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Identification of Carbonylated Protein in Frozen Rainbow Trout (*Oncorhynchus mykiss*) Fillets and Development of Protein Oxidation during Frozen Storage

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Frozen storage of fish is known to enhance lipid oxidation, resulting in the development of an unpleasant rancid taste and odor. Frozen storage of fish is also known to reduce protein solubility, and proteins are expected to be oxidatively modified; however, these oxidative mechanisms are poorly understood. Generally, protein oxidation leads to a wide range of modifications; the most studied being the formation of carbonyl groups. The present work shows, by UV spectrophometric determination of protein carbonyl groups in rainbow trout muscle, that storage at −20 °C resulted in a 2-fold increase in protein carbonylation compared to storage at −30 or −80 °C. Furthermore, low-salt-soluble proteins in fish that were either fresh or stored for 3 years at −80 °C were found to have similar extents of carbonylation. Proteome analysis and two-dimensional immunoblotting of rainbow trout low-salt- and high-salt-soluble proteins gave a detailed description of the protein carbonylation pattern. Several carbonylated proteins were identified by LC-MS/MS, such as nucleoside diphosphate kinase, adenylate kinase, pyruvate kinase, actin, creatine kinase, tropomyosin, myosin light chains 1 and 2, and myosin heavy chain. Furthermore, the results showed a reduced solubility of nucleoside diphosphate kinase in fish stored at −20 °C for 2 years compared to fish stored at −80 °C. It was observed that low-abundant proteins could be relatively more carbonylated than high-abundant proteins, thereby indicating that some proteins are more susceptible to oxidation than others, due to either their cellular localization, amino acid sequence, or biochemical function.

KEYWORDS: Fish muscle; frozen storage; protein oxidation; Western blot; LC-MS/MS

INTRODUCTION

Frozen storage is an excellent option to increase the shelf life of fish and meat products but, prolonged storage, high frozen storage temperatures, or fluctuating temperatures have a negative effect on product quality. Due to their high content of polyunsaturated fatty acids, fatty fish are especially prone to oxidation of the lipid fraction, which during prolonged storage results in the development of rancid taste and odor (*1*, *2*). However, frozen storage also leads to a decrease in protein solubility (*3*−*8*) and quality changes of the texture, such as increased toughness and loss of juiciness (*5*, *9*, *10*). Because both frozen storage of fish and protein oxidation result in protein aggregation, it is likely that there is a link between these two incidences. The development of protein oxidation in meat/fish has been studied in different radical generating model systems (*11*, *12*) inducing up to 5-fold increases (*11*). In intact muscle tissues this increase in carbonyl groups is less pronounced, with reported values of about 2-fold increases for stored beef (*11*), mackerel (*12*), and processed dry-cured ham (*13*). However, in turkey no differences in protein carbonyl levels are found after 9 days of storage (*14*).

In meat the primary initiator generating free radical species is identified as transition metal ions, especially iron and iron in heme proteins (*15*−*17*). When iron reacts with reactive oxygen species (ROS), the formation of a wide range of different radicals is initiated and a chain reaction of oxidation is started. In the Fenton reaction H_2O_2 reacts with a Fe^{2+}, resulting in the formation of both alkoxy radicals ($RO^{\bullet}$) and peroxy radicals ($ROO^{\bullet}$) (*18*). In the Haber−Weiss reaction superoxide reacts with Fe^{3+} under the formation of a hydroxyl radical ($HO^{\bullet}$) (*18*). These free radicals then react with macromolecules in their surroundings. However, the overall nature of radical reactions is very complex, and it has still has to be determined whether lipid oxidation initiates protein oxidation or protein oxidation induces lipid oxidation (*16*).

In general, protein oxidation leads to three different types of

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protein modifications, that is, (i) protein−protein cross-linkage, resulting in decreased protein solubility, for example, aggregation; (ii) peptide bond cleavage; and (iii) modifications of a wide range of different amino acids (*19*). A common feature for these three types of oxidation is that they lead to loss of protein function. Methods available for the estimation of ROS-mediated protein damage include the estimation of dityrosine, 3-nitrotyrosine, chlorine derivatives, and various mono- or dihydroxy phenylalanine derivatives and carbonyl derivatives of the amino acids proline, arginine, lysine, and threonine (*19*−*21*). Because carbonyls are formed from all ROS, they have the potential to act as markers of general oxidation (*19*, *20*, *22*, *23*).

In living cells ROS are involved in cell signaling (*24*), but at the same time they are potentially toxic byproducts originating from different electron transport systems and oxidoreductase enzymes (*17*, *19*, *22*, *25*, *26*). To cope with the deleterious ROS, the cell has a wide range of antioxidative enzymes and low molecular weight compounds to keep the intracellular redox system in balance. If the intracellular redox balance is damaged, ROS accumulate, resulting in an increase in oxidation of DNA, lipids, and proteins (*21*−*23*, *26*−*28*). When an animal is slaughtered, the intracellular redox balance is destroyed, which is likely to cause an increased oxidation level.

The study of protein oxidation in food is only at its beginning, and knowledge of the specificity of oxidized proteins will reveal where in the cells the oxidative modified proteins are localized and enable a possible explanation of possibly interactions with lipids. Results from recent studies of carbonylated protein in different foods such as chicken (*29*), milk (*30*), rainbow trout (*31*), and rice mitochondria (*32*) have contributed to the general picture that protein oxidation is a complex topic not only in relation to human health (*24*, *26*) but also with respect to the quality of the food we eat (*21*, *33*).

The aim of this study was to characterize the development of protein oxidation during frozen storage of rainbow trout. To estimate the total oxidative level, fish lipid oxidation was followed simultaneously. To enable a better understanding of the nature of protein oxidation, proteome analysis was conducted of muscle proteins fractionated as low-salt (LS) and high-salt (HS) soluble, and several of the oxidized proteins prone to oxidation were identified by LC-MS/MS.

MATERIALS AND METHODS

Materials. Unstained low molecular weight protein standard, Mark 12 (LMW), Novex colloidal blue, and See-Blue prestained standard were purchased from Invitrogen, Life Technologies (Carlsbad, CA). Bovine serum albumin labeled with 2,4-dinitrophenyl (BSA-DNP) standard was from Molecular Probes (Eugene, OR). A 2D-Quant kit for the determination of protein concentration, Pharmalyt 4−6.8, Pharmalyt 5−8, 87% glycerol, and SDS were from GE Health Science (Uppsala, Sweden). Luminol (3-aminophthalhydrazide) and *p*-coumaric acid were purchased from Fluka/Sigma (St. Louis, MO). Protease inhibitor Complete was from Roche, Mannheim, Germany. All other chemicals were of reagent/analytical grade and were purchased from either Merck (Darmstadt, Germany) or Sigma (St. Louis, MO).

Raw Material. Rainbow trout (*Oncorhynchus mykiss*) was either provided by Biomar A/S, Brande, Denmark, or purchased at the local fish market. The fish used for the frozen storage experiments were cleaned as butterfly fillets and frozen at −20, −30, or −80 °C. For protein analyses, white muscle samples were taken near the spine between the head and the dorsal fin of the fish. For all of the frozen storage experiments the fish were provided by Biomar A/S. The fish were all fed a diet with the same protein content. They were slaughtered by electronic stunning, cleaned, filleted, and stored on ice for approximately 17 h before they were frozen in a blast freezer at −40 °C. After 24 h at −40 °C, the fish were transferred to freezers with temperatures of −20, −30, or −80 °C. The fish stored at −80 °C were vacuum packed, whereas the other samples were not. It was not possible to obtain any information on feed composition or time of slaughter for the fish bought at the local fish market. These are the only fish used as "fresh fish". All experiments were conducted on a minimum of three different fish, and in total three to seven two-dimensionl (2D) gels or immunoblots were analyzed for each condition.

Measurement of Peroxide Values (PV). PV was determined as an estimation of lipid oxidation. Measurements of peroxide values were conducted on white muscle samples from the partial fillet from where the head was cut off to just after the dorsal fin. Three fish were used for each analysis. Lipids were extracted according to the methods described by Bligh and Dyer using a reduced amount of solvent (*34*). The peroxide values were determined in the lipid extract by the colorimetric ferric−thiocyanate method according to the method described by Shantha and Decker (*35*).

Muscle Fractionation. HS-soluble and LS-soluble protein samples were prepared from 500 mg of rainbow trout loin muscle as previously described (*31*). In brief, 500 mg of each sample was homogenized in 5 mL of buffer A [50 mM Tris-HCl, pH 7.4, and 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1% butylated hydroxytoluene (BHT)]. The total homogenate was centrifuged for 20 min at 600*g* at 0 °C. The supernatant was collected and used for further analysis of LS-soluble proteins. The pellet was homogenized in 10 mL of buffer A and centrifuged for 20 min at 11200*g* at 0 °C. The pellet in this final centrifugation was resuspended in 5 mL of buffer A, thereby giving a slurry containing the HS-soluble proteins. Before taking the aliquots for 2,4-dinitrophenylhydrazine (DNPH) labeling, the slurry was mixed by inversion.

DNPH Labeling of Protein Carbonyls. Protein carbonyls were measured as described by Levine et al. (*36*, *37*), and for proteomic analysis minor modifications of the labeling method were applied (*31*, *38*). In brief, 600 μL of either total, LS-soluble, or HS-soluble protein was precipitated with 600 μL of 40% (w/v) trichloroacetic acid (TCA) and centrifuged at 12000*g* for 3 min. The carbonylated proteins were labeled by adding 600 μL of 10 mM DNPH in 2 M HCl to the protein pellet, and the reaction was conducted at room temperature in the dark for 30 min. The samples were precipitated with 600 μL of 40% (w/v) TCA, and the pellets were washed three times with 1 mL of ethanol/ethyl acetate 1:1 (v/v).

UV Spectroscopy of Carbonyls. For each sample a blank without DNPH was run in parallel, and after DNPH-labeling, the pellets were dissolved in buffer B (6 M guanidine chloride in 20 mM KH_2PO_4). The carbonyl content was calculated from absorbance measurements at 370 and 280 nm (*39*). Measurements of carbonyls were conducted on three fish in the total homogenate and on seven fish for the LS-soluble protein extracts, for each for the experimental conditions respectively.

Isoelectric Focusing and 2D Gel Electrophoresis. After DNPH labeling, washed protein pellets were dissolved in buffer C (8 M urea and 2 M thiourea). Protein concentrations were measured with the 2D-Quant kit. In total, 300 μg of HS-soluble protein and 300 μg of LS-soluble-protein were applied to Immobiline Drystrips by reswelling overnight with a final volume of 350 μL. Besides the proteins the final composition of the reswelling solution was 8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% Pharmalyt 4−6.8, 1% Pharmalyt 5−8, 100 mM DTT, protease inhibitor Complete, and Orange G as dye.

2D gel electrophoresis was conducted as described by Görg (*40*). Proteins were first separated according to charge with one-dimensional Immobiline Drystrips (GE Health Science), 18 cm long, and with a linear pH gradient of 4−7. The details of isoelectric focusing and 12% (w/v) SDS-PAGE were as described (*41*). To determine both the protein and carbonyl amount two 2D gel electrophoreses were conducted twice for each sample. One 2D gel was colloidal Coomassie blue stained, and one was used for carbonyl detection by immunoblotting.

Two-Dimensional Immunoassays. Semidry Western blotting was conducted as previously described (*31*). In brief, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane 0.2 μM (Millipore, Billerica, MA) using a Hoefer TE-77 semidry transfer unit (GE Health Science). Blotting was conducted in Towbin buffer (48 mM Trizma base, 39 mM glycine, 0.037% SDS, and 20% methanol)

at a constant current of 1.1 mA/cm^2 for 70 min. The membranes were blocked in 5% skim milk in Tris-buffered saline (TBS) buffer (0.137 M NaCl and 20 mM Tris-HCl, pH 8.0) and incubated with a 1:15000 dilution of rabbit anti-DNP (DAKO, Glostrup, Denmark) in 1% skim milk, in TBS for 1 h. The membranes were washed in TBS and incubated in a 1:7500 dilution of the secondary antibody, peroxidase-conjugated swine anti-rabbit (DAKO). After washing in TBS, the blot was incubated for 1 min in 2.7 mM H_2O_2, 0.07 mM *p*-coumaric acid, and 1.25 mM luminol (3-aminophtalhydrazide) in 0.1 M Tris-HCl, pH 8.6 (*42*, *43*). Chemiluminescence was detected on hyper-film ECL (GE Health Sciences) for 10 min for both the LS-soluble and HS-soluble proteins. A minimum of three samples were analyzed for each storage condition. After immunoblotting, the gels were stained with colloidal Coomassie blue. In parallel to the 2D gel used for immunoblottinf, a 2D gel was stained with colloidal Coomassie to visualize and quantify all proteins in the sample.

Image Analysis. Colloidal Coomassie-stained 2D gels and 2D immunoblots were digitalized using a 420 OE (PDI, Bio-Rad, Hercules, CA), flatbed scanner with a pixel size of 84.7 × 84.7 pixels using PDQuest 7.1.0, Discovery Series (Bio-Rad). PDQuest was also used to locate and match protein spots among gels and blots. The quantity of each spot was calculated during spots detection and Gaussian fitting, measured as optical density/(0.1 mm)^2. To ensure a uniform quantification, the optical density/(0.1 mm)^2 of each spot in a gel was divided by 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 or blot and given a value as parts per million (ppm). The quantity of saturated spots in the 2D gels was calculated by summing the intensities of the pixels inside the defined boundary, and their quantities are comparable with regular, Gaussian-fitted spots.

In-Gel Digestion Procedure. Protein spots from the gel were in-gel digested with trypsin (Promega, Madison, WI; modified sequencing grade) according to a method modified from that of Schevchenko et al. (*44*) without previous reduction and alkylation of the proteins. Spots 2003 and 2007 were also digested by endoproteinase Glu-C (Sigma-Aldrich) by substituting trypsin with Glu-C in the digestion procedure.

Nano-LC-MS/MS. The peptides in the digestion mixture were separated by nano-LC before on-line analysis by mass spectrometry. The LC system used was a CapLC (Waters, Milford, MA) and the mass spectrometer a Q-ToF2 (Waters, Micromass, Manchester, U.K.). The peptides were trapped on a 75 μm × 15 mm precolumn and separated on a 75 μm × 150 mm analytical column, both packed with ReproSil-Pur C18-AQ 3 μm (Coricon, Knivsta, Sweden) using an in-house column packing system (Proxeon Biosystems, Odense, Denmark). Mobile phase A was 2% can (acetonitrile), 0.1% formic acid, and mobile phase B was 80% AcN, 0.1% formic acid. Peptides were loaded onto the trapping column with 0.1% formic acid with a flow rate of 5 μL min^{-1} for 10 min, followed by a gradient elution through the analytical column. The gradient was 7% B in 0−10 min, 7−20% B in 10−16 min, 20−50% B in 16−40 min, 50−100% B in 40−42 min, and 100% B in 42−57 min, and the flow rate was 250 nL min^{-1}. The nano-LC-column was connected to a silica PicoTip emitter, 10 μm tip (New Objective, Inc., Woburn, MA) in the mass spectrometer interface. The capillary voltage on the Picotip was 2.25 kV, and the cone voltage was 35 V.

Only parent ions with two or three charges were chosen for MS/MS analysis, and the data were acquired in data-dependent mode. The resulting MS/MS data were managed by Mass Lynx 4.0 software (Waters, Micromass, Manchester, U.K.).

MS Data Processing. The MS/MS dataset was processed using the ProteinLynx Global Server 2.1 software (Micromass), and the processed data were used to search the NCBInr protein database with MS/MS ion search, Mascot, Matrixscience (www.matrixscience.com) (*45*). The searches were limited to *Actinopterygii* (ray-finned fish) sequences, and the following variable modifications were used: carbamidomethyl (C), deamidation (NQ), oxidation (M), oxidation (HW), propionamide (C), and sulfone (M). The peptide tolerance was ±0.8 Da, the MS/MS tolerance was 0.6 Da, and one missed cleavage was allowed.

Some proteins were only identified by one or two peptides by the Mascot database search, and for confirmation of these results, additional peptides from each sample were de novo sequenced using the Pepseq option in the Mass Lynx software. The amino acid sequences of the peptides were matched to the amino acid sequences from the database result.

Table 1. Peroxide Value (PV)[a] for Trout Fillet Stored at Different Conditions

storage condition	temp (°C)	PV (mequiv/kg of oil)	SD[b]
frozen for 2 years	−20	12.16	0.63
frozen for 2 years	−30	1.84	1.85
frozen for 2 years	−80	0.12	1.93
frozen for 3 years	−20	24.81	3.81
frozen for 3 years	−80	0.55	0.15
fresh	ice	0.40	0.24

[a] Values given represent an average of three fish. [b] Standard deviation.

RESULTS

The aim of this study was to evaluate the overall level of protein oxidation, for example, carbonylation and the change in protein solubility and composition after prolonged frozen storage at different temperatures, and to identify fish muscle proteins prone to oxidation.

Lipid Oxidation. After 2 years of frozen storage, the peroxide values expressed in milliequivalents of peroxide per kilogram of oil were found to be about 100 times higher for fish stored at −20 °C compared to those stored at −30 and −80 °C (**Table 1**). After an additional year of frozen storage, the peroxide value for fish stored at −20 °C had doubled, whereas the fish stored at −80 °C still had a peroxide value comparable to the value found in fresh fish.

UV Spectroscopy of Carbonyls. To compare the carbonylation level in fish muscle stored at different frozen temperatures, protein carbonyls were initially measured in a total protein extract. The analysis showed (**Figure 1A**) that after 2 years of frozen storage the carbonyl content was approximately 2 times higher for fish stored at −20 °C than for fish stored at −30 or −80 °C. The carbonylation level for the LS-soluble proteins was examined separately for fish stored at −20 and −80 °C for 3 years and in fresh fish. For the LS-soluble proteins the results showed (**Figure 1B**) that fish stored at −80 °C had a carbonylation level comparable to that found in fresh fish and that the level of protein carbonylation was about 3 times higher for fish stored at −20 than at −80 °C. Overall, the results showed that high frozen storage temperature gave increased protein carbonylation levels both in whole muscle homogenates and in LS-soluble extracts (**Figure 1**).

2D Gel Electrophoresis. Protein changes in rainbow trout during frozen storage were characterized by analysis of 2D gels at two different frozen storage temperatures (**Figures 2** and **3**). A total 16 2D gels were analyzed. For fish stored at −80 °C three 2D gels with either HS- and LS-soluble protein extracts were analyzed. For fish stored at −20 °C five gels representing either HS- and LS-soluble protein extracts were analyzed. Analysis of fish muscle proteins fractionated as soluble in LS or HS buffer to 2D gel electrophoresis revealed about 600 and 400 proteins, respectively. In the HS-soluble protein fraction there was a remarkable alteration in concentration of three proteins with the sizes around 16 kDa and p*I* values of 6−7 (spots 7010, 8013, and 8014), all identified as nucleoside diphosphate kinase (NDPK), correlating with the frozen storage temperature. Further analysis of these protein spots showed that the concentration of the NDPK spots was approximately 10 times higher for fish stored at −20 °C compared to −80 °C (**Figure 4**). To eliminate the risk that this result was due to

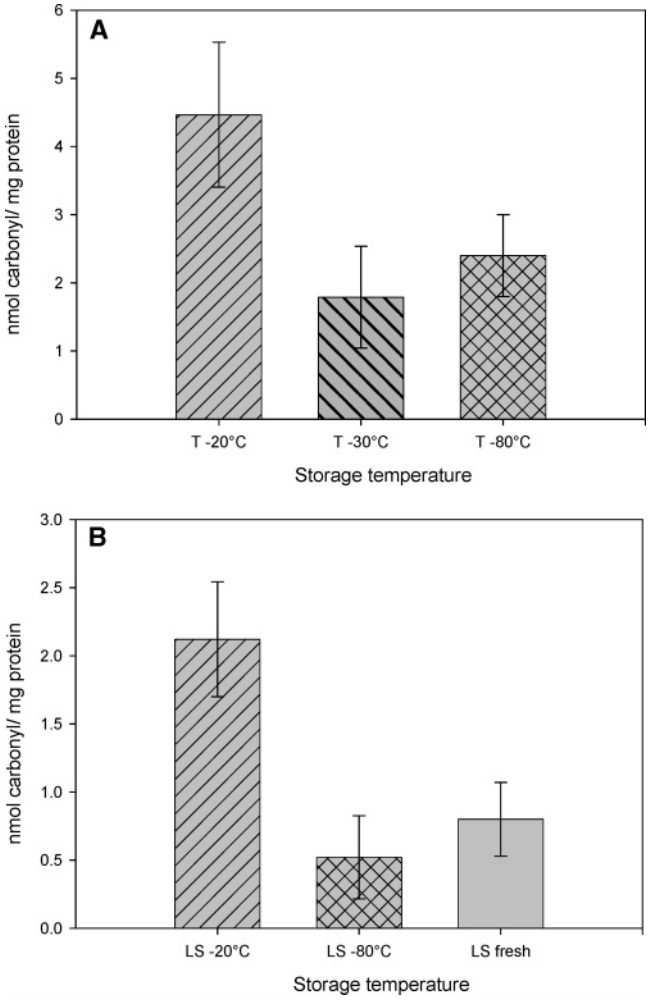

Figure 1. Spectrometric measurement of protein carbonyls given as nanomoles of carbonyl per milligram of protein: (**A**) carbonyls in a total homogenate after storage for 2 years at –20, –30, and –80 °C; (**B**) carbonyls in the LS-soluble protein fraction after storage for 3 years at –80 and –20 °C and in fresh fish. Measurements of carbonyls were conducted on three fish in the total homogenate and on seven fish for the LS-soluble protein extracts, for each for the experimental conditions respectively.

partial insufficient stained 2D gels of the −80 °C fish sample, proteins in this area of the 2D gels were thoroughly analyzed, and three of these protein spots were included in **Figure 4**. These protein spots (7004, 8001, and 8004) all had the same concentration whether the fish were stored at −20 or −80 °C. This observation was the same for all protein spots analyzed in the vicinity of these three NDPK spots. Analysis of the LS-soluble proteins showed no changes in protein concentration correlating to the different frozen storage temperatures. However, the protein spots corresponding to the HS-soluble NDPK spots (7010, 8013, and 8014), for example, LS-soluble NDPK spots 6001, 7005, and 8003, were very abundant and sometimes saturated. In the LS-extracts the concentration of these proteins was about 10 times higher than the concentration of the same proteins in the HS-extracts with intensities of 21263 (±11330), 74062 (±31510), and 21732 (±9560) for samples kept at −20 °C and 13610 (±7214), 46500 (±23085), and 16745 (±1315) for samples stored at −80 °C. None of these differences were significant ($p < 0.05$). The values are given as parts per million ± standard derivation. This rather large variation in protein spot intensities is commonly seen in 2D gels (*41*, *46*, *47*). The HS-soluble NDPK spots 7010, 8013, and 8014 had intensities of about 10% of the corresponding proteins in the LS extract, where the expected 10% decrease in intensity would be hidden by a standard variation of 50% and would be undetectable by the applied method.

2D Immunoblots. To achieve a comprehensive overview of the protein carbonylation pattern in fish muscle, 2D immunoblotting was conducted for fish stored at −20 and −80 °C (**Figures 2** and **3**). A total of 24 2D immunoblots were analyzed. For the fish stored at −80 °C six 2D immunoblots with either HS- and LS-soluble protein extracts were analyzed. For fish stored at −20 °C seven or five 2D immunoblots representing HS- and LS-soluble protein extracts, respectively, were analyzed. In general, the protein 2D immunoblots of carbonylated LS-soluble proteins were the same whether the fish were stored at −20 or −80 °C, even though the chemiluminescence signals appeared to be strongest for fish stored at −20 °C. However, due to the semiquantitative nature of chemiluminescence and Western blots (*22*, *48*, *49*), it was not possible to draw any conclusions on the degree of carbonylation on the basis of these results. For proteins soluble in HS buffer the carbonylation pattern was generally the same irrespective of storage temperature, and no differences were observed between samples. By comparison of the 2D immunoblots (**Figures 2C**,**D** and **3C**,**D**) representing the various storage temperatures, it is appears that there are some differences. However, analysis by PDQuest of all 2D immunoblots, representing a given storage condition with either HS or LS protein extracts (five to seven 2D immunoblots), showed that these differences were not consistent. This only supports the caution that most be taken in the interpretation of these semiquantitative immunoblots.

Identification of Carbonylated Proteins. Several rainbow trout proteins were analyzed and identified by nano-LC-MS/MS. The retrieved peptide sequences were 6−30 amino acids long and used for protein identification by searching all nonredundant ray-finned fish protein sequences from the NCBI database. The proteins identified by matching sequences from *O. mykiss* were cytoplasmatic carbonic anhydrase, myosin light chain (MLC) 1, and MLC 3. Because few sequences are available from this species, the other proteins were identified by matching with protein sequences from other fish species (**Table 2**). As expected, the theoretical and experimental MW and p*I* values generally were within ±0.20% and ±1.35 units, respectively (*50*, *51*). However, for some proteins the p*I* discrepancy was greater than ±1.35 units; this was expected because p*I* values are less conserved between species than MW values (*50*, *51*). In total 14 LS-soluble protein spots representing 10 different proteins and 14 HS-soluble protein spots representing 9 different proteins were identified. Furthermore, in the 2D gels of HS-soluble proteins the smear at around 116−200 kDa with p*I* values of 4−5.2 was cut in 15 pieces and analyzed separately. All were identified as MHC, but only results for spot 1603 are shown in **Table 2**. MLC 3 was also identified. Both MLC 1 and MLC 2 were found to be carbonylated, but surprisingly MLC 3 was not carbonylated; this could, however, be an artifact from the immunoblotting step, because small proteins migrate more quickly from the gel to the membrane and eventually through the membrane. However, NDPK has a similar size, and the three spots representing this protein were found to be carbonylated.

Comparison of the experimental MW and p*I* to the theoretical values for the proteins revealed that spot 8101 contained a fragment of pyruvate kinase together with adenylate kinase.

In the LS-soluble protein fraction small amounts of both actin and tropomyosin were also found.

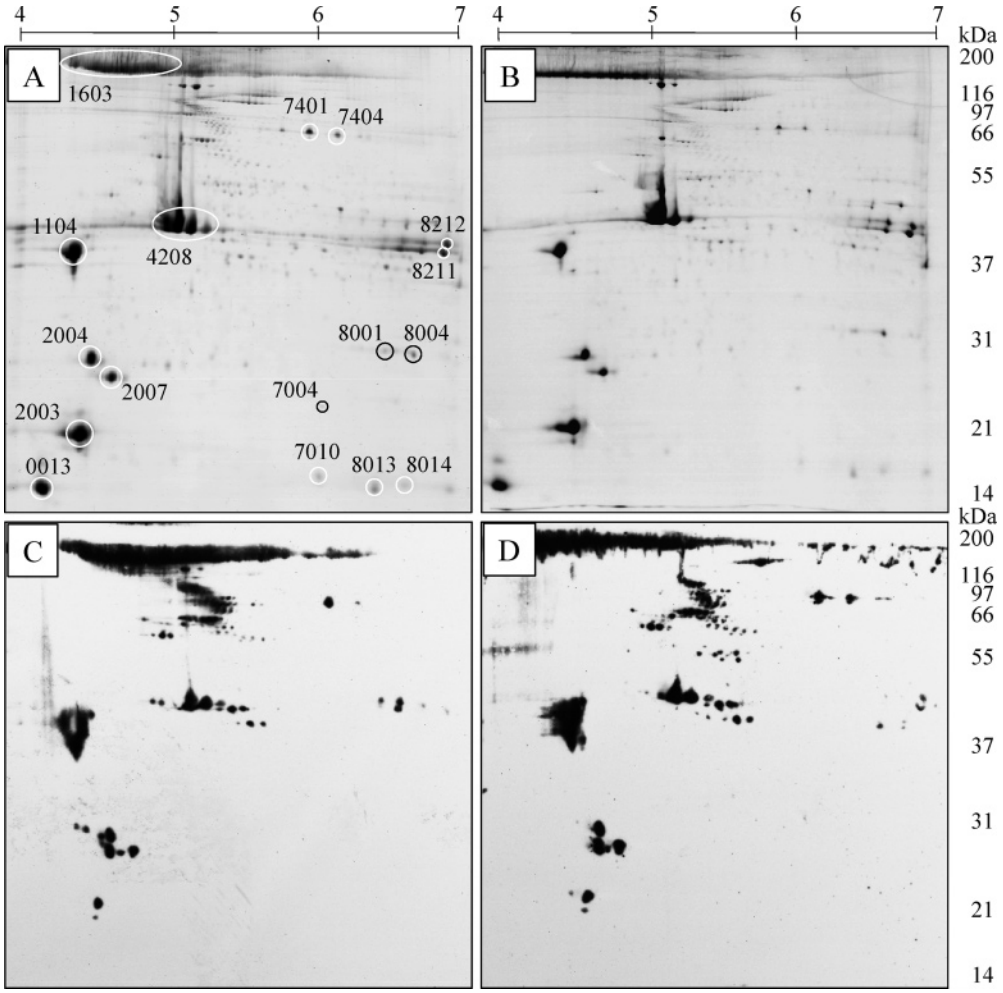

Figure 2. Two-dimensional gel electrophoresis and 2D immunoblot analysis of 300 μg of DNP-labeled carbonylated/oxidized proteins in the HS-soluble protein fractions from rainbow trout muscle after 2 years of frozen storage: (**A**, **B**) 2D gels of HS-soluble rainbow trout proteins stored for 2 years at –20 and –80 °C, respectively (gels were stained with colloidal Coomassie Blue); (**C**, **D**) 2D immunoblots of HS-soluble rainbow trout proteins stored for 2 years at –20 and –80 °C, respectively. The molecular weights from LMW marker proteins (not shown) are given to the right. Protein spots marked with a white circle have been identified by LC-MS/MS sequencing. Spots marked with a black circle are shown in **Figure 4**, but have not been sequenced.

DISCUSSION

To improve the understanding of the oxidative processes occurring during frozen storage of fish, we analyzed rainbow trout with respect to protein carbonylation levels and peroxide values as an indication of protein oxidation and lipid oxidation, respectively. Carbonyl groups were measured both by the UV spectrophotometric (*36*) and the immunoblotting method (*38*). Furthermore, changes in solubility of individual proteins were analyzed by proteomic analysis, and several oxidized proteins were identified by LC-MS/MS.

The content of carbonyl groups in whole muscle extracts from fish stored for 2 years at −20 °C were significantly higher than in fish stored at −30 and −80 °C, which had comparable carbonylation levels (**Figure 1A**). However, the UV spectrophotometric method has been reported to have its limitations, especially on crude protein mixtures from structural tissue proteins (*52*, *53*), as the evaluation of protein carbonylation is often hampered by proteins left in an insoluble pellet (*29*, *54*−*57*). Because protein oxidation reduces the solubility (*19*, *21*, *28*, *58*, *59*), proteins left in the pellet are likely to contain information about oxidation inaccessible by the applied method. To avoid the issue of insoluble protein and to verify our results, we determined the level of protein carbonyls in the LS-soluble protein fraction for fish stored at −20 and −80 °C as representatives of the two extreme storage temperatures and compared values in fresh rainbow trout (**Figure 1B**). As for the carbonylation measurements in whole muscle extracts the amount of carbonyls was highest for fish stored at −20 °C. Fish stored at −80 °C and fresh rainbow trout had comparable carbonylation levels. The difference in carbonylation between storage at −20 and −80 °C found in LS-soluble proteins was higher than in the whole muscle extract. It is difficult to make any clear conclusions on this, because carbonyl measurement on the whole muscle extract was conducted on fish stored for 2 years, and for practical reasons carbonyl measurement on the LS extract was conducted on fish that had been frozen for 3 years. It is, however, possible to conclude that carbonylation of rainbow trout increased during frozen storage at −20 °C, whereas storage at −80 °C for up to 3 years resulted in a carbonylation level for LS-soluble proteins comparable to the level observed in fresh fish. Also, 2 years of frozen storage gave a similar carbonylation level for fish stored at −30 and −80 °C, whereas there was a significant increase for fish stored at −20 °C. This development in oxidation, represented by measurement of protein carbonyls, was comparable with the trend observed for lipid oxidation, where PV values after 2 years of storage were similar for fish stored at −30 and −80 °C and about 10 times higher for fish stored at −20 °C. Comparing

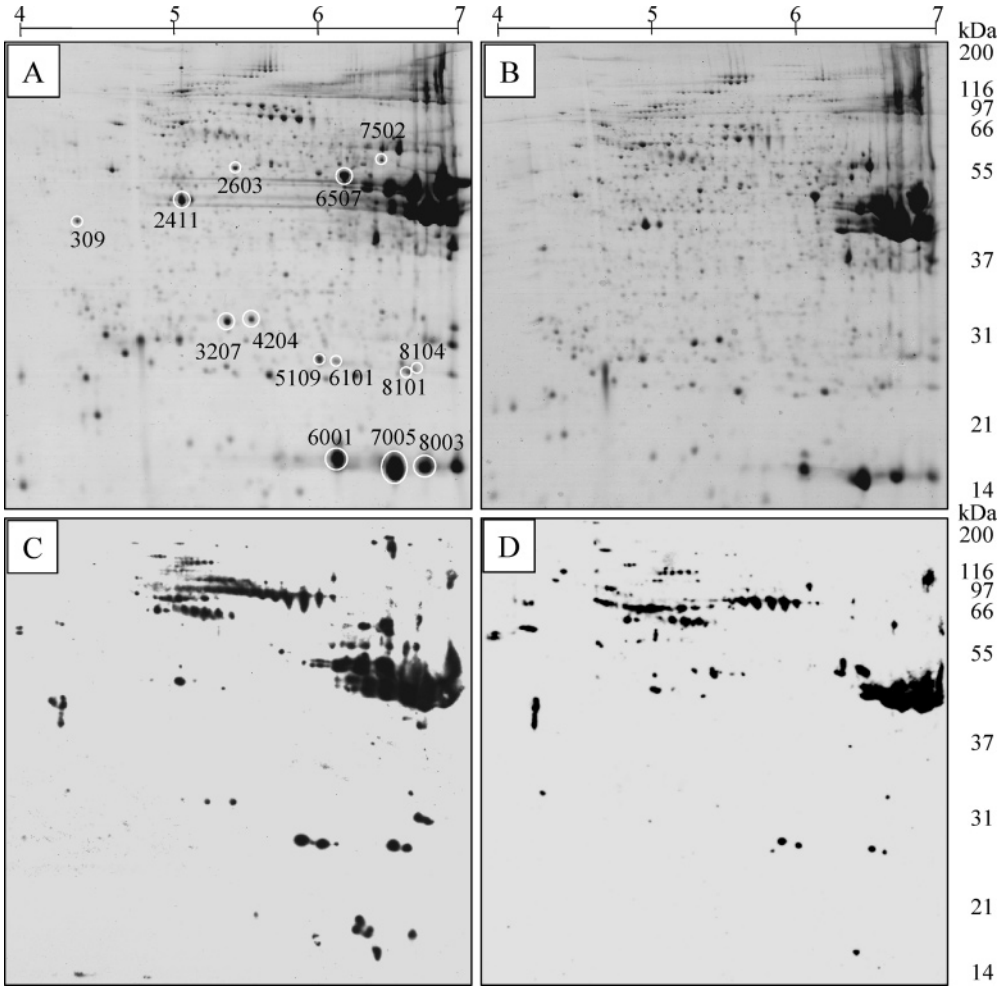

Figure 3. Two-dimensional gel electrophoresis and immunoblot analysis of 300 μg of DNP-labeled carbonylated/oxidized proteins in the LS-soluble protein fraction from rainbow trout muscle after 2 years of frozen storage: (**A**, **B**) 2D gels of LS-soluble rainbow trout proteins stored for 2 years at −20 and −80 °C, respectively (gels were stained with colloidal Coomassie Blue); (**C**, **D**) 2D immunoblots of LS-soluble rainbow trout proteins stored for 2 years at −20 and −80 °C, respectively. The molecular weights from LMW marker proteins (not shown) are given to the right. Protein spots marked with a white circle have been identified by LC-MS/MS sequencing.

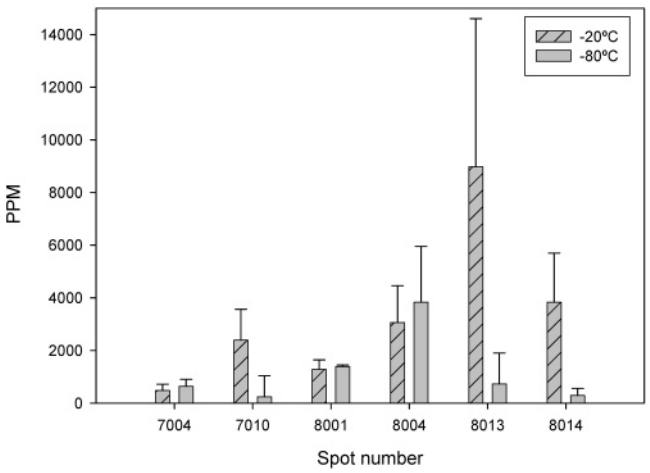

Figure 4. Spot intensity of selected HS-soluble proteins, shown according to frozen storage temperature. Error bars correspond to 95% confidence interval. The protein spot intensities are calculated as an average from five and three 2D gels representing storage at −20 and −80 °C, respectively.

the 2 and 3 years' PV results confirms that storage at −80 °C gave a lipid oxidation comparable to that found in fresh fish, whereas the PVs were doubled from 2 to 3 years of storage. Overall, the observation that prolonged storage resulted in an increase of protein carbonylation fits with previous observations (*11*, *12*, *31*). Because none of these studies were conducted on frozen samples, there seems to be a general tendency where prolonged storage, for example, a decrease in quality, leads to an increase in protein oxidation. In contrast, no increase in protein carbonyl level has been observed in fresh turkey stored for 9 days (*14*), indicating that during nonfrozen storage turkey proteins are either less sensitive to ROS or maybe more likely exposed to less ROS than beef (*11*) or mackerel (*12*). This might be ascribed to different contents of PUFA or iron.

To achieve more detailed information on the overall pattern on protein oxidation and identify the oxidized proteins, 2D immunoblotting was conducted. This method is very sensitive and enables the detection of specific carbonylated proteins, but the method is only semiquantitative (*22*, *48*, *49*), and it is difficult to make any distinct conclusions on changes in concentration of protein carbonylation. However, the tentative observation from the 2D immunoblots where the overall carbonylation seemed to increase for LS-soluble proteins (**Figure 2C**,**D**) was verified by the UV spectophometric measurements (**Figure 1B**). For the HS-soluble proteins no conclusive differences were observed whether the fish was stored at −20 or −80 °C.

Table 2. Identification of Rainbow Trout Protein Spots by Means of LC-MS/MS and Cross-Species Matching with Sequences in the Databases

description	species	accession no. NCBInr database	MW_{th};pI_{th}[a]	spot no. (high salt, HS; low salt, LS)	MW_e; pI_e[a]	Mascot score (score indicates identity or extensive homology)	peptides matched, Mascot (de novo)[b]	sequence coverage, Mascot (de novo) (%)
actin, fast myotomal muscle	*Salmo salar*	gi|10953948	41.9; 5.22	2411 (LS)	40.0; 5.25	676 (43)	14	51
				4208 (HS)	40.0; 5.25	1007 (44)	12	50
(adenylate kinase)[d]	*Tetraodon nigroviridis*	gi|47218682	21.4; 7.66	5109 (LS)	24.4; 6.03	227 (41)	5	28
				6101 (LS)	24.3; 6.15	94 (41)	3	21
				8104 (LS)	23.4; 6.59	145 (41)	4	26
				8101[e] (LS)	23.4; 6.58	54 (42)	3	17
creatine kinase, muscle	*Danio rerio*	gi|18858427	42.8; 6.32	8211 (HS)	39.2; 6.68	306 (41)	5	15
				8212 (HS)	41.0; 6.70	77 (41)	2 (+3)	4 (+11)
carbonic anhydrase, cytoplasmic	*Oncorhynchus mykiss*	gi|41059441	28.2; 5.46	4204 (LS)	28.2; 5.45	299 (42)	6	34
enolase-1, α-1	*Salmo trutta*	gi|11999265	39.9; 5.33[c]	6507 (LS)	55.0; 6.25	824 (43)	12	52
enolase-1, α-2	*Salmo trutta*	gi|11999267	39.3; 5.78[c]	7502[e] (LS)	54.4; 6.40	82 (42)	3	10
guanidinoacetate *N*-methyl-transferase	*Danio rerio*	gi|90101329	26.7; 5.88	3207 (LS)	28.0; 5.43	113 (43)	2 (+1)	9 (+6)
(myosin binding protein)[d]	*Tetraodon nigroviridis*	gi|47208602	53.6; 6.04	7401 (HS)	79.9; 5.92	42 (41)	2 (+6)	3 (+12)
				7404 (HS)	79.5; 6.07	26 (42)	1 (+7)	1 (+14)
			53.6; 6.04	2603 (LS)	65.5; 5.14	86 (40)	2 (+3)	3 (+7)
myosin heavy chain	*Oncorhynchus keta*	gi|21623523	222; 5.49	1603 (HS)	116–200; 4.0–5.2	849 (42)	25	19
myosin light chain 1	*Oncorhynchus mykiss*	gi|14335429	21.0; 4.77	2004 (HS)	21.1; 4.77	337 (42)	7	35
				2007 (HS)	20.4; 4.88	362 (44)	8	55
myosin light chain 3	*Oncorhynchus mykiss*	gi|14335431	17.2; 4.36	0013 (HS)	17.2; 4.36	151 (44)	3	31
myosin light chain 2	*Salmo salar*	gi|30141484	19.0; 4.72	2003 (HS)	18.5; 4.71	957 (43)	13	84
nucleoside diphosphate kinase	*Danio rerio*	gi|55962740	17.2 5.43	6001 (LS)	16.9; 6.20	92 (42)	2 (+2)	13 (+17)
				7005 (LS)	16.9; 6.50	96 (41)	2 (+2)	13 (+18)
				8003 (LS)	16.9; 6.20	176 (42)	2 (+2)	13 (+13)
				8014 (HS)	16.9; 6.90	41 (41)	1 (+2)	5 (+16)
	Oncorhynchus mykiss	gi|28436149	17.2; 8.76	7010 (HS)	16.9; 6.20	138 (41)	2 (+1)	17 (+9)
				8013 (HS)	16.9; 6.50	143 (41)	2 (+1)	17 (+10)
pyruvate kinase	*Takifugu rubripes*	gi|74096037	58.2; 6.35	8101[e] (LS)	23.4; 6.58	69 (42)	1 (+1)	2 (+2)
				7502[e] (LS)	54.4; 6.40	122 (42)	5	9
tropomyosin, fast myotomal muscle	*Salmo salar*	gi|409468	32.5; 4.63	0309 (LS)	37.0; 4.68	660 (42)	16	5
				1104 (HS)	37.0; 4.68	672 (44)	13	45

[a] MW_{th} and MW_e are theoretical and experimental molecular weights, respectively, given in kDa. [b] The identity was confirmed by de novo sequencing of a number of additional peptides with sizes from 6 to 21 peptides and with 63–100% homology. [c] Theoretical weight and p*I* are calculated for partial sequence. [d] Function is determined by similarity searches with BLAST. [e] Two proteins were identified from the same spot.

Quite a large number of carbonylated rainbow trout proteins were identified. In the HS-soluble protein extract the cytoskeletal proteins, for example, α-actin, tropomyosin, MLC 1, and MLC 2, a hypothetical myosin binding protein and MHC were identified. Carbonylation of actin appears to be common and has been observed in many other species and tissues such as human brain (*60*, *61*), rat muscle (*62*), chicken meat (*29*), and blue mussel gill and digestive gland (*63*), and carbonylation of MHC, MLC 1, MLC 2, and tropomyosin has previously been reported for rat muscle (*62*).

In the LS-soluble protein fraction, three kinases and two enolases were identified. Creatine kinase (spots 8211 and 8212) has also been found to be carbonylated in several species and tissues (*62*, *64*), and in the brain of people with Alzheimer's disease the level of carbonylated creatin kinase is reported to be higher than in healthy individuals (*61*). In yeast, enolase and pyruvate kinase also are found to be carbonylated (*65*).

The 2D gel electrophoresis method will always depend on a choice of different parameters such as protein extraction method, amount of applied protein, gel percentage, and p*I*, and therefore only a fraction of the total proteins will be visualized. However, it is remarkable that studies of so many different species and tissues show large similarities with respect to the identity of carbonylated proteins, which might indicate a similar oxidation pattern across species.

An emerging question is whether it is possible to predict why certain proteins are more prone to oxidation/carbonylation than others. Could it be due to their biochemical role and/or their cellular localization? It is clear that both the structural proteins in the HS-extract and the cytoplasmic proteins in the LS-extract are oxidized. Several of the structural proteins such as MHC, actin, and tropomyosin are quite heavily oxidized, but because these proteins also are quite abundant in muscle tissues, this points toward a tendency where a high protein amount correlates with a high level of carbonylation. More noteworthy is that several of the relatively weak/faint protein spots in the LS-extract such as, for example, spots 5109, 6101, 8101, and 8104, are rather strongly carbonylated. These spots are all identified as adenylate kinase, which catalyzes the transfer of a γ phosphate from ATP to AMP, thereby synthesizing two ADPs. Another interesting carbonylated protein is carbonic anhydrase, which reacts with OH^-, and thereby catalyzes the conversion of H_2O and CO_2 to HCO_3 and H^+. Recently, carbonic anhydrase has been suggested to function as a radical scavenger and to protect cells from oxidative damage (*66*). On the contrary, NDPK (spots 6001, 7005, and 8003) is very abundant in the 2D gels, but appears to be only weakly carbonylated.

The results from this study on rainbow trout oxidation show that for some of the carbonylated proteins, such as, for example, NDPK (spots 6001, 7005, and 8003) and adenylate kinase (spots

5109, 6101, 8101, and 8104), there are several oxidized proteins in the 2D gel appearing as a series of spots with differing p*I* values. Because the p*I* values of the protein series always are lower than the parent proteins' values, it can be assumed that the charge variants are a product of oxidative deamination (*65*) and that some proteins are more prone to multiple-site free radical oxidation than others (*67*). Alternatively, the spots might represent different isoforms of the proteins.

Despite the information gathered here, it is complicated to decipher a general trend for proteins prone to carbonylation, but apparently none of the heavily carbonylated proteins appear to have iron as a catalytic cofactor.

Analysis of the 2D gels of HS-soluble protein revealed that storage at −20 °C gave an increase in three NDPK spots, that is, spots 7010, 8013, and 8014 (**Figure 2A**,**B**), and none of these were oxidized, as judged by the immunoblotting analysis (**Figure 2C**,**D**). These spots correspond to the LS-soluble protein spots 6001, 8005, and 8003 (**Table 2**), and an increase of these in the HS protein fraction indicates that the proteins have obtained a reduced solubility. After 2 years of frozen storage, the intensity in the HS-soluble fraction was about 10 times higher ($p < 0.05$) for fish stored at −20 °C compared to fish stored at −80 °C (**Figure 4**). All three of these protein spots were identified as NDPK (**Table 2**). During frozen storage of cod a similar observation has been reported (*68*), where significant accumulations of a NDPK fragment was observed after 6 months of frozen storage at −20 or −30 °C. These spots do not appear on the 2D immunoblots (**Figure 2C**,**D**), and the shift in solubility of NDKP can therefore not be due to carbonylation. However, it cannot be excluded that the loss in solubility could be caused by some other oxidative modification of the protein. Generally, NDPKs were believed to be essential for the synthesis of nucleoside triphosphates (NTP) other than ATP by transferring the γ phosphate from ATP. However, recently it has been recognized that NDPKs also are involved in multiple cellular functions and play important roles in signal transduction pathways (*69*, *70*). Furthermore, NDPK peptide maps from different hake species have previously enabled a differentiation of hake species (*71*). A study comparing enzyme activity of NDPK in the white muscle of starved and fed cod has shown that the enzymatic activity is about 3 times higher in fed than starved cod (*72*). Also, this study shows that the enzymatic activity of NDKP is affected by the site of sampling, where the highest activity of NDKP is found in samples taken just behind the head of the fish. Altogether this points toward an interesting role for NDPK, where the solubility and eventually the enzymatic activity of the protein might be used to predict fish quality; however, because the activity of the enzyme is dependent on the sampling site, further analyses are required.

A previous study on the development of protein oxidation during tainting of rainbow trout (*31*) revealed a reduction in solubility for the group of LS-soluble proteins with p*I* values of 6.2−7.0 and MW of 37−55 kDa. This change in solubility does not occur during frozen storage, even though the effect is quite considerable during tainting, indicating that different biochemical reactions are involved during frozen storage and tainting.

Two spots were identified as MLC 1, spots 2004 and 2007. Because some of the obtained peptide sequences have different amino acid compositions, it is suggested that spot 2007 is not a fragment of spot 2004. For instance, from the Glu-C digest, an 11 amino acid long peptide (residues 46−56) from spot 2007 matches the database sequence (FTPDQHEDYKE), whereas H in the corresponding peptide from spot 2004 is substituted by Q (FTPDQQEDYKE). This is just one example of the differences in peptide pattern found in these two spots. The presence of two MLC 1s is observed in other species, for instance, cattle (*73*), and several fish species isoforms of MLC 1 have also been reported, for instance, arctic charr (*74*) and the tropical fish *O. andersonii* (*75*). However, in cod (*68*) and the tropical fish *O. niloticus (75*) only one MLC 1 has been observed. Because the MLCs and MHC are the major constituents of muscle fibers, differences in their composition/isoforms are likely to influence the texture of the meat and fish in question and, therefore, the food quality. With respect to farmed fish, such as rainbow trout, it would therefore be interesting to investigate if the isoforms are due to allelic variation, as reported for *O. andersonii (76*), and if selective breeding could result in fish with improved textural characteristics.

Future implications for oxidation and fish quality would therefore include more comprehensive analyses of oxidative modifications occurring during frozen storage, such as the effect of temperature, oxidative modification (carbonyl groups, dityrosines and 3-nitrotyrosine), and specifications of the amino acids in the protein chain where the oxidation occurs.

Conclusively, this study has shown that (i) the total level of carbonylated protein was the same irrespective of whether the rainbow trout was fresh or stored for 3 years at −80 °C; (ii) after 2 years frozen storage at −20 °C, a 2 times higher level of protein carbonylation and a 10 times higher level of peroxides was found than after storage at −80 °C; (iii) 2D immunoblots of proteins soluble in LS and HS buffer showed no clear differences in carbonylation pattern reflecting the frozen storage temperature (−80 or −20 °C); (iv) in both protein fractions many abundant proteins were carbonylated, although some of the less abundant proteins, such as adenylated kinase (spots 5109 and 6101) were also quite heavily oxidized, thereby indicating that different proteins have different susceptibility to carbonylation; and, finally, (v) the observation that during frozen storage the solubility of NDPK was dependent on the frozen storage temperature.

The results obtained here show that frozen storage at −20 °C of rainbow trout results in increased protein carbonylation and induces changes in protein solubility. However, there are still many unanswered questions in relation to the oxidation of fish during frozen storage, for instance, the effect of different feed compositions, protein carbonylation in vivo in the early phases of frozen storage, and the development of other protein oxidation markers such as the formation of dityrosine and nitration.

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NOTE ADDED AFTER ASAP PUBLICATION

The original posting of November 16, 2006, contained an incorrect speed of centrifugation under Muscle Fractionation. This has been corrected with the posting of November 28, 2006.

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