Proteome Analysis Elucidating Post-mortem Changes in Cod (*Gadus morhua*) Muscle Proteins

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Proteome analysis was successfully applied to study the alterations in fish muscle proteins during ice storage. The processes occurring during post-mortem metabolism are known to lead to characteristic changes in the texture and taste of fish muscle. Endogenous proteases are anticipated to play the major role in these processes, although the exact mechanisms during fish meat tenderization have yet to be depicted. Protein changes in cod (*Gadus morhua*) muscle were followed during 8 days of storage. Within the partial proteome (p*I* 3.5−8.0, MW 13−35 kDa) significant changes were found in 11 protein spots. In nine protein spots the intensity increased, and for eight of these the increases were significant ($p < 0.05$) within the first 2 h post-mortem. In contrast, two protein spots decreasing in intensity showed significant ($p < 0.03$) changes after 8 days, thereby indicating that in the fish muscle different biochemical processes are involved in the protein changes observed post-mortem.

INTRODUCTION

Fish quality depends on a variety of factors, such as genomics, developmental stage, physical condition, environment, handling prior to slaughter, and post-mortem conditions, for example, storage temperature and time.

Comparative studies of protein levels between fresh and deteriorated fish are essential in the development of biochemical markers, which can predict fish quality. Such biochemical markers are of interest both for the fishing industry and for the consumer in general.

During post-mortem storage the quality of fish decreases, and a crucial problem is the tenderization of the fish muscle. Despite substantial and intensive studies, there are still many uncertainties regarding protein changes during deterioration. Furthermore, the spoilage patterns are species dependent and seem to reflect seasonal variations (*1−4*), and therefore it should be ensured that potential biochemical markers of spoilage are reliable and reproducible during all seasons.

Known post-mortem protein changes in fish muscle include the degradation of some large cytoskeletal proteins, whereas the sarcoplasmic protein profiles seem to be rather unaffected by spoilage (*5*). Desmin is degraded post-mortem in sardine and turbot, whereas no degradation of desmin has been detected in sea bass and brown trout (*3*). Another cytoskeletal protein, α-connectin, is converted to β-connectin and a 400 kDa fragment, thereby indicating the degradation of α-connectin. In carp muscle α-connectin is completely converted into β-connectin within 2 days at 0 °C, whereas in rainbow trout muscle this conversion of α-connectin is slower and not complete after 4 days (*4*). In the case of α-actinin the protein level has been shown to decrease in carp muscle stored at 0 °C, whereas no degradation of α-actinin has been observed for rainbow trout muscle kept on ice for 14 days (*4*). In sea bass muscle 40% of the initial α-actinin was degraded after 3 days of storage at 4 °C, whereas in sea trout muscle only 12% was degraded after 6 days of storage at 4 °C (*2*). A rapid degradation of dystrophin has been reported in sea bass muscle stored at 4 °C (*6*), with a total degradation of the protein 48 h after death. Degradation of nebulin has also been reported (*4, 6*). During 7 days of storage at 5 °C of salmon a 31-kDa band appeared, which has been interpreted as a troponin-T degradation product (*7*).

It is generally accepted that endogenous proteases play a major role during the tenderization processes of meat occurring upon slaughter, and most studies have been devoted to the calcium-dependent calpains present in the cytoplasm (*2, 7−14*) and the lysosomal cathepsins (*11, 15−17*). Although these proteases have been intensively studied, there is still much controversy about how the tenderization process occurs. Until now little work has been devoted to the multicatalytic multicomplex protease 20S proteasome (*18−25*), but recent results indicate that this protease may play an important role in bovine meat tenderization (*20, 24, 25*). Despite the knowledge generated on specific protein degradations during post-mortem storage of fish, it is still unclear how the degradation of specific proteins

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relates to fish tenderization, and more investigations are needed. A dilemma in fish and meat tenderization research is the conflict between the necessary in vitro assays, elucidating what possibly can occur, and the true processes that actually occur in the muscle.

Proteome analysis is a promising technique for providing new information on biochemical changes occurring in fish during post-mortem tenderization. In fish research proteome analysis has, for instance, been applied to the comparison of myosin isoenzymes in skeletal and cardiac muscles of Arctic charr (*26*), the assessment of the effect of varying processing conditions during the washing procedure of cod mince (*27*), the study of freeze-induced protein changes in fish (*28*), and the characterization of the water-soluble protein fraction of commercial flat fish species, thereby enabling the differentiation of the different species (*29*). With respect to deterioration processes in fish, proteome analysis has been reported for sea bass, shrimps, and salmon. Proteome analysis of protein patterns in farmed sea bass (*Dicentrarchus labrax*) revealed that three protein spots of ∼16 kDa disappeared progressively in muscle extracts during storage (*30*). Proteome analysis has also been applied to evaluate proteolysis in fermented salmon (*Salmo salar*) (*31*). This study gave indications that tropomyosin could be a suitable substrate for the metabolism of *Lactobacillus sake* LAD. Furthermore, proteome analysis has been applied to a differential characterization of commercial hake species by species-specific protein identification by nanospray ion trap MS (*32*). Proteome analysis on Arctic (*Pandalus borealis*) and tropical shrimps (*Penaeus japonicus* and *Penaeus monodon*) showed different degradations in post-mortem muscle proteins in the Arctic and tropical species. The active muscle proteases seemed to be inhibited in the tropical species, but not in the Arctic, tentatively attributed to greater a difference between the living and storage temperatures for the tropical species (*33*).

The objective of the study reported here was to characterize the changes in the composition of cod muscle proteins during ice storage, with the aim of finding proteins suitable as biomarkers.

MATERIALS AND METHODS

Fish. Eight cod (*Gadus morhua*) (1−2 kg) were caught in Kattegat between Sweden and Denmark. The fish were still alive when received from a local fisherman, and immediately after slaughter, they were placed on ice. After 0.5 h of transportation, the fish were gutted and bled and muscle samples were extracted from the loin muscle, under the first dorsal fin and above the lateral line. Sampling was performed as follows: with a biopsy needle a piece of muscle was taken from the fish, the skin was removed, and the central core of the muscle was used for protein extraction, thereby ensuring that the muscle proteins used for proteome analysis had not been exposed to ice or air. The fish were kept on ice for 8 days, and muscle samples were extracted after 0, 1, 2, 4, 6, 18, 48, 110, and 190 h, referring to the time of the first sample excision as time zero. The fish were in rigor between 24 and 48 h.

Reagents. Low molecular weight protein marker (LMW) was purchased from Novex (San Diego, CA), and RNase and DNase were from Boehringer Mannheim (Mannheim, Germany). Pharmalyt 3−10, 87% glycerol, and SDS were from Amersham Biosciences (Uppsala, Sweden). All other chemicals were of reagent/analytical grade and were purchased from either Merck (Darmstadt, Germany) or Sigma (St. Louis, MO).

Protein Extraction. From each muscle sample 120 mg was homogenized (Polytron PT 1200, Kinematica) in 2 mL of 2% (w/v) sodium dodecyl sulfate (SDS), 60 mM Tris-HCl, pH 8.3, and 100 mM dithiothreitol (DTT). The samples were boiled for 2 min and incubated for 30 min at room temperature. The samples were homogenized again

and incubated for 30 min with 0.2 mg of DNase, 0.1 mg of RNase, and 50 mM MgCl$_2$. The samples were then boiled for 2 min, homogenized again, and centrifuged for 15 min at 20 °C at 20000*g*. The supernatant was collected as sample extract. Protein concentrations were determined with Peterson's (*34*) simplification of the Lowry method (*35*). To reduce the interference of lipids in the determination of protein concentration, the deoxycholate−trichloroacetic acid (TCA) precipitation step was substituted by a TCA precipitation in the presence of 0.7% (w/v) SDS and 90 mM Tris-HCl, pH 7.5, as described (*36*). The sample extracts were stored at −80 °C until the proteins were separated in the first dimension. These extracts are referred to as whole muscle extracts and are defined as all proteins soluble in 2% SDS. To ensure that the storage time of the SDS extracts did not affect the results, all samples were "randomized" so that samples from different fish and storage times were run together, ensuring that the differences observed were not due to the experimental design but true differences.

Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE). Proteins were first separated according to charge with one-dimensional (1D) Immobiline Drystrips (Amersham Biosciences), 18 cm long and with a linear pH gradient of 3−10. The 1D Immobiline Drystrips were rehydrated overnight in 8 M urea, 0.5% (w/v) 3,3-chlolamidopropyldimethylammonio-1-propanesulfonate (CHAPS), 0.25% Pharmalyt 3−10, 10 mM DTT, and Orange G as dye. The sample extracts were diluted to 0.75 μg of protein/μL in rehydration buffer [8 M urea, 1.5% (w/v) CHAPS, 2.0% Pharmalyt 3−10, 10 mM Tris-HCl, pH 8.3, 0.1% SDS (v/w)], and 60 μg of protein was loaded on the rehydrated 1D Immobiline Drystrips in cups. The dilution of sample in rehydration buffer ensured that the final SDS concentrations never exceeded 0.25% (v/w). Isoelectric focusing was carried out at 15 °C using a Multiphor II flatbed (Amersham Biosciences). The separation was performed at 300 V for 5 h, followed by 5 h during which the gradient gradually was raised to 3500 V. Separation was continued for 14 h. After separation of the proteins in the first dimension, for a total of 60000 Vh, the 1D Immobiline Drystrips were stored at −80 °C until separation in the second dimension.

The proteins were separated according to size in the second dimension on 12% (w/v) SDS-PAGE (*37*). Ten gels (19 cm × 23 cm) were cast and run simultaneously using the Hoefer DALT system (Amersham Biosciences). Prior to 2D-PAGE the 1D Immobiline Drystrips were reduced for 10 min in 10 mL of equilibration buffer [6 M urea, 50 mM Tris-HCl, pH 8.8, 30% (v/v) glycerol, 2% (w/v) SDS] with 1% (w/v) DTT, followed by a 10 min alkylation with 4.5% (w/v) iodoacetamide in 10 mL of equilibration buffer. The equilibrated 1D Immobiline Drystrips were placed on the SDS gels and sealed with 0.5% (w/v) agarose, 25 mM tris-base, 192 mM glycine, and 0.1% (w/v) SDS.

Electrophoresis was performed at 12 °C at a maximum current of 40 mA per gel. The gels were silver stained according to the method of Hochstrasser (*38*), with minor modifications. First gels were fixed in 250 mL of 11.5% (w/v) TCA and 4.5% (w/v) sulfosalicylic acid, followed by a fixing in 250 mL of 40% (v/v) ethanol and 10% (v/v) acetic acid. The gels were washed in 500 mL of water for 10 min, incubated in 250 mL of sensitizer [0.5 M sodium acetate and 0.125% (v/v) glutaraldehyde] for 30 min followed by two washes of 10 min each in 500 mL of water. The gels were incubated in 250 mL of silver solution [24 mM AgNO$_3$, 9 mM NaOH, and 0.14% (v/v) NH$_3$] followed by 5 min of washing in 500 mL of water. The gels were developed in 250 mL of 760 μM citric acid and 0.0037% (v/v) formaldehyde. The silver reaction was stopped in 250 mL of 30% (v/v) ethanol and 7% (v/v) acetic acid. Molecular masses were predicted from coelectrophoresis with molecular weight marker proteins. Isoelectric points were estimated on the basis of the linearity of the 1D Immobiline Drystrips.

Image Analysis. Silver-stained 2D gels were digitized using a 420 OE (Argus II, AGFA, Mortsel, Belgium), flatbed scanner with a pixel size of 84.7 μm × 84.7 pixels μm. The 2D analysis image software [PDQuest 6.2 Discovery series (Bio-Rad)] was used to locate protein spots and match spots among gels. Spot matching was accomplished by manually defining ∼100 anchor spots followed by automated matching of the remaining spots.

The quantification of each spot was calculated during spot detection and Gaussian fitting, measured as optical density/(0.1 mm)^2. To ensure

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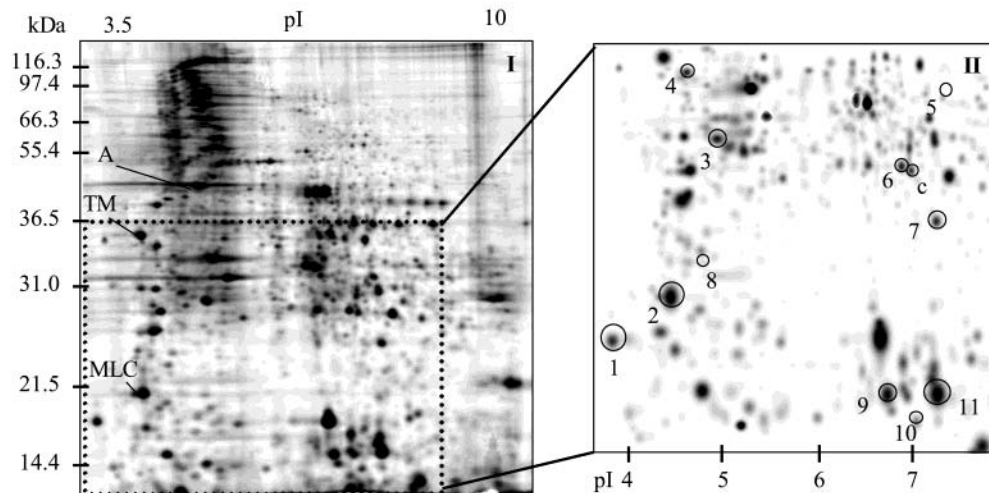*J. Agric. Food Chem.,* Vol. 51, No. 14, 2003 **3987**

Figure 1. Two-dimensional gel electrophoresis of cod muscle proteins. (I) Representative 2D electrophoresis gel of cod muscle proteins, extracted at time zero. The extracted muscle proteins (60 μg) were first separated by linear pH 3–10 IPG immobile Drystrips, followed by a size separation on 12% SDS gels. The gels were silver stained. The dotted line square specifies the area in the gels used for further proteome analysis. The proteins marked are tentatively identified as (A) actin, (MLC2) myosin light chain type 2, by comparison to the results found in cod (*27*), and (TM) tropomyosin, by comparison to the mouse gastrocnemius muscle 2D database database. (II) Computer-generated standard gel. The gel represents the protein spots included in the analysis of all 74 gels. During comparative post-mortem analysis the intensities of the marked spots have been found to change significantly ($p < 0.05$).

a uniform quantification, the optical density/(0.1 mm)^2 of each spot in a gel was divided by the optical density/(0.1 mm)^2 of all matched and unsaturated spots (peak intensity values are within the linear range of the scanner) in the gel and given a value as parts per million (ppm). Spots that were saturated (peak intensity values were higher than the linear range of the scanner) in one gel were excluded from all gel images in the following analysis.

RESULTS AND DISCUSSION

Differences in the level of muscle proteins, due to biological, post-mortem processes, were studied in eight cod (*G. morhua*) kept on ice for 8 days. The proteins in whole muscle extracts were separated by 2D-PAGE, and silver staining revealed 1500−2000 protein spots. A representative 2D gel is shown (**Figure 1**). Reported here is the analysis of the partial proteome with 338 ± 39 SD spots, corresponding to protein spots with p*I* values of 3.5−8.0 and molecular weights of 13−35 kDa; 102 spots were represented in all gels. For the protein spots with a higher molecular weight the resolution became rather blurred and the reproducibility decreased. Therefore, the studies were concentrated on the partial proteome with the optimal resolution. The experiment followed the parallel conditions in all eight individuals. A total of 74 gels were analyzed, corresponding to 8 post-mortem storage times from the 8 fish and 10 extra gels to estimate the reproducibility.

A reproducibility test was performed on the same sample analyzed on different gels. Three gels were run in parallel, during both first and second dimensions, thereby producing true doublets. Separating the same samples during different 1D and 2D electrophoresis runs produced other doublets. When the intensity of a certain spot on different gels varied by a factor of <2, the spot quantity was regarded as constant. For the true duplicates the intensity varied with a factor of 1−2 for 70−79% of the protein spots, decreasing to 62−76% when the proteins from the same sample were separated during different 1D and 2D runs. For samples extracted at the same post-mortem storage times but from different fish, 61−78% of the protein spots varied by a factor 1−2. This decreased to 49−63% for samples extracted from the same fish at different storage times,

thereby indicating that changes occurred in the overall protein composition during ice storage. These calculations were performed on 24 normalized gels from 4 fish and 4 different storage times and are similar to the reproducibility reported elsewhere (*39*). Such results emphasize the semiquantitative aspect of proteome analysis, which to a large degree comes from the protein-staining techniques (*40*). Different proteins have a tendency to stain differently, and, furthermore, minor day-to-day variations can also affect the results. However, this is compensated for by the strength of 2D-PAGE, the visualization of several thousand proteins, which would otherwise not be accessible. Taking this reproducibility variation of the intensities into account, the spots studied here were all well separated and present in all gels, and the intensity of each spot represented an average of four or eight fish. Thorough analysis of the gels revealed 11 protein spots showing significant alterations at the protein level along the time scale, where the proteins are investigated. A decrease ($p < 0.03$) was observed in two protein spots (9 and 11) and an increase ($p < 0.05$) in nine protein spots (1−8, 10) (**Figures 1** and **2**). The estimated molecular weight and p*I* values of the proteins are listed (**Table 1**).

For proteins increasing in intensity the changes in protein level were significant within the first 2−18 h, whereas for the two proteins being degraded (spot 9 and 11) the effect was significant after 190 h of storage (**Figure 2**). Four of the eight fish were followed for up to 2 weeks on ice, and here the overall degradation pattern became more pronounced (results not shown). An enlarged region of the gel is shown in **Figure 3**, focusing on protein spots 6, 7, and C. Protein spot C is highlighted as a reference point. This protein spot is the neighbor to protein spot 6, where the intensity doubled within the first 2 h from 8693 ± 3349 to 16942 ± 2449, increasing to 18414 ± 2452 after 6 h on ice ($p < 0.05$). The values are given as ppm ± standard deviation (SD). The intensity in spot C, with the values 8611 ± 2365, 10723 ± 4226, and 7331 ± 961 at 0, 2, and 6 h, respectively, was considered not to change. Protein spot 7 also changed significantly ($p < 0.05$). Here the intensity increased from 10044 ± 2378 to 19517 ± 2426 and to 20740
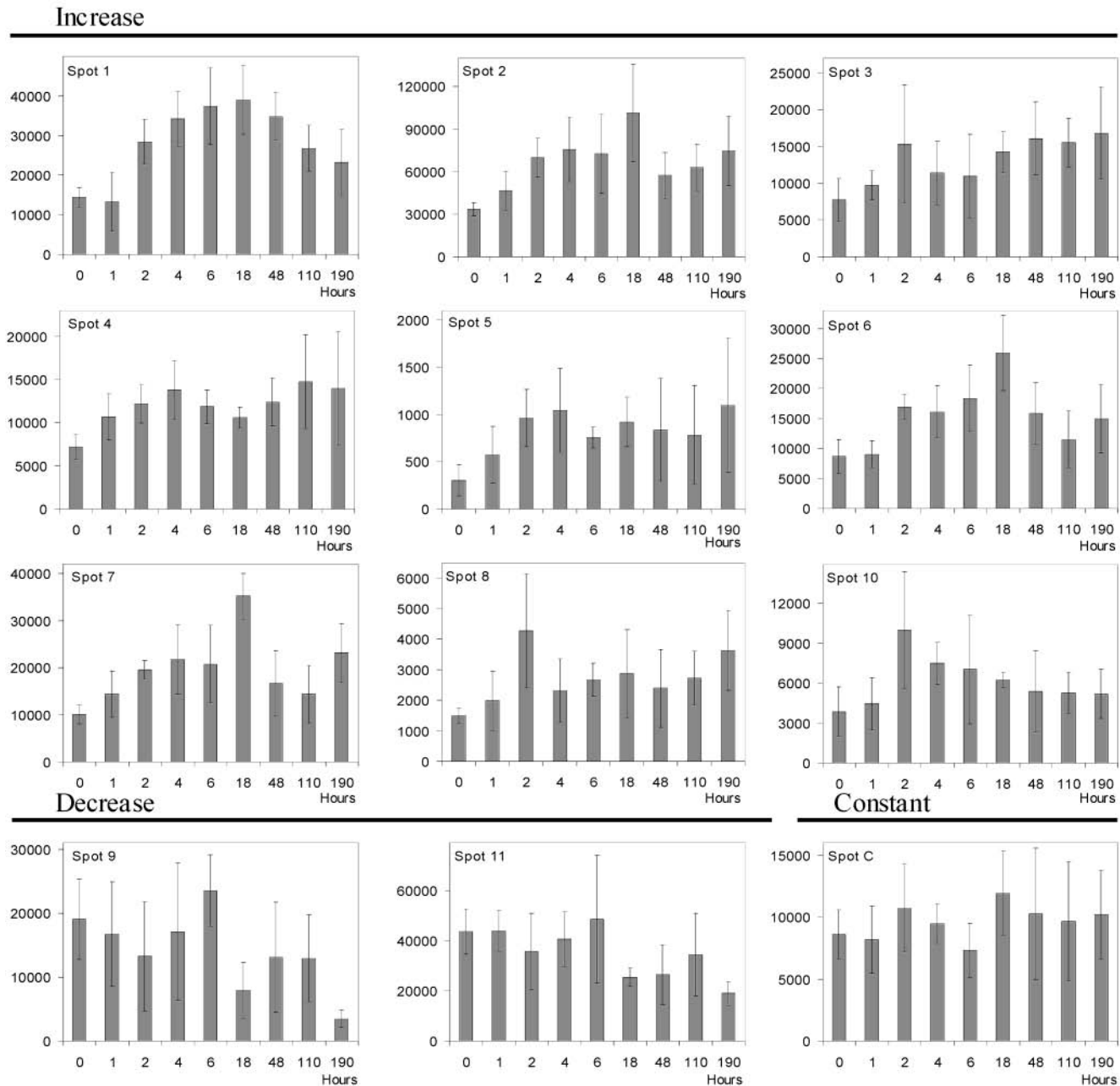
Figure 2. Expression profiles of 12 post-mortem proteins. The proteins are highlighted in **Figure 1**, and protein spots 6, 7, and C are also highlighted in the partial 2D gel shown in **Figure 3**. Along the *x*-axis each of the 12 histograms shows the relative abundance at the nine post-mortem times, 0, 1, 2, 4, 6, 18, 48, 110, and 190 h. For 0, 1, 2, 4, 48, 110, and 190 h, every bar is an average of eight animals; for 6 and 18 h, the bar is an average of four animals. The error bars correspond to the 95% confidence interval. The *y*-axis corresponds to the intensity expressed as ppm.

Table 1. Estimated Protein Weight and p*I* Values

spot	MW	p*I*	spot	MW	p*I*
1	17.2	3.6	7	23.2	7.2
2	19.2	4.4	8	20.9	4.7
3	28.5	4.9	9	15.1	6.6
4	33.7	4.6	10	14.1	6.9
5	32.7	7.3	11	15.1	7.2
6	26.7	6.8	C	26.4	6.9

± 3678 at 0, 2, and 6 h, respectively. These three proteins are all shown in an enlarged region of the gel (**Figure 3**) of cod muscle samples at 0, 2, and 6 h post-mortem.

After slaughter there will be a shift in metabolism induced by the cessation of blood circulation with subsequent disruption of oxygen supplies to the muscle. The immediate consequence is a decrease in ATP concentration followed by ATP synthesis from ADP and creatine phosphate via the enzyme adenylate kinase (myokinase) (*41*). However, the main prerigor anaerobic route for ATP synthesis is glycolysis, and it is likely that enzymes involved in these metabolic processes will be up-regulated (*42*). Therefore, the proteome changes in post-mortem cod muscle are likely to reflect either synthesis of new proteins, protein degradation, changes in solubility, or a combination of these factors. For instance, the translation of stress proteins such as heat shock proteins and protein kinases involved in signaling events (*43*, *44*) is likely to change.

Analysis of the normalized gels revealed that the pattern of changes at the protein level of cod muscle divides into three groups: (i) decreasing; (ii) increasing; and (iii) initial increase followed by a decline/stagnation.
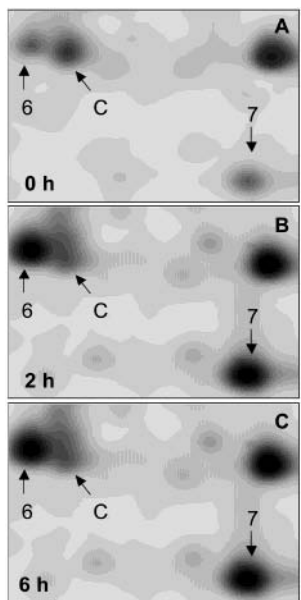
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Figure 3. Partial 2D gel demonstrating alterations in the protein level of one cod, extracted at time zero (0 h) and 2 (2 h) and 6 h after slaughter (6 h), respectively. The three partial 2D gels are from the same individual. The apparent increase in the level of protein spots 6 and 7 is compared to the constant protein level of spot C.

Known protein degradations in fish muscle include the degradation of some large cytoskeletal proteins, α-connectin (2500 kDa) (*4*), nebulin (800 kDa) (*4, 6*), dystrophin (400 kDa) (*6*), α-actinin (100−140 kDa) (*2, 4, 14*), and desmin (55 kDa) (*3*). All of these proteins have larger molecular weights than the proteins studied in the partial proteome described here and are therefore not observed in this experiment. However, some of the protein spots with increasing levels might be degradation products from these large proteins. An increase in protein level for the first 2−18 h post-mortem, followed by a decrease or stagnation, could reflect an initial increase in protein translation due to a shift in metabolic processes. Another explanation would be found in the synergistic model of action between different proteases (*45*). Here it is proposed that in the post-mortem muscle the calpains are first activated due to increasing calcium levels in the muscle. Later the cathepsins are activated due to decreasing pH, and depletion of ATP during rigor mortis results in degradation of proteins by the proteasome. As post-mortem time progresses this shift in activity from one group of proteases to another would explain the pattern where there is an initial increase in protein spot intensity, for example, an accumulation of degradation product, followed by a decline/stagnation in protein level.

Recently a similar proteome analysis on protein changes in post-mortem sea bass (*Dicentrachus labrax*) (*30*) and salmon (*31*) has been reported. In sea bass the gradual degradation of a 16 kDa sacroplasmic protein spot has been found; here 94% of the protein is degraded after 96 h (*30*). In cod (**Table 1**) we found that two protein spots of similar sizes, that is, spot 9 (M_r 15.1, p*I* 6.6) and spot 11 (M_r 15.1, p*I* 7.2), also were degraded. Especially protein spot 9 showed a remarkable decrease in intensity after 190 h of storage, when the values given as ppm ± SD decreased from 18995 ± 7525 at time zero to 3236 ± 1765 after 8 days on ice. This corresponds to a degradation of 83% of the initial protein after 190 h of storage. Myosin has been found to degrade in cold-water shrimp (*Penaeus borealis*) but not in warm-water shrimp *(P. japonicus* and *P. monodon)* (*33*). Likewise, a proteomic study on post-mortem protein

degradation in pig meat has shown the accumulation of degradation fragments generated from the structural proteins myosin heavy chain, actin and troponin-T, indicating that these proteins are degraded (*46*). In cod protein spot 2 is tentatively identified as myosin light chain type 2 (**Figure 1**) by comparison with the cod proteome (*27*). In the results reported here on cod this protein spot 2 increased from 33524 ± 5687 ppm at time zero to 72569 ± 12304 ppm at 6 h. An increase in the myosin light chain could be indicative of a degradation of the myosin heavy chain (200 kDa) that might lead to an increase in solubility of the myosin light chains. Due to the size of the myosin heavy chain, it was not resolved in the proteome analysis reported here.

Abundant studies of fish and mammalian post-mortem proteolytic processes have proved the endogenous proteases calpains (the calcium-dependent cytoplasmic proteases) to be involved in post-mortem tenderization (*7, 13*). It is generally accepted that this group of enzymes (especially μ-calpain) catalyzes limited proteolysis of proteins located in the myofibrils and plays a major role in post-mortem tenderization of mammalian muscle cells. However, the exact biochemical mechanisms of the calpains are still unclear, as is the importance of these proteases in relation to meat tenderization (*13, 47, 48*). Another group of proteases involved in post-mortem proteolyses are the lysosomal cathepsins (*8, 9, 17, 42*); however, there is some controversy about how lysosomal cathepsins are released from the lysosomes and reach the cytoplasm to degrade the myofibrillar proteins. Recent results show that the proteasomes also participate in post-mortem tenderization of bovine muscle (*20, 24, 25*), and further studies in this field are crucial for improving the understanding of the processes involved in post-mortem tenderization of meat and fish. Although inconclusive, it appears that the calpains, the cathepsins, the proteasome, and maybe other proteases, yet to be discovered, act synergistically in the degradation of myofibrillar proteins.

As for other studies regarding protein changes in fish muscle during storage (*7, 30, 31*) we observed relatively few degradations in cod muscle as compared to mammalian muscle (*49, 50*), indicating that relative little proteolysis occurs in fish muscle proteins with the size and p*I* values studied here. These differences are likely to reflect the variation in fish and mammalian muscle largely due to the different environments in which fish live (*51*); also the majority of muscle tissue in fish is white in contrast to the red muscle tissue found in mammalians (*1*), giving fundamental differences in metabolic processes. The parallel post-mortem processes in mammalian and fish muscles are likely to take place via different mechanisms, as a consequence of differences in proteolytic activities after death (*52*). In contrast to fish, weakening of the mammalian muscle is desirable because it improves tenderness.

The results observed here for the changes of 11 cod muscle protein spots draw the attention toward proteins spots that would be of interest to compare with proteins in other fish. The logical proteomic approach for protein identification would be mass spectroscopy/MS Maldi followed by database searches, because it is essential to know both the identities and functions of the differently expressed proteins. Identification by mass spectroscopy depends mainly on database searches of peptide masses. Fish genomes are largely underrepresented in the sequencing programs, and hence few fish sequence data are available; this limits the changes of protein identification. In the nonredundant Swiss-prot database a total of 119029 sequences are represented, 895 of these are fish sequences, of which 213 are cod sequences. In the redundant 731773 TrEMBL sequences 4953 fish se-

quences are present, 991 of these being cod sequences. To identify the cod protein spots here found to change in protein level, it is therefore necessary to generate sequence information, either by Edman degradation of the protein spots or by MS/MS sequencing as has been successfully reported for the identification of hake proteins (*32*).

The long-term objective of this study is to produce reference maps of cod proteins and thereby focus on the effect of the genomic and environmental factors relating to fish quality. This is an important step in the development of biochemical markers, thereby enabling the prediction of fish quality. Also, it is essential to disclose other areas of the cod proteome and thereby gain insight to the protein alterations at higher molecular masses. Because it is of utmost importance to identify the spots changing in protein level, further research is required.

ACKNOWLEDGMENT

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