

RESEARCH ARTICLE

Effects of *postmortem* storage temperature on sea bass (*Dicentrarchus labrax*) muscle protein degradation: Analysis by 2-D DIGE and MS

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Storage conditions are known to be important for postmortem deterioration of fish muscle, and temperature is one of the factors with the strongest impact on this process. In order to shed light on the influence of temperature on the status of sea bass (*Dicentrarchus labrax*) muscle proteins during postmortem storage, a 2-D DIGE and mass spectrometry study was performed on fish kept at either 1 or 18°C for 5 days. As expected, the greatest alterations in sea bass filet protein composition were observed upon postmortem storage at 18°C, with distinct changes appearing in the 2-D protein profile after 5 days of storage at this temperature. In particular, degradation of the myofibrillar protein myosin heavy chain and of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, among the most abundant muscle proteins, could be clearly observed upon storage at higher temperatures. Although to a lesser extent, however, several proteins were observed to vary in abundance also upon storage for 5 days at 1°C. In particular, one of the most interesting observations was the rapid and significant decrease in the abundance of nucleoside diphosphate kinase B and phosphoglycerate mutase 2, which was observed also at low storage temperatures and appeared to be temperature-independent. The results of this study offer new knowledge on changes occurring in sea bass muscle proteins during postmortem storage at different temperatures and provide indications on protein degradation trends that might be useful for monitoring freshness of fish and quality of storage conditions.

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1 Introduction

Freshness is considered to be the most important quality of fish products, and the development of reliable methods for its assessment is of outstanding interest in the field of food science. One of the most unfavorable changes related to fish

freshness is the postmortem tenderization of muscle, which promotes the progressive loss in quality. Therefore, the biochemical processes that are involved here have been extensively studied in order to identify the potential quality indicators or to control postmortem degradation.

Postmortem aging of fish normally follows four stages: rigor mortis, resolution of rigor, autolysis (loss of freshness), and bacterial spoilage. These stages can vary, depending upon a range of factors that include species, physiological

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Abbreviations: DIA, differential in-gel analysis; FDR, false discovery rate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MHC, myosin heavy chain; MLCs, myosin light chains

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condition of fish, stress prior to death, and temperature of postmortem storage, the last factor being the one with the strongest impact [1]. The autolytic processes catalyzed by endogenous enzymes represent the initial steps of fish muscle deterioration. Different proteolytic systems within the muscular fibers seem to be involved in the disorganization of the myofibrillar structure, including the lysosomal cathepsins, cytosolic calpains, cytoplasmatic aminopeptidases, multicatalytic proteasome, as well as some connective tissue hydrolytic enzymes [2]. Several fish muscle proteins were shown to be essentially affected by postmortem proteolysis, such as titin and nebulin [3], which anchor myofilaments to Z-disks; α -actinin, which cross-links elastic filaments to thin filaments [4]; troponin T in thin filaments; desmin, a myofibrillar packaging protein [5, 6]; dystrophin, an essential component of the costameric structure, which represents more than 5% of sarcolemma cytoskeletal proteins [7]; tropomyosin, a myofibrillar component [3, 8]; and myosin heavy chain (MHC), the large protein component of striated muscle myofibrils [9].

Aquaculture is currently contributing nearly half of the fish consumed by the human population worldwide. This sector has grown extensively in the last 50 years and is still growing more rapidly than other animal food-producing sectors [10]. The growth of this market is leading to the steady increase in the amount of fish that is produced, stored, and transported, and new problems concerning quality issues are being generated. A higher quality is needed to maintain an elevated economical value of the aquacultured product, and the identification of markers enabling to monitor this parameter is a growing concern. The increasing importance of aquaculture is also reflected by the increasing number of proteomic studies that have been performed on commercial fish species in the recent years [2, 4, 5, 11–17].

The recent developments in cutting-edge technologies such as proteomics can provide better insight into the biochemical processes occurring postmortem in fish, thus facilitating the identification of markers of fish quality. One of the most popular methods of differential proteome analysis combines protein separation by high-resolution 2-DE with MS identification of selected protein spots [18]. Indeed, these technologies probably represent the best strategy for poorly characterized animal species: they offer a comprehensive approach for studying biochemical systems by expanding the level of investigation from a single protein to a wide range of proteins present in a cell or a tissue at once, without 'a priori' knowledge [10, 11]. Another advantage offered by the combined 2-D PAGE/MS identification approach relies on the fact that 2-DE is very well suited for studies on degradation patterns, as this is the only proteomics setup that reads proteins (and not just peptides) with a high precision. In addition to protein identification data, PAGE/MS enables to obtain information also on molecular weight and isoelectric point of the spots of interest, and to

compare these data with the theoretical parameters of the intact, unmodified protein.

Multiplexing methods such as fluorescent 2-D DIGE [12, 18, 19] represent a further improvement of this analytical strategy, since they substantially reduce the variability by displaying two or more complex protein mixtures labeled with different fluorescent dyes on the same 2-D gel [20]. In the specific field of our investigation, the implementation of the multiplexing approach enables a better comparison of protein profiles generated at different times of storage. Moreover, by loading the samples to be compared into the same gel together with an internal standard, and by subjecting them to exactly the same focusing and PAGE conditions, the 2-D DIGE approach enables robust and reliable sample-to-sample and gel-to-gel comparisons by means of dedicated software tools.

In fish, proteomic technologies have been applied primarily to model organisms, such as zebrafish (*Danio rerio*). However, many teleosts of interest in biological research and with potential application in aquaculture have unique physiological characteristics (farmed fish are species that reach a large adult size with important muscle hyperplasia) that cannot be directly investigated from the study of small laboratory fish models [12]. As a consequence, large-scale proteomic studies are being increasingly applied to farmed and wildfish species of economical relevance, such as farmed sea breams (*Sparus aurata*) [17] and wild European hake (*Merluccius merluccius*) [21].

Here, 2-D DIGE and MS/MS were used to trace changes in the protein profiles of aquacultured sea bass (*Dicentrarchus labrax*) muscle proteins over 5 days of postmortem storage at different temperatures, with the aim of identifying potential biochemical markers enabling monitoring and evaluation of fish filet quality.

2 Materials and methods

2.1 Animals and experimental protocol

The experiment was carried out at the Department of Biotechnology and Molecular Sciences of the University of Insubria (Varese, Italy) based on our previously described protocol [22]. One month before slaughter, sea bass (*D. labrax*) were randomly distributed into three tanks of 1 m³ each with 40 fish per tank, and fed Hendrix-Skretting® Power Excel feed for marine fish. The tanks were connected to a sea water recirculation system with the following water conditions: temperature 20 ± 2°C, pH 7, total ammonia < 0.2 mg/L, and dissolved oxygen level over 99% of the saturation. At the start of the experiment and after 4 days of starvation, six fish (average body weight 710 ± 157.87 g) were removed from water and sacrificed by asphyxia/hypothermia (immersion in ice-cold water at a fish:ice ratio: 2:1). Fish were then divided into two subgroups of three fish each, which were stored unguessed

for up to 5 days postmortem either in a refrigerator at 1°C or in a thermostat at 18°C. The first sampling was performed immediately after death (T0) by taking a fragment of white muscle tissue (2 × 2 × 2 cm) from the lateral–dorsal (epiaxial) muscle quadrant of each fish. The next sampling was performed similarly after 5 days of post-mortem storage at the respective temperatures. In this way, the first three fish were sampled two times: at T0 and after 5 days of storage at 1°C (T5₁) and the other three ones were sampled at T0 and after 5 days of storage at 18°C (T5₁₈). When taking the muscle fragments, about 1 cm of tissue was left between one fragment and another to avoid contamination. Furthermore, throughout the trial, fish were all kept flat on the belly to prevent contact with the dorsal walls of the container or with other fish in the group. All muscle fragments taken in this way were immediately frozen at –80°C and stored at this temperature until the proteomic analysis. All procedures were approved by the Animal Care Committee of the University of Insubria and conducted according to the guidelines of the Italian Committee on Animal Care.

2.2 Protein extraction

Proteomic experiments were carried out at the Proteomics Laboratory of Porto Conte Ricerche (Tramariglio, Alghero). Proteins were extracted from frozen muscle tissues using a TissueLyser mechanical homogenizer (Qiagen, Hilden, Germany) as reported previously [23]. A small portion (50 mg) of frozen fish muscle was minced with a sterile scalpel, placed in a 2-mL Eppendorf safe-lock tube (Eppendorf, Hamburg, Germany), and then immersed in lysis buffer (8 M urea, 2% CHAPS, and 0.5% IPG buffer; GE Healthcare, Little Chalfont, UK) at a 5% w/v ratio. Three cycles of 1.5 min at 30 cycles/s in the TissueLyser mechanical homogenizer were employed for each sample. All extracts were then clarified for 15 min at 12 000 × g at 4°C, quantified by the Bradford method (Sigma-Aldrich, St. Louis, MO, USA), tested for the quality and quantity by SDS-PAGE as described previously [23], and then stored at –80°C until analysis.

2.3 Sample labeling with DIGE fluorophores

Sixty micrograms of proteins extracted from six muscle fragments sampled at the time of death (T0) and after 5 days of postmortem storage at either 1°C (*n* = 3) or 18°C (*n* = 3) were labeled with 400 pmol *N*-hydroxysuccinimidyl-ester of cyanine dye Cy-3 and Cy5 (GE Healthcare), respectively. A pooled sample comprising equal amounts of each of the specimens in the study (T0+T5₁ and T0+T5₁₈), which served as internal standards, was labeled with Cy-2. Samples were maintained under dark conditions and on ice during 30 min for labeling. After quenching with 10 mM lysine

solution (Sigma-Aldrich), the labeled proteins of T0, T5₁, T5₁₈, and those of the internal standard were mixed in suitable combinations and adjusted to a concentration of 1%. IPG buffer (GE Healthcare) and Destreak Rehydration Solution (GE Healthcare) were added to a final volume of 450 µL (Table 1).

2.4 2-D gel electrophoresis

First-dimension isoelectric focusing (IEF) was performed using 24 cm precast IPG strips (pH 3–11 NL; GE Healthcare). Mixtures of samples (450 µL) were applied onto the strips by passive rehydration overnight at room temperature. Six strips were run together on an IPGphor equipped with the Ettan™ IPGphor™3 loading manifold (GE Healthcare). The strips were focused at 20°C for a total of about 90 000 Vh.

After IEF, the strips were sequentially incubated in a freshly prepared solution of 1% DTT and 2% iodoacetamide in 50 mM Tris-HCl (pH 8.8), 6 M urea, 20% glycerol, and 2% SDS for 10 min.

The second-dimension SDS-PAGE was conducted on six 8–16.5% polyacrylamide gradient gels, using an Ettan™ DALTwelve electrophoresis system (GE Healthcare), for 30 min at 5 W/gel and then for 5 h at 17 W, at 25°C.

2.5 Image acquisition and analysis

After 2-D electrophoresis, gels were scanned on a Typhoon Trio⁺ image scanner (GE Healthcare) at 100 µm of resolution and with the photomultiplier tube (PMT) voltage was set between 500 and 700 V. The scanned gel images were then transferred to the ImageQuant V5.2 software package (GE Healthcare), cropped, and exported to the DeCyder Batch Processor and differential in-gel analysis (DIA)

Table 1. Experimental design of sample preparation for quantitative analysis by 2-D DIGE

Gel number	Cy2	Cy3	Cy5
1	Pooled sample T0+T5 ₁	T0 sample 1	T5 ₁ sample 1
2	Pooled sample T0+T5 ₁	T0 sample 2	T5 ₁ sample 2
3	Pooled sample T0+T5 ₁	T0 sample 3	T5 ₁ sample 3
4	Pooled sample T0+T5 ₁₈	T0 sample 4	T5 ₁₈ sample 4
5	Pooled sample T0+T5 ₁₈	T0 sample 5	T5 ₁₈ sample 5
6	Pooled sample T0+T5 ₁₈	T0 sample 6	T5 ₁₈ sample 6

modules (GE Healthcare) for statistical analysis. To compare protein spots across gels, a match set was created from the images of all gels. The statistical analysis of protein level changes was performed by the DeCyder-BVA (Biological Variation Analysis, v.6.5) module. Preparative cyan-dye-labeled gels were scanned and matched with the master gel in order to assign the right correspondence for spot picking.

Calibration of the electrophoretic gels for protein molecular weight (M_r) and isoelectric point (pI) was performed by using markers for 2-DE electrophoresis (Sigma-Aldrich) and Precision Plus All Blue Protein Standards (Bio-Rad Laboratories, Hercules, CA, USA). Specifically, seven 7-cm IPG strips (pH 3–11, NL) were loaded as follows: one strip was loaded only with 2-DE markers, three strips were loaded with 100 μ g of proteins from samples T0, T5₁, and T5₁₈, and three strips were loaded with a combination of each sample with 2-DE markers. All strips were then subjected to IEF as above for a total of about 30 000 V h and to SDS-PAGE in 15% polyacrylamide gels. All samples were analyzed in the same experiment in order to ensure the uniformity of running conditions. After staining with the Simply Blue Safe Stain (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions; gels images were digitalized with Image Scanner II (GE Healthcare). Image analysis, including spot detection, matching, and calibration, was performed using the Image Master 2-D Platinum v6.0.1 software (GE Healthcare). The M_r and pI values for the respective proteome patterns were determined by interpolation with the Image Master 2-D Platinum v6.0.1 calibration tools using M_r/pI markers associated with each gel. Isoelectric points of experimental samples were then cross-checked by using the method described for Immobiline DryStrip gels in the GE Healthcare Data File no. 18-1177-60 AB.

2.6 Spot picking and in situ tryptic digestion

For protein identification, preparative 2-D PAGE gels were set up by overnight rehydration loading of 300 μ g of protein extract into 3–11 NL 24-cm IPG strips. Strips were then focused and subjected to 2-DE as described above. All blue molecular weight markers (Bio-Rad Laboratories) were also loaded on an electrode wick and run together with the isoelectrofocussed strips for calibrating molecular weight across the gel. After electrophoresis, the gel slab was subjected to mass-compatible silver staining as described previously [24]. Visible protein spots of interest were manually excised from the gels, destained with 15 mM $K_3Fe(CN)_6$ in 50 mM $Na_2S_2O_3$, washed with water, and then stored in ACN. The spots were then subjected to an O/N tryptic digestion at 37°C in 50 mM $(NH_4)HCO_3$, pH 8.0, by using trypsin in the range from 40 to 100 ng, depending on spot intensity. Peptide mixtures were then collected by squeezing with ACN and centrifugation. Peptides were

subsequently acidified with 20% TFA, dried, resuspended in formic acid 0.2%, and then stored at $-20^\circ C$.

2.7 MS/MS analysis

LC-MS/MS analysis was performed on an XCT Ultra 6340 ion trap equipped with a 1200 HPLC system and a chip cube (Agilent Technologies, Palo Alto, CA, USA). After loading, samples were concentrated and desalted at 4 μ L/min on a 40-nL enrichment column (75 μ m $\times$ 43 mm, Agilent Technologies), with 0.2% formic acid. Peptides were then fractionated on a C₁₈ reverse-phase capillary column at a flow rate of 300 nL/min, with a linear gradient of eluent B (0.2% formic acid in 95% ACN) in A (0.2% formic acid in 2% ACN) from 3–60% in 20 min.

ESI parameters were as follows: capillary voltage 1730 V; dry gas (N_2), 5.00 L/min; dry temperature, 325°C; trap drive, 100; skimmer 30 V; lens 1, -5.00 V; octopole RF amplitude, 200 Vpp; capillary exit, 90 V. The ion-trap mass spectrometer was operated in a positive-ion mode. Trap ICC smart target was 30 000 units and maximal accumulation time was 100 ms. MS/MS was operated at a fragmentation amplitude of 1.3 V, and threshold ABS was 6000 units. Scan speed was 8100 UMA/s in MS and 26 000 UMA/s in MS/MS scans. Peptide analysis was performed scanning from m/z 250 to m/z 2200 in AutoMS (n) precursor selection mode of the three most intense ions (fragmentation mass range from 100 to 2200 m/z). Dynamic exclusion was used to acquire a more complete survey of the peptides by automatic recognition and temporary exclusion (0.15 min) of ions from which definitive mass spectral data had previously acquired. Data analysis software, provided by the manufacturers, was used to analyze MS/MS spectra and to generate a peak list that was introduced in the in-house MASCOT MS/MS ion search software (Version 2.3, Matrix Science, Boston, MA, USA) for protein identification in NCBI database using the chordata (vertebrates and relatives) taxonomy. Search parameters were as follows: peptide tolerance 300 ppm, MS/MS tolerance 0.6 Da, charge state +2 and +3, enzyme trypsin, allowing one missed cleavage.

2.8 Statistical analysis

The results related to T0 and 5 days of postmortem storage at either 1°C (T5₁) or 18°C (T5₁₈) were compared and statistically evaluated using univariate analysis one-way ANOVA. One-way ANOVA was performed using the DeCyder-BVA module and applying the false discovery rate (FDR) to minimize the number of false positives. A q -value of 0.05 was specified corresponding to the lowest FDR at which the protein is called significant. Data were normalized for computing the fold changes. Protein spots with a statistically significant variation ($p \leq 0.05$), showing an over

twofold difference in volume, were selected as differentially expressed and analyzed by MS/MS.

3 Results and discussion

The ultimate freshness of fish muscle depends on the extent of proteolysis of key target proteins within muscle fibers, which takes place during the postmortem period and promotes disintegration of the muscular structure [2, 4, 7]. This process is known to proceed much faster in fish than in other muscle-based foods, such as mammalian and avian meats. Furthermore, while in pork, beef, and poultry weakening of the muscular structure is desirable as it improves tenderness, this is not the case in fish, in which 'filet tenderization' stands for 'lack of freshness', and 'low-quality product'. Given the recognized importance of storage conditions for postmortem deterioration of fish muscle, this study has focused on the storage temperature, which is considered the factor with the strongest impact in this process. Differences in the abundance of muscle proteins, due to biological postmortem processes, were studied by means of 2-D DIGE and MS/MS analysis in sea bass (*D. labrax*).

3.1 Changes in the protein profile of fish muscle after 5 days of postmortem storage at 1°C

In order to assess the modifications introduced in sea bass muscle proteins by postmortem storage, a 2-D DIGE analysis of the total muscle protein profiles at death (T0) and after 5 days of postmortem storage at 1°C (T5₁) was performed. The overlay signal of a representative map is reported in Fig. 1. In general, the 2-D profile appeared well preserved, and the extent of degradation was minimal after 5 days of storage at 1°C. Upon DeCyder analysis, spots 1–7 showed a significant quantitative variation (higher than twofold) during storage and were subjected to LC-MS/MS identification (Table 2). Spots 1, 2, 3, and 4 (Fig. 1), identified as fructose-bisphosphate aldolase A, glycogen phosphorylase, and phosphoglucosmutase-1, showed a significant increase in T5₁ with respect to T0 ($p < 0.05$), whereas spots 5, 6, and 7, identified as nucleoside diphosphate kinase B (NDP kinase B), fatty acid-binding protein, and parvalbumin, respectively, decreased at the same postmortem time (Table 2). As seen from the data presented in Table 2, NDP kinase B showed the highest abundance decrease during storage at 1°C, with an average ratio of -20.13 in T5₁ with respect to T0 ($p < 0.04$), whereas the spots identified as fructose-bisphosphate aldolase A (1 and 2) showed the highest abundance increase at the same conditions ($p < 0.03$).

The observation of an increasing spot upon storage might appear surprising. It should be taken into account, however, that the apparent increase in the abundance of a spot in the

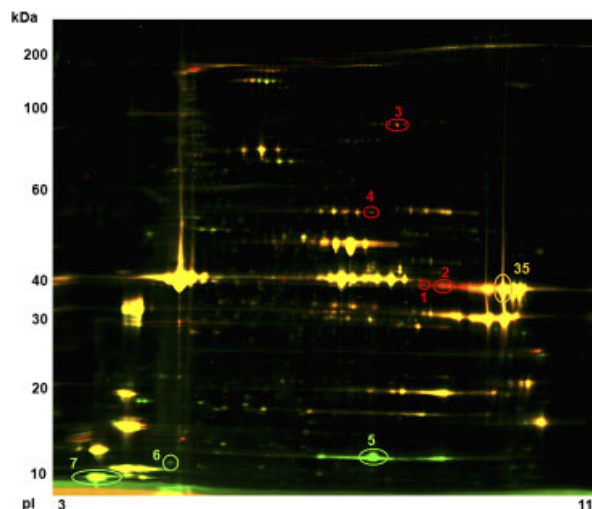


Figure 1. Overlay 2-D DIGE profile of protein patterns corresponding to sea bass muscle at death (T0) and after 5 days of postmortem storage at 1°C (T5₁). Equal amounts (60 µg) of two pools of proteins extracted from T0 ($n = 3$) and (T5₁) ($n = 3$) samples (green and red signals, respectively), together with a standard mix of equimolar amounts of the six aforementioned biological replicates, were loaded on the same gel. Proteins undergoing a significant variation in abundance (> 2 -fold change) are numbered, circled, and indicated in red (increase) or green (decrease). The resulting overlapping colors (from red to green, through shades of yellow) reflect the expression of each protein along the time scale of investigation.

Table 2. List of protein spots undergoing significant variations in abundance (> 2 -fold change) after 5 days of postmortem storage at 1°C (T5₁) with respect to the time of death (T0)

Spot no.	Protein name	Average ratio T5 ₁ /T0	1-ANOVA
1	Fructose-bisphosphate aldolase A	3.06	0.03
2	Fructose-bisphosphate aldolase A	2.9	0.03
3	Glycogen phosphorylase	2.12	0.05
4	Phosphoglucosmutase-1	2.15	0.03
5	Nucleoside diphosphate kinase B	-20.13	0.04
6	Fatty acid-binding protein	-3.63	0.04
7	Parvalbumin	-3.16	0.044

Differences were determined by one-way analysis of variance (1-ANOVA) ($p < 0.05$).

2-D map can depend on protein modifications occurring as a result of postmortem biochemical processes, such as dephosphorylation, oxidation, or loss of charged amino acid side chains, leading to a shift in the isoelectric point. This might be the case of proteins for which an increase in only one spot of the isoelectric series was detected. As an

example, for fructose biphosphate aldolase A (spots 1 and 2), glycogen phosphorylase (spot 3), and phosphoglucosylase-I (spot 4) the appearance of 'laterally shifted' spots is evident when examining the DIGE overlay patterns. Most interestingly, these 'shifted' forms were detected at the same molecular weight of the 'parent' spots (i.e. spots 1 and 2 with spot 35, having experimental *pI*s of 7.66 and 7.76 versus 8.10, respectively). Conversely, most interesting was the dramatic disappearance of NDP kinase B at 5 days of storage (−20.13 in fold change), despite the relative stability of the muscle protein pattern in general.

3.2 Changes in the protein profile of fish muscle after 5 days of postmortem storage at 18°C

In order to investigate the trend in protein degradation at higher storage temperatures, and to compare it with the degradation trends observed upon storage at 1°C, a 2-D DIGE analysis of the protein profiles of sea bass muscle at death (T0) and after 5 days of postmortem storage at 18°C (T5₁₈) was also performed. Results are presented in Fig. 2. As expected, a higher number of spots showed a significant quantitative variation (higher than twofold, $p < 0.05$) compared with the T0 samples (Table 3). When subjected to MS identification, many of these spots did not produce statistically significant results (spots 9, 10, 13, 14, and 16).

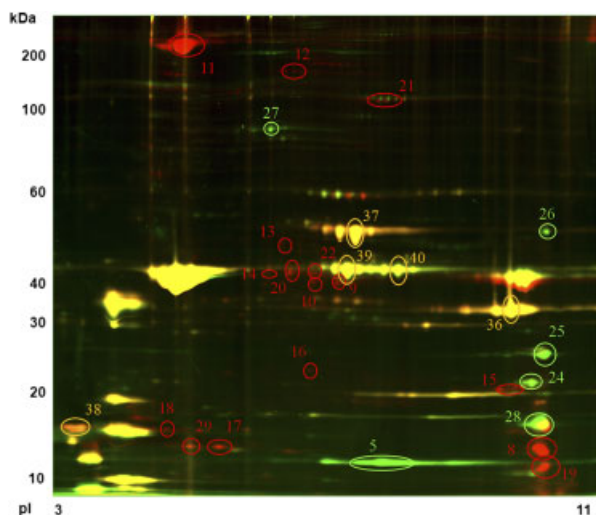


Figure 2. Overlay 2-D DIGE profile of protein patterns corresponding to sea bass muscle at death (T0) and after 5 days of postmortem storage at 18°C (T5₁₈). Equal amounts (60 µg) of two pools of proteins extracted from T0 ($n = 3$) and (T5₁₈) ($n = 3$) samples (green and red signals, respectively), together with a standard mix of equimolar amounts of the six aforementioned biological replicates, were loaded on the same gel. Proteins undergoing a significant variation in abundance (>2-fold change) are numbered, circled, and indicated in red (increase) or green (decrease). The resulting overlapping colors (from red to green, through shades of yellow) reflect the expression of each protein along the time scale of investigation.

Table 3. List of protein spots undergoing significant variations in abundance (>2-fold change) after 5 days of postmortem storage at 18°C (T5₁₈) with respect to the time of death (T0)

Spot	Protein name	Average ratio T5 ₁₈ /T0	1-ANOVA
8	Glyceraldehyde-3-phosphate dehydrogenase	47.01	0.0021
11	Myosin heavy chain	13.88	0.004
12	Myosin-binding protein C	8.44	0.0021
15	Triosephosphate isomerase B	7.22	0.0081
17	Myosin light chain 2	6.06	0.019
18	Eukaryotic translation initiation factor 5A-1	5.22	0.012
19	Myosin heavy chain	5.14	0.0031
20	Enolase	4.19	0.0071
21	Glycogen phosphorylase	3.48	0.012
22	Enolase	3.1	0.045
29	Myosin light chain 2	2.27	0.012
5	Nucleoside diphosphate kinase B	−15.52	0.033
24	Phosphoglycerate mutase 2	−3.84	0.0057
25	Phosphoglycerate mutase 2	−3.21	0.011
26	Elongation factor 1-α	−3.6	0.0059
27	Myosin heavy chain	−3.07	0.017
28	Troponin I	−2.66	0.0046

Differences were determined by one-way analysis of variance (1-ANOVA) ($p < 0.05$).

This might be an indication that this constellation of spots might be represented by a mixture of proteolytic fragments derived from the most abundant muscle proteins, being each present at amounts too low to enable MS identification with the instrumentation used in this work. Other differentially abundant spots produced MS identifications corresponding to proteins having higher molecular weights than those calculated based on spot location, suggesting that they were fragments of larger proteins (spots 8, 17, 19, 20, 22, 27, and 29, Table 4). Among these, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and MHC were identified in two strongly increased spots in the basic, low-molecular-weight region, in agreement with proteolytic events occurring during storage (GAPDH in spot 8, at 18 versus 36.2 kDa and *pI* 8.23 versus 8.7, and MHC in spot 19, at 13 versus 222 kDa and *pI* 8.24 versus 5.57) (Table 4). Similarly, spots 20 and 22 (45 kDa, *pI* 6.27 and 6.30, respectively) were identified as α-enolase, with the 'parent' spot located at higher molecular weight and isoelectric point (spot 37, 54 kDa, *pI* 6.98).

The major changes in the 2-D DIGE analysis of T5₁₈ versus T0 samples concerned GAPDH and myosin, which are among the most abundant protein components of striated fish muscle. Myosin is a protein of approximately 500 kDa composed of six subunits: two MHCs and four myosin light chains (MLCs). It is assembled into thick filaments that form the functional myosin in muscle. Some studies have indicated postmortem proteolysis of fish MHCs

Table 4. Tandem mass spectrometry protein identifications

Spot	Identified protein	Acc. no. ^{a)}	Species	Theoretical <i>M_r</i> /pI ^{b)}	Observed <i>M_r</i> /pI ^{c)}	Score ^{d)}	QM ^{e)}	% C ^{f)}
1	Fructose-bisphosphate aldolase A	P04075	<i>H. sapiens</i>	39.851/8.30	46.00/7.66	58	3	5
2	Fructose-bisphosphate aldolase A	P04075	<i>H. sapiens</i>	39.851/8.30	46.00/7.76	251	11	13
3	Glycogen phosphorylase	P11217	<i>H. sapiens</i>	97.487/6.57	107.00/7.26	498	17	10
4	Phosphoglucosmutase-1	Q08DP0	<i>B. taurus</i>	61.836/6.36	67.00/7.12	582	18	7
5	Nucleoside diphosphate kinase B	P85292	<i>M. magellanicus</i>	14.280/5.70	15.00/7.17	406	13	23
6	Fatty acid-binding protein	Q4TZH2	<i>B. grunniens</i>	14.827/6.73	17.00/4.56	33	1	5
7	Parvalbumin	B6UV97	<i>H. molitrix</i>	11.624/4.46	13.00/4.0	189	7	36
8	Glyceraldehyde-3-phosphate dehydrogenase	Q9PTW5	<i>P. olivaceus</i>	36.190/8.70	18.00/8.23	254	4	13
9	Unidentified							
10	Unidentified							
11	Myosin heavy chain	D5JZ19	<i>D. labrax</i>	202.759/5.61	187.00/5.39	2032	71	17
12	Myosin-binding protein C	Q90688	<i>G. gallus</i>	143.054/5.96	141.00/6.08	87	2	1
13	Unidentified							
14	Unidentified							
15	Triosephosphate isomerase B	Q90XG0	<i>D. rerio</i>	27.096/6.45	28.00/8.00	392	13	29
16	Unidentified							
17	Myosin light chain 2	Q9IB31	<i>P. argentata</i>	19.153/4.73	17.00/5.45	385	9	67
18	Eukaryotic translation initiation factor 5A-1	Q6EWQ7	<i>B. taurus</i>	17.049/5.08	19.00/5.40	55	2	5
19	Myosin heavy chain	Q9IBD7	<i>S. dumerili</i>	222.534/5.57	13.00/8.24	134	3	2
20	Enolase	Q6PC12	<i>D. rerio</i>	47.481/6.16	45.00/6.27	995	44	35
21	Glycogen phosphorylase	P11217	<i>H. sapiens</i>	97.487/6.57	107.00/7.22	498	17	10
22	Enolase	Q6TH14	<i>D. rerio</i>	47.841/6.25	45.00/6.30	508	34	20
24	Phosphoglycerate mutase 2	Q32HV0	<i>B. taurus</i>	28.838/8.99	29.00/8.14	56	3	31
25	Phosphoglycerate mutase 2	Q32KV0	<i>B. taurus</i>	28.838/8.99	36.00/8.20	56	3	6
26	Elongation factor 1- α	P68103	<i>B. taurus</i>	50.451/9.10	60.00/8.21	295	27	18
27	Myosin heavy chain	P13538	<i>G. gallus</i>	223.976/5.63	96.00/6.05	40	2	0
28	Fast skeletal muscle troponin I	P48788	<i>H. sapiens</i>	21.496/8.87	22.00/8.23	95	7	4
29	Myosin light chain 2	Q9DEA8	<i>T. chalcogramma</i>	19.152/5.73	19.00/5.20	708	31	2
34	Desmin	Q62654	<i>B. taurus</i>	53.556/5.21	61.00/5.62	84	3	5
35	Fructose-bisphosphate aldolase A	Q803Q7	<i>D. rerio</i>	40.228/8.35	45.00/8.10	596	20	37
36	Glyceraldehyde-3-phosphate dehydrogenase	Q511Z5	<i>D. labrax</i>	36.041/8.56	40.00/8.01	449	24	48
37	α -Enolase	P06733	<i>H. sapiens</i>	47.481/6.25	54.00/6.98	651	23	28
38	Myosin light chain 2	Q9IB25	<i>T. trachurus</i>	19.136/4.71	17.00/3.91	803	301	84
39	Creatine kinase M-type	C7ASM1	<i>S. chuatsi</i>	43.123/6.32	46.00/6.90	685	29	33
40	Creatine kinase M-type	C1BIK3	<i>O. mordax</i>	42.991/6.32	47.00/7.24	286	13	22

a) Accession number.

b) Theoretical molecular weight/isoelectric point of full-length protein.

c) Molecular weight/isoelectric point of full-length protein calculated upon calibration of electrophoretic gels.

d) Score: probability score in MASCOT program (the probability that the observed match between the experimental data and mass values calculated from a candidate peptide sequence is a random event).

e) Queries matched indicates the number of matched peptides in the database search.

f) Percent coverage: the minimum coverage of the matched peptide in relation to the full-length sequence.

[25], particularly when stored at room temperature [3]. Others have found that MHCs are fairly stable when fish muscle is stored in ice for a prolonged period [26–28].

In our study, the sequence coverage of protein spots 19 and 27, both identified as MHC, was very low (Table 4). This, together with their detection in different areas of the 2-D DIGE gel for samples at T0 and T5₁₈ (Fig. 2), at lower molecular weights (5.57 and 5.63 versus 222 kDa, respectively), and at higher isoelectric points (8.24 and 6.05 versus 5.6, respectively) than expected (Table 4), indicates that they are likely fragments of a protein with a high molecular weight, as sequence coverage is calculated relative to the full-length protein. In particular, the concentration of the

MHC fragment (spot 19) increased, thereby implying an MHC breakdown concurrent with 5-day storage of sea bass muscle at 18°C. The increase seen in MLC 2 (spots 17 and 29) could also be indicative of myosin degradation. Interestingly, in the study of Kjærsgård et al. [29], MLC 2 was found to increase about threefold during ice storage of cod. A similar study on postmortem storage of pig for 72 h showed the concentration of MLC 2 to increase about fivefold during postmortem storage [30].

On the other hand, the abundance of several spots decreased significantly during storage at 18°C. In particular, six spots showed high fold change values and were subjected to MS analysis. Namely, protein identities were NDP kinase

B (spot 5), phosphoglycerate mutase 2, elongation factor 1- α , MHC, and troponin I (spots 24 and 25, 26, 27, and 28, respectively). As opposite to spots showing a strong increase upon storage, all spots showing a statistically significant decrease upon storage did all produce protein identifications compatible with the molecular weights predicted from their sequences.

3.3 Influence of postmortem storage temperature on muscle protein profile

When observing proteins that decreased during storage, a consistency of data between the two storage times could be seen. In order to evaluate the differences in protein degradation occurring upon storage at two different temperatures, and to pinpoint possible markers of cold storage by means of an overall comparison, a 2-D DIGE analysis was performed on sea bass muscle after 5 days of postmortem storage at 1 and 18°C (Fig. 3). As a result, several protein spots that differed significantly in intensity were detected (Table 5).

Among others, some spots showed interesting increasing trends, such as spots 8 and 19, which as discussed above, did likely correspond to degradation fragments of GAPDH and MHC, respectively (fold change), as well as those corresponding to MLC 2 (spots 17 and 29). Conversely, spots identified as phosphoglycerate mutase 2 (spot 25), desmin (spot 34), myosin-binding protein C (spot 12), and MHC (27) showed a stronger degradation upon storage at 18°C when compared with storage at 1°C ($p < 0.05$). Interestingly, almost all of the spots with a decreasing trend provided MS identifications compatible with the expected molecular weight of the protein. The only protein identified among spots of decreasing abundance that did not match the expected molecular weight was MHC in spot 27 (96 versus 222 kDa, and pI 6.05 versus 5.63); however, it should be

Table 5. List of protein spots undergoing significant variations in abundance (>2 -fold change) after 5 days of postmortem storage at 1°C with respect to those stored at 18°C

Spot	Protein name	Average ratio (T5 ₁ /T5 ₁₈)	1-ANOVA
8	Glyceraldehyde-3-phosphate dehydrogenase	>60	<0.05
19	Myosin heavy chain	>60	<0.05
17	Myosin light chain 2	56.56	0.009
29	Myosin light chain 2	51.16	0.013
12	Myosin-binding protein C	23.21	0.007
27	Myosin heavy chain	−5.23	0.015
34	Desmin	−3.36	0.022
25	Phosphoglycerate mutase 2	−2.24	0.044

Differences were determined by one-way analysis of variance (1-ANOVA) ($p < 0.05$).

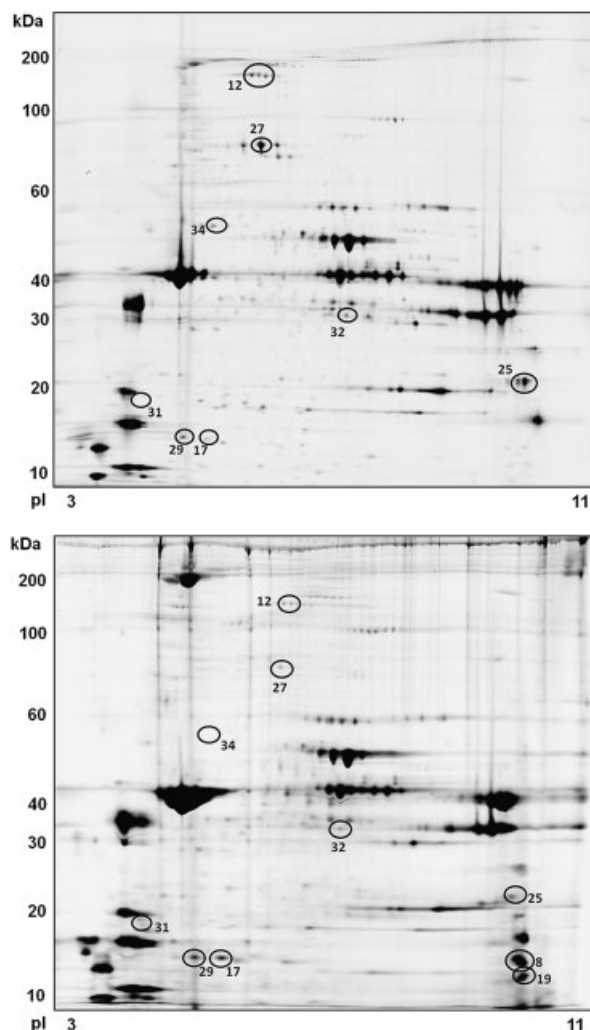


Figure 3. Protein profiles of 5 days postmortem protein extracts from sea bass muscle stored at 1°C (T5₁) (top) and stored at 18°C (T5₁₈) (bottom). Proteins undergoing a significant variation in abundance (>2 -fold change) are numbered and circled.

noted that MHC was detected at the same position in the map generated from samples at T0, as well as in fresh sea bream muscle [17], and might justify this apparent discrepancy.

In addition, the comparative analysis showed that sea bass myosin may degrade even at low temperatures of storage. Similarly, Wang et al. [9] found MHC breakdown in myofibrillar proteins of cod stored at temperatures between 0 and 6°C. With respect to deterioration processes, proteome analysis has also been reported for shrimps [31]. In this study, proteome analysis of Arctic (*Pandalus borealis*) and tropical shrimps (*Penaeus japonicus* and *Penaeus monodon*) revealed differences in postmortem degradation of muscle proteins between cold-water and warm-water species. Muscle proteases seemed to be inhibited during storage in the tropical species, but not in the Arctic species; such a

result was tentatively attributed to a significantly higher difference between living and storage temperatures in the case of tropical shrimps. Other studies in fish have well established that the stability of myofibrillar proteins differs postmortem, depending on the habitat temperature of the species. During iced and frozen storage, the thermal characteristics of myosin subunits deteriorate faster in cold- than in warm-water fish [32, 33].

The myofibrillar packaging protein desmin (spot 34) was found to strongly decrease after 5 days of storage at 18°C, although a decrease was also observed upon storage at 1°C. Desmin is the predominant protein of the intermediate cytoskeleton of mature muscle filaments [34], being thus essential for maintaining myofibril, myofiber, and whole muscle tissue structural and functional integrity [35]. Owing to its role in maintaining the integrity of the muscle cell, desmin degradation is believed to have a significant impact on meat quality [5, 36]. Abundant studies have reported postmortem proteolysis of desmin during aging of muscle in pork [37–39], in beef [5, 36, 40], and in fish [6, 15].

The most striking observation of this study was the consistent decrease in abundance of NDP kinase B observed after 5 days of storage at both temperatures. When comparing the abundance decrease of NDP kinase B at T₅₁ with that at T₅₁₈, it can be clearly observed how variation of this proteins is strongly susceptible to the storage time and occurs very rapidly independent of the storage temperature. Indeed, the average ratio of NDP kinase B did not differ significantly between T₅₁ (–20.13) and T₅₁₈ (–15.52), and after 5 days of storage the protein was almost undetectable in both maps. In a study published in 2001 [26], Verrez-Bagnis et al. reported the postmortem evolution of protein patterns in farmed sea bass muscle. The protein pattern was monitored by SDS-PAGE and by 2-DE after 0, 2, 4, and 6 days of cold storage. SDS-PAGE revealed the gradual disappearance of a protein band of 16 kDa after fish death. The loss of this polypeptide was related to the time of postmortem storage; 94% of degradation was observed after 96 h. This is in agreement with our results, which highlight a steep decrease in NDP kinase B with storage time. However, the identity of the 16-kDa sarcoplasmic protein described by Verrez-Bagnis et al. remained unknown as it did not significantly match any known proteins present in the protein databases of that year. We hypothesize that the NDP kinase B identified in our study might correspond to the spot reported by Verrez-Bagnis et al. [26]. This suggests that NDP kinase B may represent a possible biomarker for fish freshness.

4 Concluding remarks

In conclusion, the results of the present study demonstrated a relatively more stable protein pattern in sea bass muscle as compared with mammalian muscle. This indicates that sea bass muscle proteins might be proteolysed to a relatively less degree. The greatest alterations in sea bass filet protein

composition were ascribed to the 18°C storage, and distinct changes appeared after 5 days of storage at this temperature. Although to a lesser extent, also storage at 1°C introduced modifications in the total protein profile of sea bass muscle, especially concerning NDP kinase B, for which a fast and significant decrease was observed at both storage temperatures. The results presented here provide important indications on the events occurring in sea bass muscle proteins during storage and provide an important basis for future studies aimed to determine how these changes are related to quality traits of fish filet.

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5 References

- [1] Ocaño-Higuera, V. M., Marquez-Ríos, E., Canizales-Dávila, M., Castillo-Yáñez, F. J. et al., Postmortem changes in cazon fish muscle stored on ice. *Food Chem.* 2009, **116**, 933–938.
- [2] Delbarre-Ladrat, C., Chéret, R., Taylor, R., Verrez-Bagnis, V., Trends in post mortem aging in fish: understanding of proteolysis and disorganization of the myofibrillar structure. *Crit. Rev. Food Sci. Nutr.* 2006, **46**, 409–421.
- [3] Astier, C., Labbe, J. P., Roustau, C., Benyamin, Y., Sarcoplasmic disorganization in post-mortem fish muscles. *Comp. Biochem. Physiol. B* 1991, **100B**, 459–465.
- [4] Papa, I., Alvarez, C., Verrez-Bagnis, V., Fleurence, J., Benyamin, Y., Post mortem release of fish white muscle alpha-actinin as a marker of disorganization. *J. Sci. Food Agric.* 1996, **72**, 63–70.
- [5] Taylor, R. G., Geesink, G. H., Thompson, V. S., Koohmaraie, M., Goll, D. E., Is Z-disk degradation responsible for post-mortem tenderization? *J. Anim. Sci.* 1995, **73**, 1351–1367.
- [6] Campus, M., Addis, M. F., Cappuccinelli, R., Porcu, M. C. et al., Stress relaxation behaviour and structural changes of muscle tissues from Gilthead Sea Bream (*Sparus aurata* L.) following high pressure treatment. *J. Food Eng.* 2010, **96**, 192–198.
- [7] Papa, I., Taylor, R. G., Astier, C., Ventre, F. et al., Dystrophin cleavage and sarcolemma detachment are early post mortem changes on bass (*Dicentrarchus labrax*) white muscle. *J. Food Sci.* 1997, **62**, 917–921.
- [8] Ogata, H., Aranishi, F., Hara, K., Osatomi, K., Ishihara, T., Proteolytic degradation of myofibrillar components by carp cathepsin L. *J. Sci. Food Agric.* 1998, **76**, 499–504.
- [9] Wang, P. A., Martinez, I., Olsen, R. Z. L., Myosin heavy chain degradation during post mortem storage of Atlantic cod (*Gadus morhua* L.). *Food Chem.* 2009, **115**, 1228–1233.

- [10] FAO. *The State of World Fisheries and Aquaculture 2010*. FAO 2010. Rome, Italy, 197p. <http://www.fao.org/docrep/013/i1820e/i1820e.pdf>.
- [11] Alves, R. N., Cordeiro, O., Silva, T. S., Richard, N. et al., Metabolic molecular indicators of chronic stress in gilthead sea bream (*Sparus aurata*) using comparative proteomics. *Aquaculture* 2010, 299, 57–66.
- [12] Fornè, I., Abia, J., Cerda, J., Fish proteome analysis: model organisms and non sequenced species. *Proteomics* 2010, 10, 858–872.
- [13] Parrington, G., Coward, K., Use of emerging genomic and proteomic technologies in fish physiology. *Aquat. Living Resour.* 2002, 15, 193–196.
- [14] Goll, D., Dayton, W., Singh, I., Robson, R., Studies on alpha-actinin/actin interaction in the Z-disk by using calpain. *J. Biol. Chem.* 1991, 266, 8501–8510.
- [15] Seki, N., Watanabe, T., Connectin content and its post-mortem changes in fish muscle. *J. Biochem.* 1984, 95, 1161–1167.
- [16] Verrez-Bagnis, V., Noel, J., Sautereau, C., Fleurence, J., Desmin degradation in post mortem fish muscle. *J. Food Sci.* 1999, 64, 240–242.
- [17] Addis, M. F., Cappuccinelli, R., Tedde, V., Pagnozzi, D. et al., Proteomic analysis of muscle tissue from gilthead sea bream (*Sparus aurata* L.) farmed in offshore floating cages. *Aquaculture* 2010, 309, 245–252.
- [18] Chevalier, F., Highlights on the capacities of “Gel-based” proteomics. *Prot. Sci.* 2010, 8, 23.
- [19] Marouga, R., David, S., Hawkins, E., The development of the DIGE system: 2-D fluorescence difference gel analysis technology. *Anal. Bioanal. Chem.* 2005, 382, 669–678.
- [20] Ünlü, M., Morgan, M. E., Minden, J. S., Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. *Electrophoresis* 1997, 18, 2071–2077.
- [21] Gonzalez, E. G., Krey, G., Espiñeira, M., Diez, A. et al., Population proteomics of the European Hake (*Merluccius merluccius*). *J. Proteome Res.* 2010, 9, 6392–6404.
- [22] Terova, G., Preziosa, E., Marelli, S., Gornati, R. et al., Applying transcriptomics to better understand the molecular mechanisms underlying fish filet quality. *Food Chem.* 2011, 124, 1268–1276.
- [23] Addis, M. F., Tanca, A., Pagnozzi, D., Crobu, S. et al., Generation of high-quality protein extracts from formalin-fixed, paraffin-embedded tissues. *Proteomics* 2009, 9, 3815–3823.
- [24] Addis, M. F., Tanca, A., Pagnozzi, D., Rocca, S., Uzzau, S., 2-D PAGE and MS analysis of proteins from formalin-fixed, paraffin-embedded tissues. *Proteomics* 2009, 9, 4329–4339.
- [25] Busconi, L., Folco, E. J., Martone, C. B., Sanchez, J. J., Post mortem changes in cytoskeletal elements of fish muscle. *J. Food Biochem.* 1989, 13, 443–451.
- [26] Verrez-Bagnis, V., Ladrat, C., Morsel, M., Noel, J., Fleurence, J., Protein changes in post mortem sea bass (*Dicentrarchus labrax*) muscle monitored by one- and two-dimensional gel electrophoresis. *Electrophoresis* 2001, 22, 1539–1544.
- [27] Jasra, S. K., Jasra, P. K., Talesara, C. L., Myofibrillar protein degradation of carp (*Labeo rohita* (Hamilton)) muscle after post mortem unfrozen and frozen storage. *J. Sci. Food Agric.* 2001, 81, 519–524.
- [28] Tsuchiya, H., Seki, N., Action of calpain on alpha-actinin within and isolated from carp myofibrils. *Nippon Suisan Gakk.* 1991, 57, 1133–1139.
- [29] Kjærsgård, I. V. H., Jessen, F., Proteome analysis elucidating post mortem changes in cod (*Gadus morhua*) muscle proteins. *J. Agric. Food Chem.* 2003, 51, 3985–3991.
- [30] Lametsch, R., Karlsson, A., Rosenvold, K., Andersen, H. et al., Postmortem proteome changes of porcine muscle related to tenderness. *J. Agric. Food Chem.* 2003, 51, 6992–6997.
- [31] Martinez, I., Friis, T. J., Careche, M., Postmortem muscle protein degradation during ice-storage of Arctic (*Pandalus borealis*) and tropical (*Penaeus japonicus* and *Penaeus monodon*) shrimps: a comparative electrophoretic and immunological study. *J. Sci. Food Agric.* 2001, 81, 1199–1208.
- [32] Davies, J. R., Ledward, D. A., Bardsley, R. G., Poulter, R. G., Species dependence of fish myosin stability to heat and frozen storage. *Int. J. Food Sci. Technol.* 1994, 29, 287–301.
- [33] Hastings, R. J., Rodger, G. W., Park, R., Matthews, A. D., Anderson, E. M., Differential scanning calorimetry of fish muscle: the effect of processing and species variation. *J. Food Sci.* 1985, 50, 503–510.
- [34] Greaser, M., An overview of the muscle cell cytoskeleton. *Proc. Recip. Meat Conf.* 1991, 44, 1–5.
- [35] Capetanaki, Y., Milner, D. J., Weitzer, G., Desmin in muscle formation and maintenance: knockouts and consequences. *Cell Struct. Funct.* 1997, 22, 103–116.
- [36] Huff-Lonergan, E., Mitsushahi, T., Beekman, D. D., Parrish, F. C., Jr. et al., Proteolysis of specific muscle structural proteins by calpains at low pH and temperature is similar to degradation in post mortem bovine muscle. *J. Anim. Sci.* 1996, 74, 993–1008.
- [37] Christensen, M., Henckel, P., Purslow, P. P., Effect of muscle type on the rate of post-mortem proteolysis in pig. *Meat Sci.* 2004, 66, 595–601.
- [38] Kristensen, L., Purslow, P. P., The effect of ageing on the water-holding capacity of pork: role of cytoskeletal proteins. *Meat Sci.* 2001, 58, 17–23.
- [39] Morrisson, E., Mielche, M., Purslow, P. P., Immunolocalisation of intermediate filaments proteins in porcine meat. Fiber type and muscle-specificity variations during conditioning. *Meat Sci.* 1998, 50, 91–104.
- [40] Takahashi, K., Structural weakening of skeletal muscle tissue during post mortem ageing of meat: the non-enzymatic mechanism of meat tenderization. *Meat Sci.* 1996, 43, S67–S80.