 1 min. This homogenate was centrifuged at 10000 x g (GR 20.22, Jouan, France) for 40 min at 10°C. The pellet was collected and resuspended in buffer containing 0.04 M potassium di-hydrogen phosphate, 0.04 M di-potassium hydrogen phosphate and 0.6 M potassium chloride at pH 6. The protein concentration of the different samples was adjusted to 4 mg/ml.

144

145 **2.7. Purification of calpains from muscle**

146 The whole procedure was carried out at 4°C. The chromatographic column (Phenyl
147 Sepharose, ϕ 26 mm, L 10.5 cm) was balanced with equilibration buffer composed of 50%
148 buffer A and 50% buffer B (buffer A with 1 M NaCl).
149 Fifty milliliters of sarcoplasmic extract with 0.5 M NaCl (final concentration) were directly
150 run onto the chromatographic column. The non-absorbed proteins, including calpastatin, the
151 endogenous inhibitor of calpains, were washed with the equilibration buffer. The calpain
152 active fractions were then eluted in batch with buffer A. These different protein peaks were
153 collected in ice.

154

155 **2.8. Determination of proteins**

156 The amount of proteins was evaluated by Biuret method according to Gornall et al. [11]
157 with bovine serum albumin solution as the standard. The values were the means of three
158 measurements for each sample.

159

160 **2.9. Calpain activity measurement**

161 Calpain activity was determined in triplicate at 30°C in a 303 µl reaction mixture
162 containing 3 µl of 0.5 M $CaCl_2$, 6 µl of 5 % CHAPS {3-[3-(cholamidopropyl)-dimethyl-
163 ammonio]-1-propanesulfonate} and 5 µl of 20 mM synthetic fluorogenic substrate SucLT (N-
164 Succinyl-Leu-Tyr-7-amido-4-methylcoumarin) prepared in methanol. The reaction was
165 initiated by adding 255 µl of enzymatic sample. During a fourty minutes reaction,
166 fluorescence was monitored in microplate wells using the spectro-photo-fluorometer
167 FLUOstar OPTIMA POLARstar OPTIMA reader (BMG LABTECH, Champigny sur Marne,
168 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. Activity was expressed in FU (fluorescence units) per minute per g of muscle. The values were expressed as mean ± Standard Deviation.

2.10. Quantification of calpastatin inhibitory activity

Calpastatin inhibitory activity was measured with a calpain-active sample produced separately from a whole fish white muscle (the non-absorbed proteins, including calpastatin), as described above. 55 µl of calpastatin sample (or buffer for the control) was mixed with 200 µl of calpain sample 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. Calpastatin activity was expressed in FU (fluorescence units) per minute per g of muscle. The values were the means of three measurements for each sample.

2.11. Activity measurement of lysosomal enzymes

2.11.1. Cathepsin D

Cathepsin D activity was determined with hemoglobin as the substrate according to Anson's method [12]. Activity was determined at 37°C on a 8 ml reaction mixture containing 2 ml of 0.2 M acetate/ acetic acid (pH 4) buffer, 10 mM β-mercaptoethanol, 1 mM EDTA and 2 ml of 2 % (w/v) denatured hemoglobin. The reaction was initiated by adding 4 ml of sarcoplasmic protein extract and stopped by adding at different interval times 300 µl of 10% trichloroacetic acid (TCA) to 300 µl of mixture reaction sampled. After an overnight incubation at 4°C, the sample was centrifuged at 18000 × g for 15 min at 10°C. 150 µl of supernatant reacted with 150 µl of Bio-Rad Protein assay (BIO-RAD Laboratories GmbH, München, Germany) for the quantification of TCA-soluble peptides released by digestion. Absorbance was measured

spectrophotometrically at 595 nm. For each samples, blanks containing 40 µl of 0.01 M isovalerypepstatin, an effective inhibitor of cathepsin D were prepared. Cathepsin D activity was obtained by the difference with and without isovalerylpepstatin. The activity was expressed in µg/ml of peptides liberated per minute per g of muscle. The values were the means of three measurements for each sample.

2.11.2. Cathepsins B and L

B and L cathepsin activities were determined at 30°C in a 298 µl reaction mixture containing 70 µl of 0.2 mM acetate/ acetic acid (pH 4) buffer, 10 mM β-mercaptoethanol, 1 mM EDTA, 6 µl of 5 % CHAPS, 1 µl of 1.40 M 2-mercaptoethanol, 16 µl of 5 % (w/v) Brij® 35 and 5 µl of synthetic fluorogenic substrate prepared in methanol at 20 mM. Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride, Z-Phe-Arg-7-amido-4-methylcoumarin hydrochloride are used as the substrates for cathepsin B and cathepsins (B + L) respectively. The reaction was initiated by adding 200 µl of protein extract. A control with buffer A instead of enzymes was run in parallel. The activity was expressed in FU (fluorescence units) per minute per g of muscle. The values were expressed as mean ± S.D. of three measurements for each sample.

2.12. SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on myofibrillar and sarcoplasmic protein extracts according to the method of Laemmli [13]. Protein concentrations were adjusted in denaturing buffer (10 mM Tris-HCl pH 8, 2% SDS, 0.1 M DTT, 0.01% bromophenol blue and 1 mM EDTA).

Electrophoresis for the sarcoplasmic proteins were carried out using a Phast System horizontal apparatus (Amersham Biosciences, Uppsala, Sweden) using 15.5%

polyacrylamide gels (SDS-PAGE, Phast gel, Amersham Biosciences, Uppsala, Sweden). Electrophoresis conditions were 10 mA, 50 V and 3.0 W, at 15°C for 45 minutes. 5 µg of proteins were loaded into a well. Protein bands were stained with Coomassie blue.

Electrophoresis of the myofibrillar proteins were carried out in a Mini-protean dual slab cell (Bio-Rad, Richmond, CA, USA) with 10 % (w/v) polacrylamide as the separating gel and 5% (w/v) as the stacking gel. 24.5 µg of proteins were loaded into a well. Gel was stained with Coomassie blue.

Two molecular weight marker sets were used: high molecular weight range markers SDS-6H (205 kDa, 116 kDa, 97.4 kDa, 66 kDa, 45 kDa, 29 kDa) (Sigma markers, Saint Louis, USA) and low molecular weight range markers SDS-7 (66 kDa, 45 kDa, 36 kDa, 29 kDa, 24 kDa, 20 kDa, 14.2 kDa) (Sigma markers, Saint Louis, USA).

3. Results and discussion

3.1. Effect of high pressure and storage on the calpain and calpastatin activities

The figure 2 illustrates the evolution of the activity of the calpains after the high-pressure treatment through the time of *post-mortem* storage. When the muscle has not been previously submitted to high-pressure, global calpain activity was stable during seven days *post-mortem.* Conversely, Delbarre-Ladrat et al. [4] have showed a significant decline in *post-slaughter* sea bass m-calpain activity. This difference might be explained by an important inter-individual variability which is erased in our experiment. Initial calpain activity is not affected by pressurization at 100 MPa; but it increased during the first two days of storage after treatment before decreasing on the following days. The initial increase of activity could be due to a structural modification which allows a better affinity between enzyme and substrates. In the muscle pressurized at 300 MPa , the calpain activity was very low even immediately after the pressure treatment. It has been proved that the high-pressure treatment led to structural

244 modifications and a dissociation of the two calpain subunits [14, 15], which can in certain
245 cases induce a loss of activity related to a further autolysis of dissociated calpain [16]. In our
246 experiments, we observe this calpain inhibition only after a 300 MPa treatment.
247 Furthermore, as seen in figure 3, the inhibiting potential of calpastatin is slightly reduced
248 during *post-mortem* storage. Delbarre-Ladrat et al. [4] have shown that the calpastatin activity
249 remained rather constant during *post-mortem* storage. The calpains may be responsible for the
250 proteolytic degradation of the calpastatin and also this inhibitor loosed this activity [17]. This
251 phenomenon may explain this result. Figure 3 shows that application of pressure on white fish
252 muscle does not affect the calpastatin activity. Moreover, Goll at al. [18] studies established
253 that this inhibitor was a heat-stable protein which is resistant to denaturing agents such as
254 urea, SDS and trichloroacetic acid [18]. All these data show that calpastatin is a remarkably
255 stable protein including under high pressure treatment.
256
257 **3.2. Effect of high pressure and storage on the cathepsins activities**
258 The figures 4, 5 and 6 show the evolution, respectively, of cathepsins D, B and (B+L)
259 activities upon the refrigerated storage following high-pressure treatment. As for the calpains,
260 significant modifications were observed according to the high-pressure treatment and the
261 period of storage at 4°C. First of all the activity of day 0 increased with the pressure level. For
262 the three cathepsins, this increase is probably linked to the damaging of the lysosomal
263 membrane: the physical constraints generated by the high-pressure treatment lead to the
264 rupture of membrane and consequently to a release of these enzymes [19, 20].
265 During the refrigerated storage, the activity of the cathepsin D increased for the control
266 samples: the cathepsins are released during the storage because the lysosomal membrane
267 damaged naturally. The liberation of the proteases was often explained by a fall of the pH
268 [21], but in our study, the pH did not evolve significantly (data not shown). During the *rigor*

development, the ATP stores drop. This loss of ATP provokes a failure of ionic membranous pumps and finally leads to a damaging of lysosomal membrane, as described by Hopkins (2000) relayed by Sentandreu et al. [22].

For the pressurized samples, the activity of cathepsin D increased at the 2nd day of storage, then diminished at the 7th day. At the 7th day, at the loss final cathepsin D activity increased with the level of pressure. High-pressure treatment induces an important release of the proteases from lysosomes, but probably provokes a partial denaturation of the cathepsin D structure.

During the storage, the activity of cathepsin B decreased at the 2nd day of storage and then, the activity remained constant for the five remaining days of storage. For the cathepsins (B+L), the activity decreased lightly after two days of storage and then remained also stable. For both enzymes, the initial activity increases with the high pressure treatment; this shows the destruction of lysosomes.

3.3. Effect of high-pressure and storage on the sarcoplasmic and myofibrillar proteins.

The figure 7 A and B present whole sarcoplasmic and myofibrillar proteins in the dorsal white fish muscle. The sarcoplasmic proteins are mainly composed of enzymes which play a role in the energy-producing metabolism [23]. 12 major bands are present in the electrophoretic profiles and are also described by Nakagawa et al. [24]. The different proteins bands can be listed: 97 kDa, a doublet at 60 kDa, 51 kDa, a 41-39 kDa huge bands. These last were probably, respectively, creatine kinase and aldolase [24]. The 36 kDa components assumed to be glyceraldehydes-3-phosphate dehydrogenase. The supplementary bands were noticed at 34, 27, 25, 21.5 and 17 kDa. Two small proteins bands, at 13 and 12 kDa, were also observed.

294 On the electrophoresis of myofibrillar proteins, 8 bands were mainly observed: myosin (at
295 about 200 kDa), α-actinin, desmin, actin, tropomyosin, 32 kDa band (this band may result
296 from the degradation of tropomyosin by cathepsin B and L [25]), 22 and 16 kDa bands.
297 SDS-PAGE profiles of sarcoplasmic proteins after different high-pressure treatments and
298 different times of storage are show**n** on figures 8A and 8B. Some bands present different
299 intensities. The proteins of 21.5, 51 and 97 kDa molecular weight are more intense with the
300 high-pressure treatment. Therefore the sarcoplasmic proteins are more efficiently extracted
301 with the increased pressure of the treatment. At the death time, for the control, the bands
302 (doublet at 20.5 kDa and 30.5 kDa) are not present, but they appeared after two days of
303 storage. After the high-pressure treatment, the sarcoplasmic proteins remain stable during the
304 *post-mortem* storage. Several studies showed that the major sarcoplasmic proteins are
305 conserved during the storage [25, 26].
306 On the SDS-PAGE profiles on the figures 9A and 9B, it can be observed that the
307 myofibrillar proteins do not change during the storage. The only changes are due to the high-
308 pressure treatment. The profiles are different for the sample treated at 300 MPa. The myosin
309 heavy chain (MHC), the doublet (nearby 150 kDa), the troponin-T (37 kDa), the 32 kDa
310 protein and the 20 kDa protein are less intense and also some bands disappeared in a time
311 dependent way. These observations can be explained by a denaturation of the myofibrillar
312 proteins or a modification of their structure with the high pressure, which could modify their
313 extractability.
314

315 **4. Conclusions.**

316 Our results have shown that the activities of the calpains were diminished in contrast to
317 those of cathepsin which increased by a high-pressure treatment. But no significant changes
318 on the myofibrillar studied proteins during the storage are noticed. Electrophoresis are run

under denaturing conditions, and may not allow detection of all the modifications. If the native structures of the proteins are changed, it is not observable in our experiments. The studies on the bigger myofibrillar proteins such as titin, nebulin or extracellular proteins such as collagen, elastin should also be considered in order to gain a further insight in pressure induced modification of muscular proteins.

5. Literatured cited .

1. Aoki T, Ueno R (1997) Food Res Int 30 (8): 585-591

2. Jiang ST (2000) Food Sci Agric Chem 2(2): 55-74

3. Ladrat C, Chaplet M, Verrez-Bagnis V, Noël J, Fleurence J (2000) Comp Biochem Physiol B 125 (1): 83-95

4. Delbarre-Ladrat C, Verrez-Bagnis V, Noël J, Fleurence J (2004) Food Chem 84 (3): 441-446

5. Goll DE, Otsuka Y, Nagainis PA, Shannon JD, Sathe SK, Muguruma M (1983) muscle and J Food Biochem 7: 137-141

6. Yuste J, Capellas M, Pla R, Fung DYC, Mor-Mur MM (2001) J Rapid Meth Automation Microbiol 9: 1–10

7. Deliza R, Rosenthal A, Abadio FBD, Silva CHO, Castillo C (2005) J Food Eng 67(1-2): 241-246

8. Angsupanich K, Ledward DA (1998) Food Chem 63 (11): 39–50

9. Ashie INA, Simpson BK (1996) Food Res Int 29 (5–6): 569–575

10. Ohshima T, Ushio H, Koizumi C (1993) Trends Food Sci Technol 4: 370–375

11. Gornall AG, Bardawill CJ, David MM (1949) The Journal of Biological Chemistry 177: 751-766

12. Anson ML, (1938) J Gen Physiol 22: 79-89

344 13. Laemmli UK (1970) Nature 227 : 680-685
345 14. Bessière P, Bancel F, Cottin P, Ducastaing A (1999) Biochem Mol Biophys 47(1): 25-35
346 15. Bessière P, Cottin P, Balny C, Ducastaing A, Bancel F (1999) Biochim Biophys Acta
347 1430: 254-261
348 16. Saido TC, Sorimachi H, Suzuki K (1994) FASEB J 8: 814-822.
349 17. Mellgren RL, Mericle MT, and Lane RD (1986) Arch Biochem Biophys 246(1): 233-239
350 18. Goll DE, Thompson VF, Li H, Wei W, Cong J (2003) Physiol Rev 83: 731-801
351 19. Ohmori T, Shigehisa T, Taji S, Hayashi R (1992) Biosci Biotech Biochem 56 (8): 1285-
352 1288.
353 20. Jung S, de Lamballerie-Anton M, Taylor RG, Ghoul M (2000) J of Agric Food Chem 48
354 (6): 2467-2471
355 21. Duston TR (1983) J Food Biochem 7: 223-245
356 22. Sentandreu MA, Coulis G, Ouali A (2002) Trends Food Sci Technol 13 (12): 400-421
357 23. Nakagawa T, Watabe S, Hashimoto K (1988) Nippon Suisan Gakkaishi 54 (6): 999-1004
358 24. Nakagawa T, Watabe S, Hashimoto K (1988) Nippon Suisan Gakkaishi 54 (6): 993-998
359 25. Ladrat C, Verrez-Bagnis V, Noel J, Fleurence J (2003) Food Chem 81: 517-525.
360 26. Verrez-Bagnis V, Ladrat C, Noelle J, Fleurence J (2002) J Sci Food Agric 82: 1256-1262
361

362 **Acknowledgement**

363 The authors thank the Comunidad de Madrid (CAM) for Ms. Hernández´ predoctoral
364 fellowship.

6. Figure captions

Figure.1. Simplistic representation of experiments.

Figure.2. Evolution of calpain activities after 0, 2, 4 and 7 days *post-mortem* for the different samples pressurized: 0.1 MPa (□), 100 MPa (▨), and 300 MPa (▩). Results are means (± S.D.) of three measurements; the vertical bars represent the standard deviation. The activities are expressed in fluorescence units per minute per g of muscle.

Figure.3. Evolution of calpastatin activities after 0, 2, 4 and 7 days *post-mortem* for the different white fish muscle pressurized: 0.1 MPa (□), 100 MPa (▨), and 300 MPa (▩). Results are means (± S.D.) of three measurements; the vertical bars represent the standard deviation. The activities are expressed in fluorescence units per minute per g of muscle.

Figure.4. Evolution of cathepsin D activities after 0, 2, 4 and 7 days *post-mortem* for the different samples pressurized: 0.1 MPa (□), 100 MPa (▨), and 300 MPa (▩). Results are means of three measurements; the vertical bars represent the standard deviation. The activities are expressed in µg/ml of peptides liberated per minute per g of muscle.

Figure.5. Evolution of cathepsin B activities after 0, 2, 4 and 7 days *post-mortem* for the different samples pressurized: 0.1 MPa (□), 100 MPa (▨), and 300 MPa (▩). Results are means of three measurements; the vertical bars represent the standard deviation. The activities are expressed in fluorescence units per minute per g of muscle.

Figure.6. Evolution of cathepsins B+L activities after 0, 2, 4 and 7 days *post-mortem* for the different samples pressurized: 0.1 MPa (□), 100 MPa (▨), and 300 MPa (▩). Results are

means of three measurements; the vertical bars represent the standard deviation. The activities are expressed in fluorescence units per minute per g of muscle.

Figure. 7. Annotated major sarcoplasmic (A) and myofibrillar proteins (B).

Figure.8. Effect of high pressure and *post-mortem* storage on the sarcoplasmic proteins analysed by Coomassie blue SDS-PAGE: day 0 and 2: A; day 4 and 7: B.

Figure.9. Effect of high pressure and *post-mortem* storage on the myofibrillar proteins analysed by Coomassie blue SDS-PAGE: day 0 and 2: A; day 4 and 7: B.

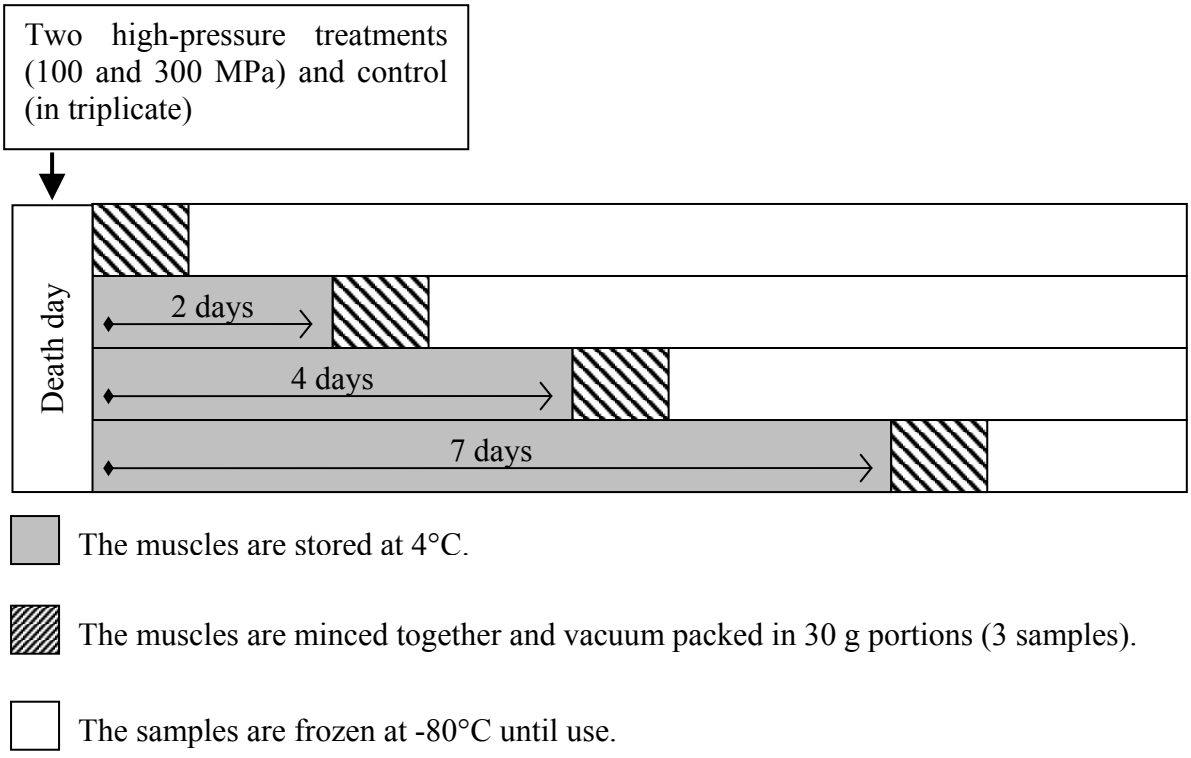

Two high-pressure treatments (100 and 300 MPa) and control (in triplicate)
Death day
2 days
4 days
7 days
The muscles are stored at 4°C.
The muscles are minced together and vacuum packed in 30 g portions (3 samples).
The samples are frozen at -80°C until use.

Fig.1.

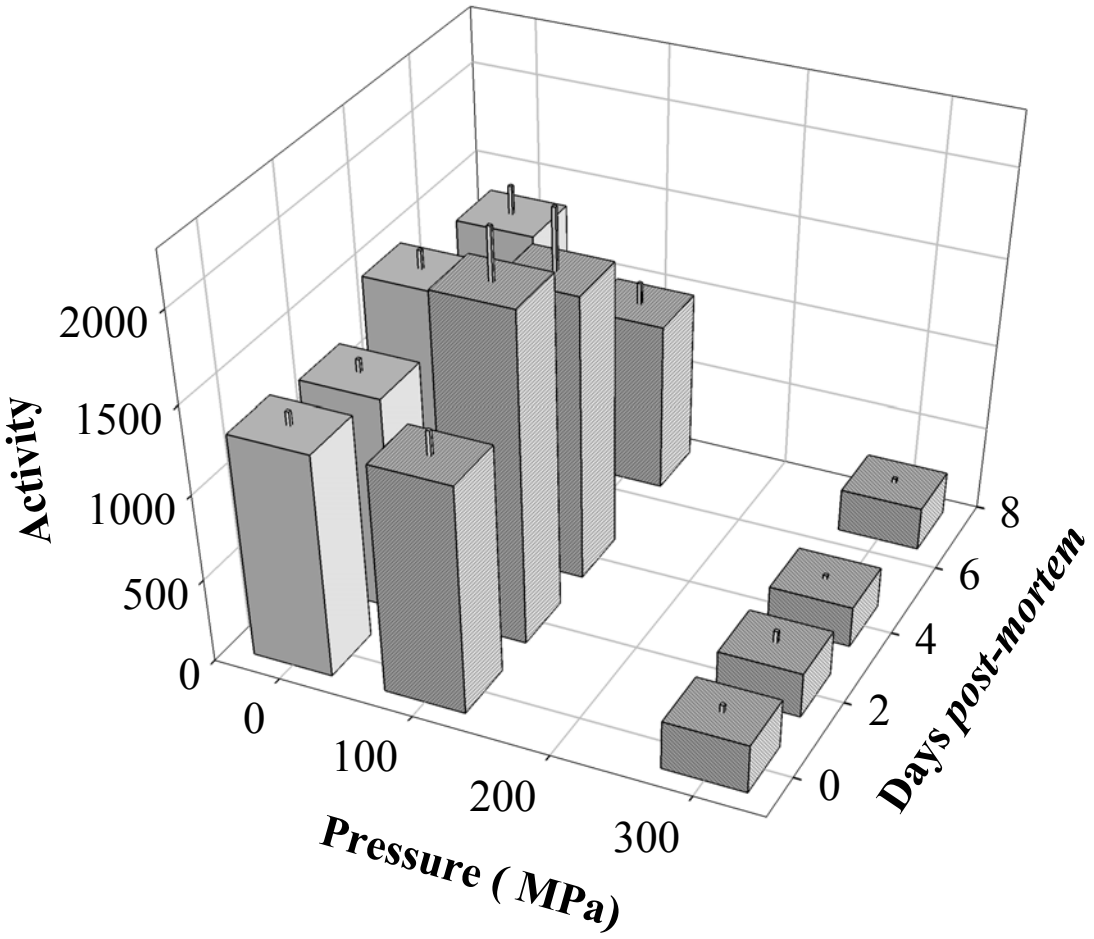

Activity
2000
1500
1000
500
0
0
100
200
300
Pressure (MPa)
0
2
4
6
8
Days post-mortem

Fig.2.

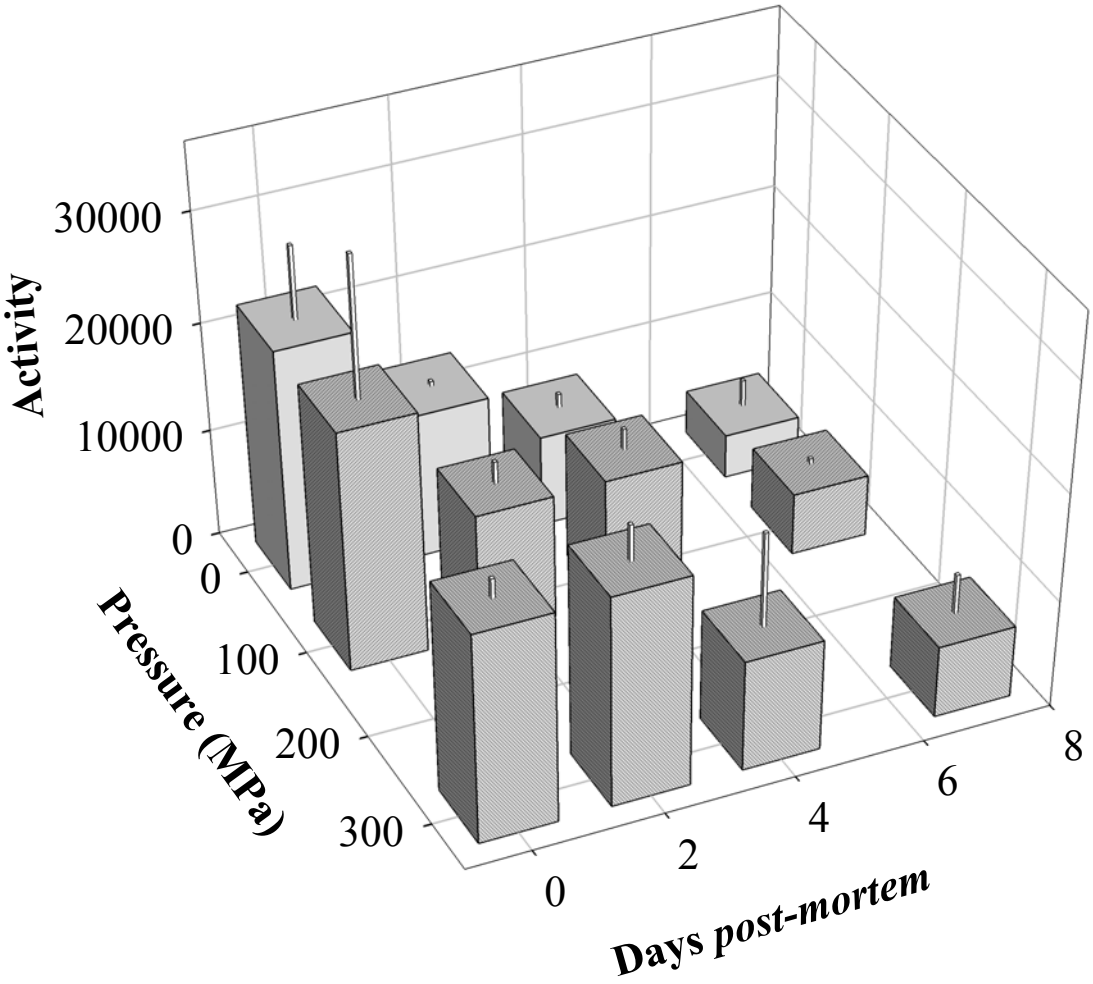

Activity
30000
20000
10000
0
Pressure (MPa)
0
100
200
300
Days post-mortem
0
2
4
6
8

Fig 3.

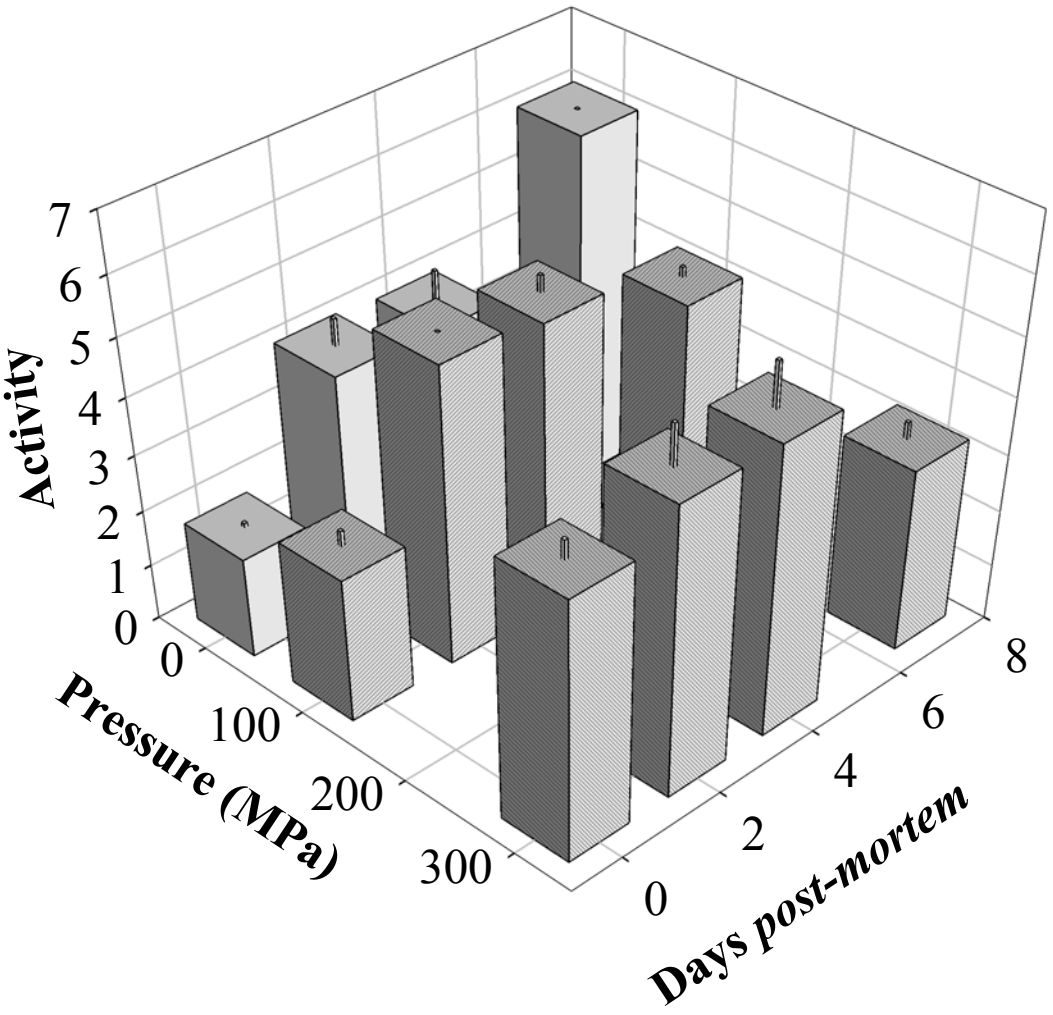

Activity
7
6
5
4
3
2
1
0
0
100
200
300
Pressure (MPa)
0
2
4
6
8
Days post-mortem

Fig.4.

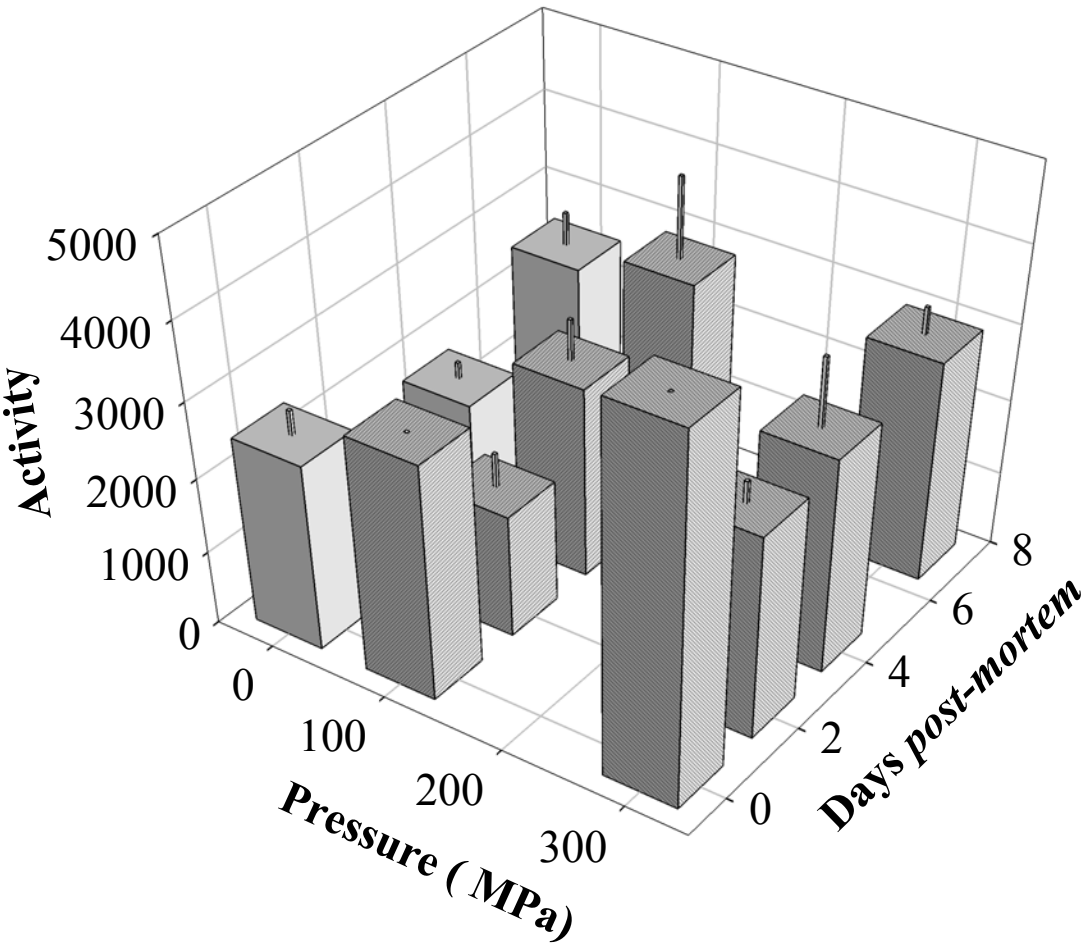

Activity
5000
4000
3000
2000
1000
0
0
100
200
300
Pressure (MPa)
0
2
4
6
8
Days post-mortem

Fig.5.

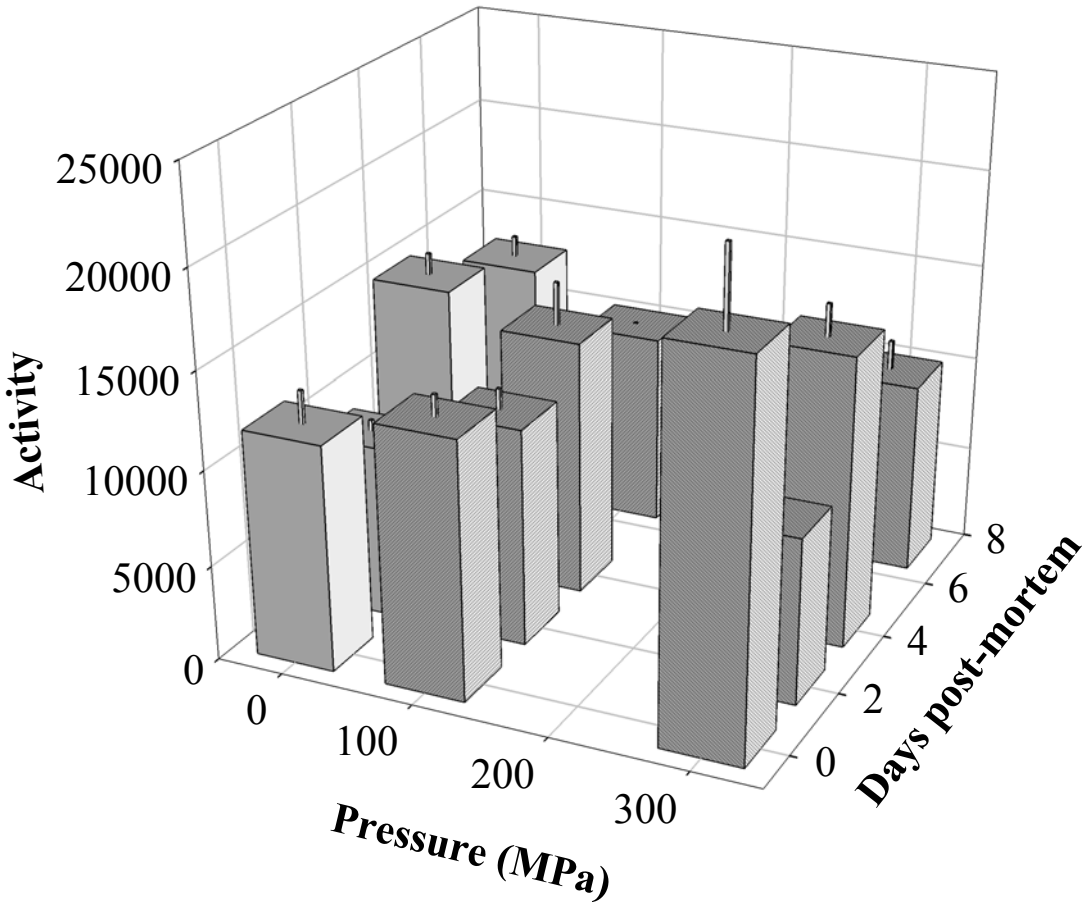

25000
20000
15000
10000
5000
0
Activity
0
100
200
300
Pressure (MPa)
0
2
4
6
8
Days post-mortem

Fig.6.

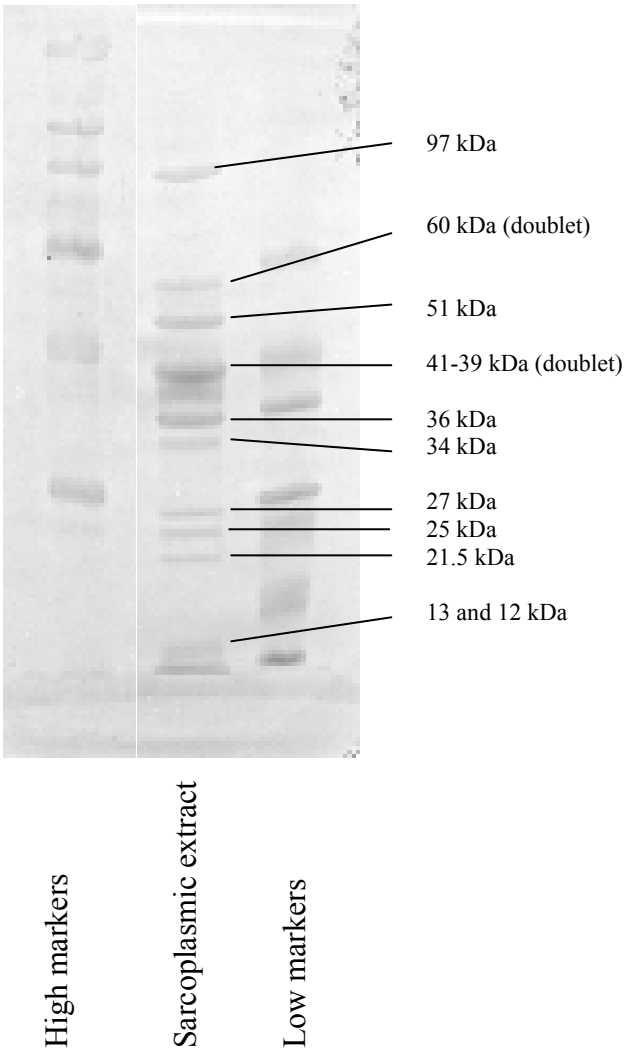

97 kDa
60 kDa (doublet)
51 kDa
41-39 kDa (doublet)
36 kDa
34 kDa
27 kDa
25 kDa
21.5 kDa
13 and 12 kDa
High markers
Sarcoplasmic extract
Low markers

Fig.7A.

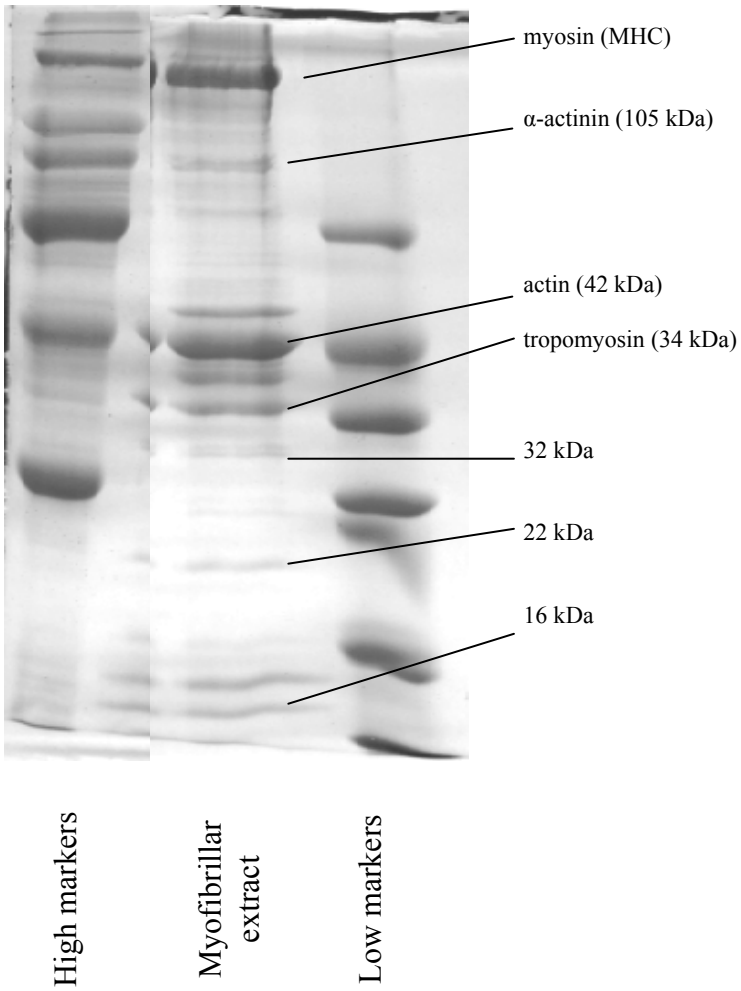

myosin (MHC)
α-actinin (105 kDa)
actin (42 kDa)
tropomyosin (34 kDa)
32 kDa
22 kDa
16 kDa
High markers
Myofibrillar extract
Low markers

Fig.7B.

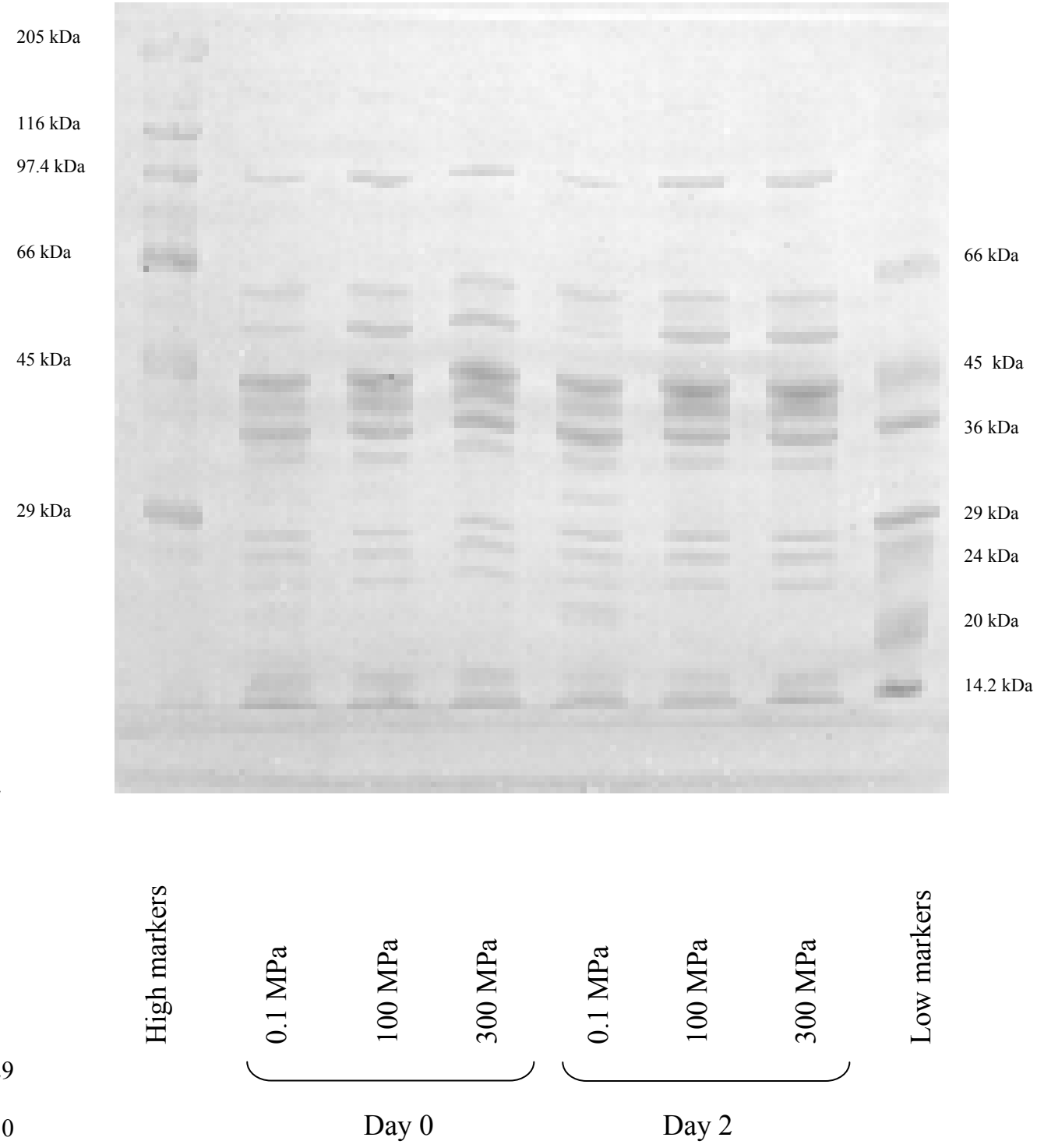
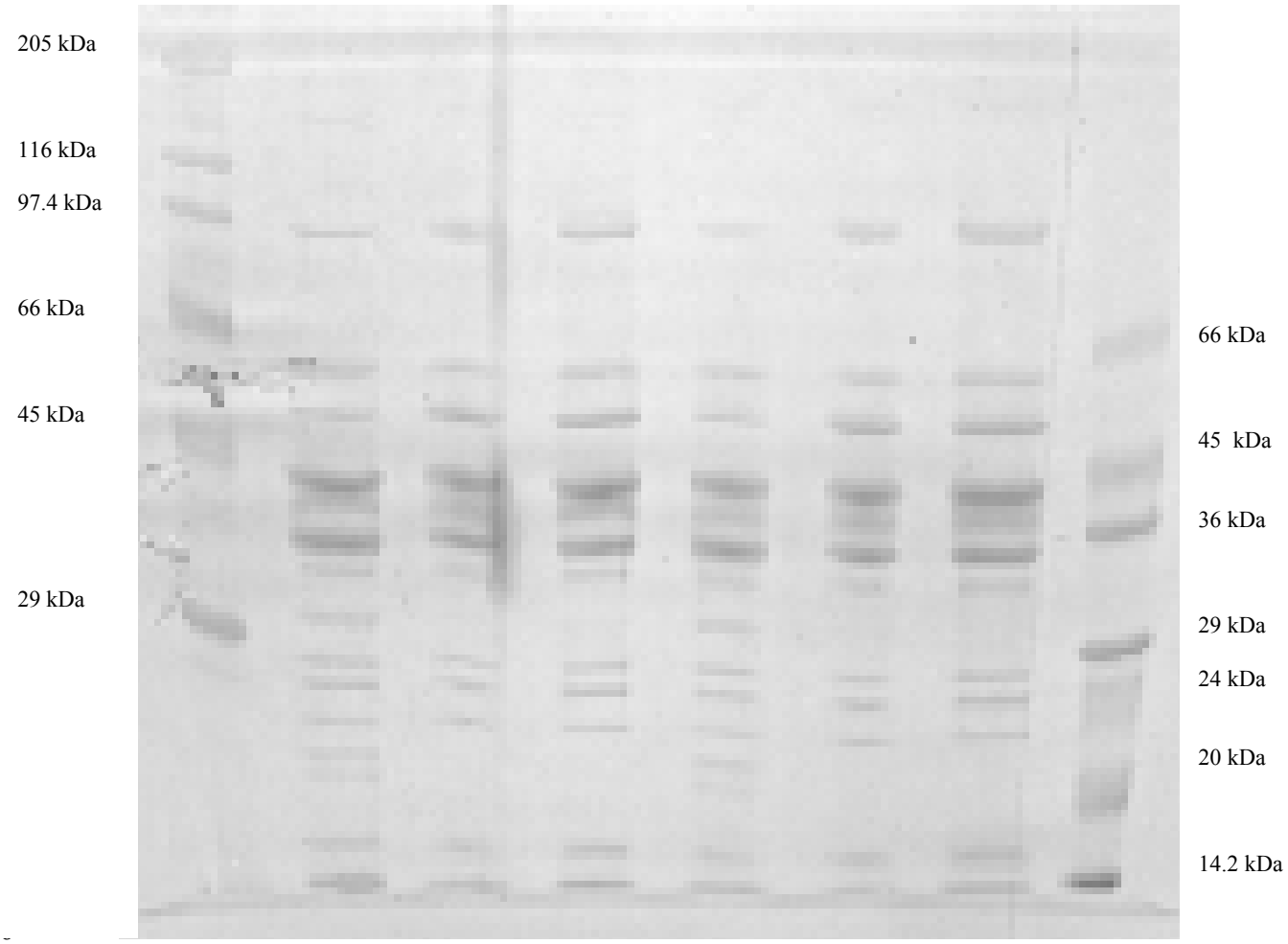

205 kDa
116 kDa
97.4 kDa
66 kDa
45 kDa
29 kDa
66 kDa
45 kDa
36 kDa
29 kDa
24 kDa
20 kDa
14.2 kDa
High markers
0.1 MPa
100 MPa
300 MPa
0.1 MPa
100 MPa
300 MPa
Low markers
Day 0
Day 2

Fig. 8A.

35



3

37

38

39 Fig. 8B.

40

41

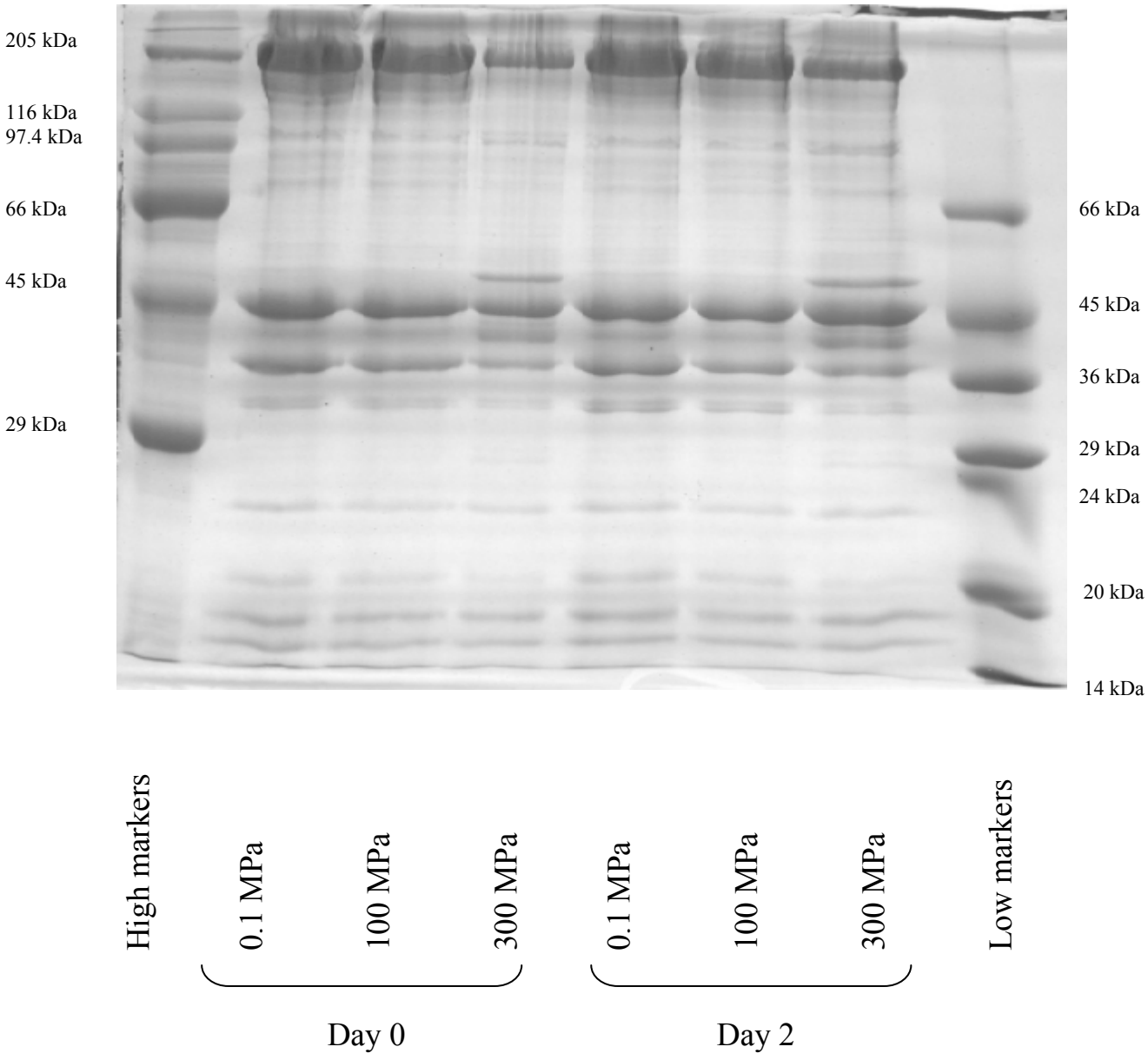

205 kDa
116 kDa
97.4 kDa
66 kDa
45 kDa
29 kDa
66 kDa
45 kDa
36 kDa
29 kDa
24 kDa
20 kDa
14 kDa
High markers
0.1 MPa
100 MPa
300 MPa
0.1 MPa
100 MPa
300 MPa
Low markers
Day 0
Day 2

Fig.9A.

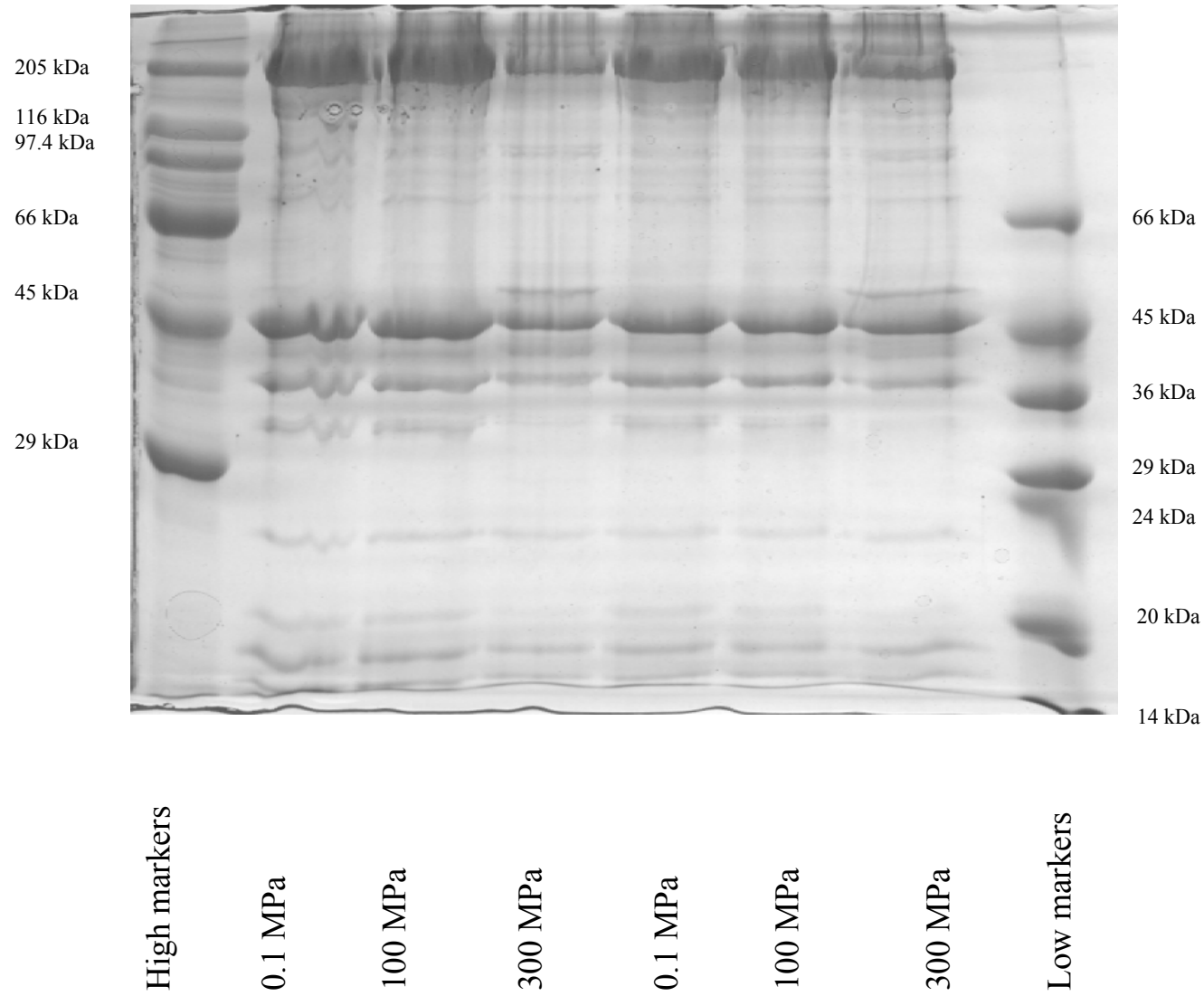

205 kDa
116 kDa
97.4 kDa
66 kDa
45 kDa
29 kDa
66 kDa
45 kDa
36 kDa
29 kDa
24 kDa
20 kDa
14 kDa
High markers
0.1 MPa
100 MPa
300 MPa
0.1 MPa
100 MPa
300 MPa
Low markers
Day 4
Day 7

Fig.9B.