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Two-Dimensional Gel Electrophoresis Detection of Protein Oxidation in Fresh and Tainted Rainbow Trout Muscle

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Protein oxidation is evaluated in rainbow trout muscle by labeling protein carbonyls with 2,4-dinitrophenyl hydrazine (DNPH) followed by immunoblotting of proteins separated by SDS-PAGE or two-dimensional gel electrophoresis (2D-GE). The carbonylation level is accessed on proteins in a whole muscle homogenate or proteins soluble in a high-salt or low-salt buffer. Spoilage-related changes in carbonylation are followed in the high-salt-protein and low-salt-protein fractions by 2D immunoblotting, which reveals increases regarding total number and intensity of carbonylation in both protein fractions for fish kept at room temperature for 48 h. The major amount of carbonylated proteins is found among the high-salt-soluble proteins, and this protein fraction is also responsible for the biggest increase in carbonylation during fish tainting. The results give an estimate of the level of protein carbonylation in rainbow trout and reveal that oxidation increases for a distinct number of proteins during tainting.

KEYWORDS: Protein oxidation; 2D immunoblot; fish muscle; carbonylation; proteomic; myofibrillar; sarcoplasmic

Reactive oxygen species (ROS) such as O_2^-, $HO^\bullet$, and H_2O_2 are formed in all living organisms during normal living conditions. Their capability to destroy DNA, lipids, and proteins has been recognized since the mid-1950s, and within the past decade they have been found to play an important role in signal transduction (*1*). To keep the system in balance, the living organism has a wide range of antioxidative enzyme systems capable of reducing ROS. When an animal is slaughtered, there is a shift in metabolic and catabolic processes and the cellular antioxidative capability is reduced. This results in an accumulation of ROS followed by an increase in oxidation of DNA, lipids, and proteins.

Fish meat, that is, the muscle, is mainly composed of proteins and lipids. Lipid oxidation is a well-described and recognized problem that results in a rancid taste, and due to a substantial content of polyunsaturated fatty acids, fish lipids are highly susceptible to oxidation (*2*). This is especially problematic during storage of fatty fish, where the rancidity increases and, subsequently, the product quality decreases. However, lipid oxidation cannot explain the textural changes accumulated during storage such as loss of juiciness and increased toughness.

Despite an increasing interest in protein oxidation, it is unclear whether lipid oxidation induces protein oxidation or if the opposite is the case (*3*). In meat, potent oxidative catalysts, with respect to lipid and protein oxidation, are protein-bound transition metals (*4*). The importance of these transition metals is supported by recent studies of hemoglobins from different animal species (*5*−*7*), which suggest lipid oxidation rates in various muscle foods to reflect a species-dependent ability of the different hemoglobins to promote lipid oxidation (*5*).

ROS give rise to a large range of protein oxidations caused by various amino acid modifications (*4*), which can be detected by different methods. The most common method is based on protein carbonyls (aldehydes and ketones) formed on proline, arginine, lysine, and threonine by reaction with ROS. These carbonyls are formed from all ROS and are chemically stable, which is an analytical advantage, during both sample preparation and detection. The aromatic molecule 2,4-dinitrophenyl (DNPH) binds to carbonyls, thereby forming a stable dinitrophenyl hydrazone (DNP) product. The DNP-labeled proteins can be analyzed by UV absorption or by anti-DNP antibodies for either enzyme-linked immunosorbent assay (ELISA) or immunoblotting techniques (*4*, *8*, *9*). In fish muscle, the level of protein oxidation has been studied by bulk methods giving a good overall impression of the level of protein oxidation, which increases both during storage of whole fish and during incubation with different free-radical-generating systems (*10*−*13*). However, because these studies have been conducted on protein bulk preparations, they cannot give any information about the total number, size, and cellular localization of the oxidized proteins.

Combining the methods of muscle fractionation, labeling of oxidized protein with DNPH, gel electrophoresis, and immunoblotting facilitates the generation of new knowledge on protein oxidation in fish muscle and meat, in general, during storage or tainting. Two methods of gel electrophoresis can be

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implemented, either SDS-PAGE or two-dimensional gel electrophoresis (2D-GE). Fractionation of cellular proteins prior to gel electrophoresis gives a more comprehensive pattern of the protein composition, thereby enabling detailed studies on the biochemical process occurring in the different localizations of the cell. Fish muscle proteins are often fractionated in low-salt-soluble (mainly sarcoplasmic proteins) and high-salt-soluble (mainly myofibrillar proteins) proteins (*14*).

Previously, protein oxidation/carbonylation of fish muscle has been studied by a variety of different methods (*3*, *10*−*12*, *15*) but not 2D immunoblotting of DNPH-labeled protein carbonyl groups. However, this technique has recently been applied to detect carbonylated proteins in mainly mammalian brain tissue (*16*, *17*), thereby revealing new information on actual number, p*I*, and mass of proteins prone to oxidation during the development of Alzheimer's disease. Recently, proteomics combined with mass spectrometry has also been applied to elucidate protein carbonylation in aged mouse brain (*18*) and carbonylated glycoproteins in human blood plasma from patients with Alzheimer's disease (*19*).

The intentions of the experiments described here were to elucidate the pattern of protein oxidation in fish muscle, that is, to reveal if certain proteins are more carbonylated than others. Another aim was to reveal the expected span of protein oxidation in fish and thereby get an impression of the level of protein oxidation in fish suited for human consumption and in totally tainted fish.

MATERIALS AND METHODS

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

Rainbow trout (*Oncorhynchus mykiss*) provided by Biomar A/S were cleaned and frozen at −40 or −80 °C. Fish that had been frozen for 4 months at −40 °C or for 9 months at −80 °C were used for both total muscle extracts and muscle fractionations. The two different frozen storage conditions did not influence the edibility of the fish or the level of protein oxidation. To induce a high level of protein oxidation, the fish were left in the fume hood at room temperature for 48 h. The illumination followed the natural photoperiod throughout the incubation time. All experiments were conducted on three different fish, and in total three to five immunoblots and gels were analyzed for each condition. Samples were extracted from freshly defrosted fish and from tainted fish that had been incubated at room temperature for 48 h.

Total Muscle Extracts and Muscle Fractionation. Muscle extracts were prepared from rainbow trout loin muscle immediately after defrosting in cold water, and all steps were conducted at 4 °C or on ice. Alternatively, muscle extracts were prepared from rainbow trout loin muscle, after the fish had been kept at room temperature for 48 h. The samples for the two different time points were extracted from the same fish. Total muscle extracts were prepared from 500 mg of muscle, which was homogenized (Polytron PT 1200, Kinematica) in 5 mL of buffer A [50 mM Tris-HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.1% butylated hydroxytoluene (BHT)] three times for 30 s with a pause on ice for 30 s.

High-salt-soluble and low-salt-soluble protein samples were prepared from 500 mg of rainbow trout loin muscle. For each sample 500 mg was homogenized in 5 mL of buffer A. The muscle was homogenized as described above. The total homogenate was centrifuged for 20 min at 11200*g* at 0 °C. The supernatant was collected and used for further analysis of low-salt-soluble proteins. The pellet was homogenized in 10 mL of buffer A and centrifuged at 600*g* for 15 min at 0 °C. The pellet in this final centrifugation was resuspended in 5 mL of buffer A, thereby giving a slurry containing the high-salt-soluble proteins. Before aliquots for DNPH labeling were taken, the slurry was mixed by inversion.

Derivation of Oxidized Proteins with DNPH for SDS-PAGE. The protein concentration was measured after the addition of an equal volume of 12% SDS to the protein slurry in buffer A, giving buffer B (25 mM Tris-HCl, 0.5 mM EDTA, 0.05% BHT, and 6% SDS). This dissolved all proteins in the slurry, and protein concentration was determined with a bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Labeling of oxidized protein was conducted according to the method of Levine et al. (*8*). To a total of 35−120 μg protein in 60 μL buffer B was added 60 μL 20 mM DNPH dissolved in 10% (v/v) trifluoroacetic anhydride (TFA). The reaction was conducted for 30 min in the dark and stopped by the addition of 60 μL of 1.85 M Trizma-base, 28% glycerol, and 100 mM dithiothreitol (DTT). As negative controls, samples were processed similarly, but DNPH was omitted in the 10% (v/v) TFA. A total of 8−28 μg of each sample was applied to precast 10% Tris−glycine Novex gels (Invitrogen, Life Technologies, Paisley, U.K.), and gels were run according to the manufacturer's recommendations. Two gels were run in parallel: one was Coomassie stained, and one was used for immunoblotting.

Derivation of Oxidized Proteins with DNPH for 2D-GE. Carbonyls were labeled with DNPH in HCl, as described (*8*, *16*). For each high-salt- or low-salt-soluble protein fraction 600 μL was precipitated with 600 μL 20% (w/v) trichloracetic acid (TCA). The samples were left on ice for 15 min and centrifuged at 13000*g* for 15 min at 4 °C. The pellet was resuspended in 600 μL 10 mM DNPH in 2 mM HCl. The reaction was conducted in the dark for 30 min at room temperature with constant shaking. The proteins were precipitated on ice for 10 min with a final concentration of 20% (w/v) TCA and centrifuged for 15 min at 13000*g* at 4 °C. The pellets were washed three times with 1 mL ethanol−ethyl acetate and centrifuged for 5 min at 13000*g* at 4 °C. The protein pellets were stored at −80 °C for a maximum of 2 weeks.

Solvent for DNPH. Typically two different solvents are used for DNPH, that is, either HCl or TFA (*8*, *16*). For immunolabeling of carbonylated proteins followed by SDS-PAGE, the method with DNPH dissolved in either HCl or TFA was tested. The DNPH−HCl method gave a very high background, in the immunoblot above 66 kDa (results not shown) for whole muscle and high-salt-soluble proteins. Prior to SDS-PAGE the pellets were dissolved in 8.5 M urea, centrifuged for 10 min at 13000*g*, and diluted with 1 volume of sample buffer [100 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 40 mM DTT]. The background smear was not observed in the low-salt-soluble proteins, thereby indicating the phenomena to be solubility related. Using the DNPH−TFA method eliminated the background smear completely, probably due to the higher final SDS concentration (4%) in the assay, also during DNPH labeling. Because high concentrations of SDS are not compatible with the isoelectric focusing step in 2D-GE (*20*) the approach with DNPH dissolved in TFA was not attempted for 2D-GE. However, the high background was not observed to be a problem in 2D-GE, maybe due to the presence of thiourea, 3,3-chlolamidopropyl-dimethylammonio-1-propanesulfonate (CHAPS), and pharmalytes, which increase protein solubility (*21*).

Isoelectric Focusing and 2D-GE. After DNPH labeling, washed protein pellets were dissolved in 8 M urea and 2 M thiourea; hereafter, protein concentrations were measured with the 2Dquant kit (Amersham Biosciences). In total, 350 μg high-salt-soluble protein (corresponding to 120 mg of muscle) and 500 μg low-salt-soluble protein (corresponding to 180 mg of muscle) were applied to Immobiline Drystrips (Amersham Biosciences), 18 cm long and with a linear pH gradient of 4−7, 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, and Orange G as dye.

2D-GE was conducted as described by Görg (*22*). Proteins were first separated according to charge with one-dimensional Immobiline

Drystrips, and isoelectric focusing was carried out at 15 °C using a Multiphor II flatbed (Amersham Biosciences). The separation was performed at 100 V for 5 h, followed by 5 h during which the gradient gradually was raised to 3500 V. Hereafter, separation continued for 18 h. After separation of the proteins in the first dimension, for a total of 74000 Vh, the proteins were separated according to size in the second dimension on 14% (w/v) SDS-PAGE as described previously (*23*). To determine both the protein and carbonyl amounts, 2D-GE was conducted at least twice for each sample. One 2D gel was silver stained with $AgNO_3$ according to the method of Hochstrasser (*24*), and one was used for carbonyl detection by immunoblotting. The details of 2D-GE and silver staining are described elsewhere (*23*).

Immunoblotting. Proteins were transferred to a poly(vinylidene difluoride) (PVDF) membrane with a pore size of 0.2 μM (Millipore, Billerica, MA) using a Hoefer TE-77 semidry transfer unit (Amersham Biosciences). 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 blotting efficiency was controlled by colloidal Coomassie staining (Invitrogen Life Technologies) of the 2D-gel after protein transfer by immunoblotting and by staining of the membrane in Ponceau S in 1% (v/v) acetic acid (results not shown). Hereafter, 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) overnight at 4 °C. The membrane was incubated with a 1:15000 dilution of rabbit anti-DNP (DAKO, Glostrup, Denmark) in 1% skim milk, in TBS buffer for 1 h. The membranes were rinsed with TBS buffer once and washed three times for 10 min in TBS buffer. The membranes were incubated in a 1:7500 dilution of the secondary antibody, peroxidase-conjugated swine anti-rabbit (DAKO) for 1 h, and washed two times for 10 min with TBS buffer and once with 20 mM Tris-HCl, pH 8.1, for 10 min. 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-aminophthalhydrazide) in 0.1 M Tris-HCl, pH 8.6 (*25*, *26*). The chemiluminescence was detected on hyperfilm ECL (Amersham Biosciences) for 10 min for both the low-salt-soluble and high-salt-soluble proteins.

Data Analysis. Silver-stained 2D gels and 2D immunoblots were digitized using a 420 OE (PDI, Bio-Rad, Hercules, CA), flatbed scanner with a pixel size of 84.7 × 84.7 pixels. The 2D analysis image software PDQuest 7.1.0 Discovery Series (Bio-Rad) was used to locate protein spots and match spots among gels and blots. Spot matching was accomplished by manually defining anchor spots followed by automated matching of the remaining spots. The program generates a synthetic gel, which corresponds to a standard gel. In the standard gel each protein spot is represented, thereby enabling the comparison of the different gels.

RESULTS AND DISCUSSION

Detection of oxidized, more specifically, carbonylated, rainbow trout proteins separated on SDS-PAGE and 2D-GE has been successfully accomplished. The aim of the applied study was to compare the level of protein oxidation in edible fish (here defrosted) and tainted fish to get an impression of the alterations in protein oxidation level during deterioration. Immunolabeling of carbonylated proteins separated on SDS-PAGE (**Figure 1**) confirms the presence of carbonylated proteins in fish muscle. To validate the obtained signals, a negative control is applied; the proteins are treated as the DNPH-labeled proteins, but without DNPH in the TFA reaction step. No immunolabeling signal is found in these lanes (**Figure 1B**, lanes 5−7), thereby confirming the signals to reflect real protein carbonylations and not unspecific antibody binding or interactions of heme proteins with *p*-coumaric acid and luminol during the final chemiluminence detection step. Three different fractions are applied to the Coomassie-stained gel and the immunoblot, showing the level of protein carbonylation in whole muscle homogenate, in low-salt-soluble proteins, and in high-salt-soluble proteins. It is evident that the proteins present in the high-salt-soluble fraction are responsible for the major part of protein carbonylation in rainbow trout muscle. The amount of protein applied is 28 μg of whole protein homogenate, 8 μg of low-salt-soluble protein, and 18 μg of high-salt-soluble protein. In SDS-PAGE immunolabeling of carbonylated rainbow trout proteins the strongest signals are found at ∼200 kDa or above; however, some distinct bands with sizes around 37−57 kDa are also visible. The Coomassie-stained SDS-PAGE shows a parallel gel, where the same amount of protein is applied as in the gel used for the immunoblot. Comparing the Coomassie-stained gel with the immunoblot reveals that, even though the levels of proteins above 200 kDa are relatively low, a major amount of protein carbonyls is found in this region. An estimate of the carbonylation level can be achieved by comparing the carbonyl standard of 8 ng of DNP-labeled BSA in the immunoblot (**Figure 1B**, lane 1) with the carbonylation signals. In the SDS-PAGE immunoblot (**Figure 1**, lane 1) the protein band at 200 kDa (representing ∼1 μg of protein) shows a carbonylation level comparable to 8 ng of DNP−BSA.

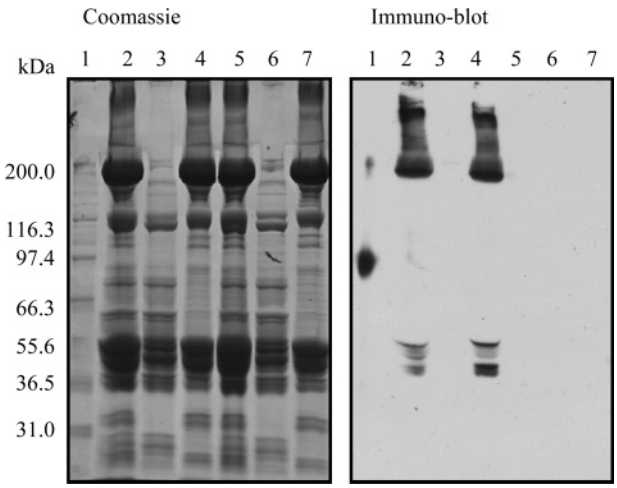

Figure 1. Immunoblot analysis detecting carbonylated proteins in rainbow trout muscle extract separated on 10% SDS-PAGE: (**A**) Coomassie-stained parallel SDS-PAGE showing the amount of protein applied; (**B**) immunoblot showing the DNP-labeled proteins. DNPH was included in the reaction mixture in lanes 2–4 and omitted in lanes 5–7. The protein fractions applied are 28 μg of whole muscle homogenate (lanes 2 and 5), 8 μg of low-salt-soluble protein (lanes 3 and 6), and 18 μg of high-salt-soluble protein (lanes 4 and 7). A molecular weight standard is shown in lane 1 of the Coomassie-stained gel, and the molecular weights of the marker proteins are given to the left. In lane 1 in the immunoblot 8.0 ng of DNP-labeled bovine serum albumin is shown as a reference for the level of protein carbonylation.

A much more detailed pattern of both protein composition and oxidation/carbonylation emerges from 2D-GE and 2D immunoblotting of fractionated muscle proteins (**Figures 2** and **3**). The level of protein carbonylation was investigated in muscle from freshly defrosted rainbow trout and in muscle from the same fish after 48 h at room temperature. It is obvious (**Figures 2C**,**D** and **3C**,**D**) that for both the high-salt-soluble and low-salt-soluble proteins an increase in number of oxidized proteins occurs after 48 h at room temperature. The experiments were conducted on fish that had been frozen either for 4 months at −40 °C or for 9 months at −80 °C. These different frozen storage conditions did not affect the level of oxidized proteins.

Silver staining of high-salt-soluble proteins applied to 2D-GE (**Figure 2A**,**B**) reveals ∼1500 protein spots where of some of the most abundant proteins can be tentatively identified as

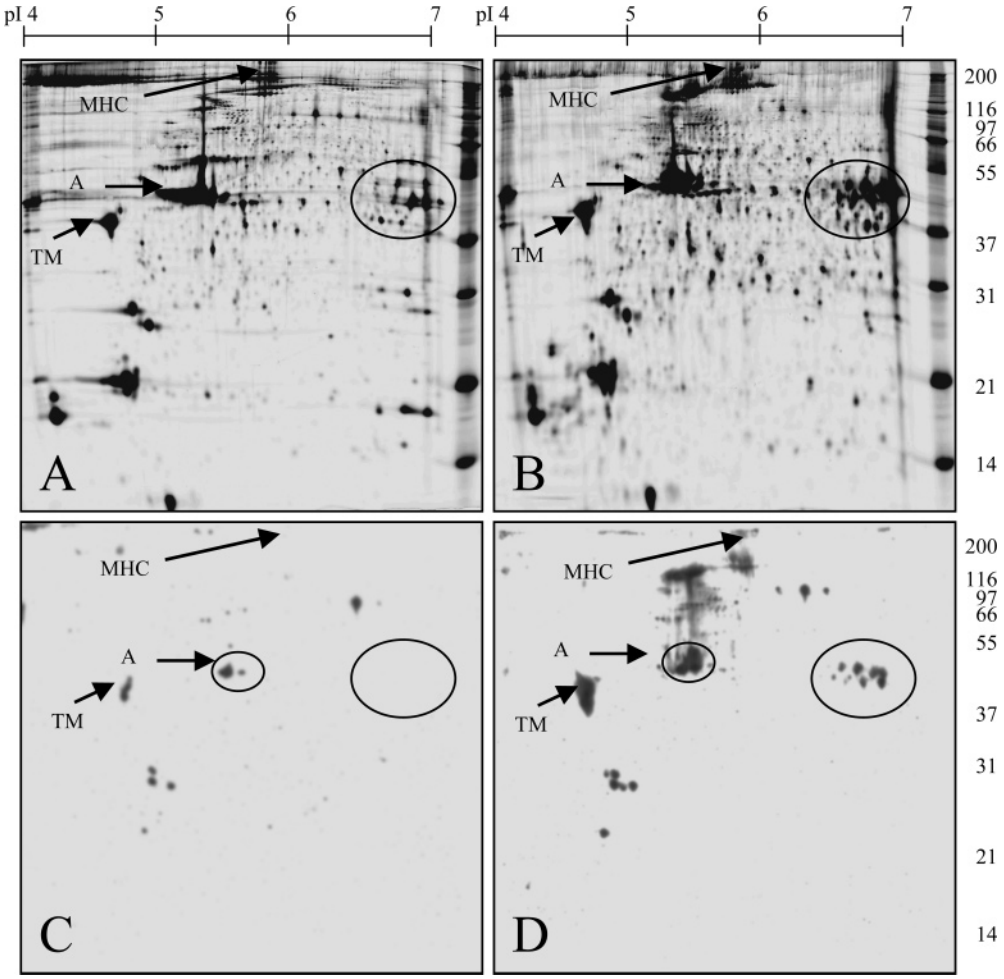

Figure 2. 2D-GE (**A**, **B**) and immunoblot (**C**, **D**) analysis of 350 μg of DNP-labeled carbonylated/oxidized proteins in the high-salt-soluble protein fractions from rainbow trout muscle either freshly defrosted (**A**, **C**) or after 48 h at room temperature (**B**, **D**). The proteins are separated by use of linear pH 4–7 IPG Immobiline Drystrips, followed by a size separation on 14% SDS-PAGE, and finally blotted to a PVDF membrane. The marked proteins are tentatively identified as actin (A), tropomyosin (TM), and myosin heavy chain (MHC) (*28*, *38*). A molecular weight standard is shown to the left of the silver-stained gel, and the molecular weights of the marker proteins are given to the left.

myosin heavy chain, tropomyosin (*27*), and actin (*28*). For rainbow trout the molecular mass and p*I* values for actin are 37.84 kDa and 5.65, respectively. The values have been calculated from the given actin sequence (*28*) with the pI/Mw program on the ExPASy homepage (*29*). The overall 2D-GE protein pattern in rainbow trout high-salt-soluble fraction is similar to the pattern found in cod (*27*) and shrimp (*30*). However, due to the relatively high amount of rainbow trout protein applied combined with silver staining, more spots are visualized in the gels presented here. Silver-stained 2D gels with low-salt-soluble protein (**Figure 3A**,**B**) reveal ~2000 protein spots; unfortunately, it is not possible to make any tentative identifications within this group of proteins.

After 48 h at room temperature, the silver-stained gels show alterations in protein composition (**Figures 2A**,**B** and **3A**,**B**). Proteome analysis has previously revealed changes in protein composition due to deterioration processes in fermented salmon (*31*), farmed sea bass (*32*), shrimp (*30*), and cod (*23*). However, none of these studies were conducted under such extreme conditions as those applied in this study. It is evident (**Figure 2A**,**B**) that in the high-salt-soluble protein fraction there is an increase in the amount of proteins, especially with sizes between 14 and 55 kDa and p*I* 5−7 after 48 h at room temperature. Among the low-salt-soluble-proteins the biggest difference from just thawed to 48 h at room temperature is found for proteins with sizes of 14−21 kDa and p*I* 5−6, where there is a decrease in intensity from the just thawed fish to the tainted fish. The differences in protein number and intensity due to decay are explicit in all evaluated silver-stained gels. The increase in high-salt-soluble 2D-GE (**Figure 2A**,**B**) proteins is possible due to an accumulation of degradation products during fish decay, but may also represent proteins from the low-salt-soluble fraction, which have changed solubility concurrently with tainting of the fish.

The 2D immunolabeling of high-salt-soluble proteins isolated from freshly defrosted rainbow trout shows ~10 nicely separated carbonylated proteins, all with sizes between 21 and 55 kDa and 6−7 proteins with sizes of 66−97 kDa. Furthermore, there are some carbonylated proteins with sizes >97 kDa. Some of the oxidized proteins can be identified as tropomyosin (*27*) and actin (*28*). After incubation for 48 h at room temperature, there is a strong increase in the total number of carbonylated high-salt-soluble proteins. Myosin heavy chain has a size of ~220 kDa and a p*I* value of ~5.5; therefore, carbonylation in this region indicates that myosin heavy chain has become carbonylated during the decay period. The smear of carbonylated proteins with sizes between 200 and 97 kDa and with p*I* values of ~5.5 could be myosin heavy chain fragments that have become carbonylated. A recent study on protein oxidation of chicken breast myosin with $FeCl_3/H_2O_2$/ascorbate has shown a decrease in concentration of myosin heavy chain with concurrent appearance of new peptide bands with lower molecular masses,

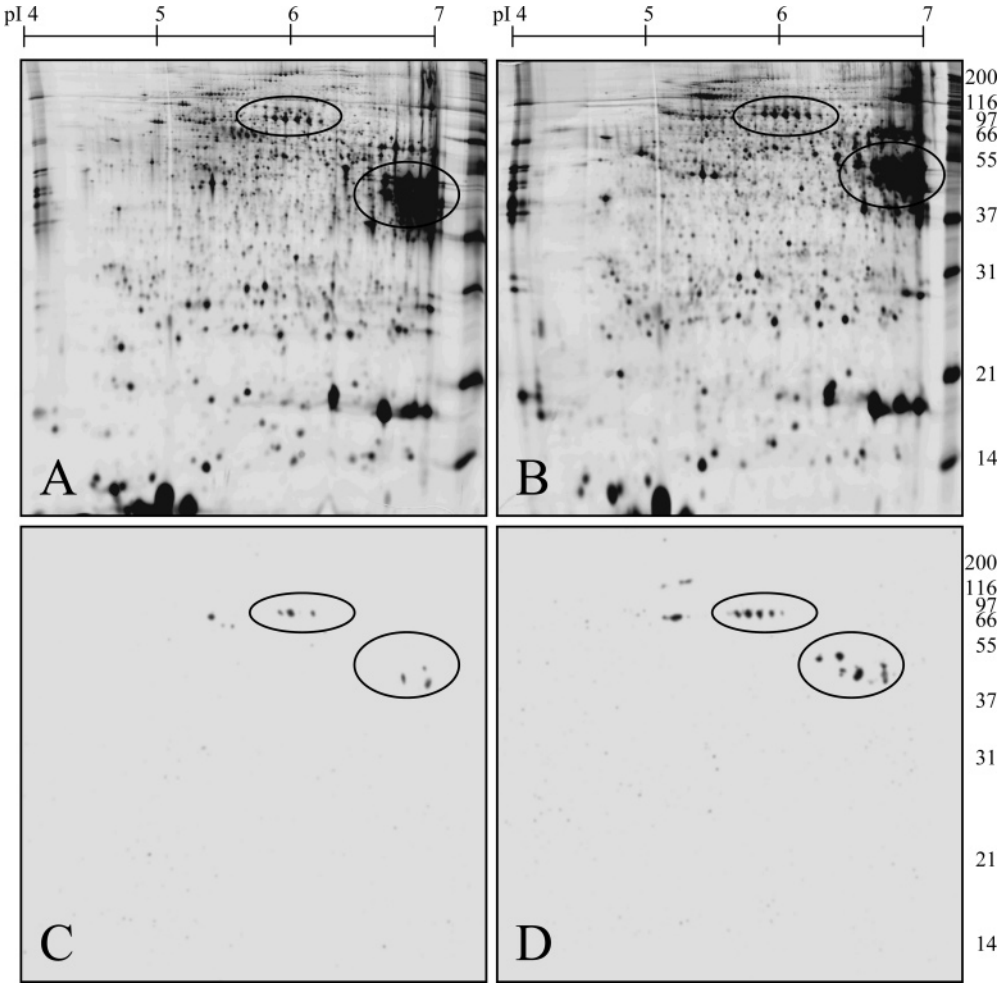

Figure 3. 2D-GE (**A**, **B**) and immunoblot (**C**, **D**) analysis of 500 μg of DNP-labeled carbonylated/oxidized proteins in the low-salt-soluble protein fractions from rainbow trout muscle either freshly defrosted (**A**, **C**) or after 48 h at room temperature (**B**, **D**). The proteins are separated by use of linear pH 4–7 IPG Immobiline Drystrips, followed by a size separation on 14% SDS-PAGE, and finally blotted to a PVDF membrane. A molecular weight standard is shown to the left of the silver-stained gel, and the molecular weights of the marker proteins are given to the left.

mostly in the range of 97−200 kDa (*33*). When a protein is degraded, the fragments will have a smaller size than the original protein, but also there will often be a change in p*I* value. Among rainbow trout high-salt-soluble proteins it is obvious (**Figure 2C**,**D**) that tropomyosin is more carbonylated after the decay. Furthermore, a group of about eight proteins at p*I* 6.5−7.0 and sizes of 36−55 kDa are carbonylated after 48 h at room temperature. Comparing the silver-stained gels with the immunoblots reveals that the total concentration of this group of proteins increases in the high-salt-soluble protein fraction. Because oxidation is known to decrease protein solubility (*10*, *13*, *34*), it is possible that some of the low-salt-soluble proteins in just defrosted fish are found in the high-salt-soluble fraction after 48 h at room temperature. In the low-salt-soluble protein fraction the silver-stained gels show this group of proteins with p*I* 6.5−7.0 and sizes of 36−55 kDa to be most abundant, but it is not possible to detect a decrease in the concentration from the sample in the just defrosted fish to the tainted fish. However, in the low-salt soluble 2D immunoblot there are also increases in protein carbonylation for this group after tainting, indicating that protein carbonylation, occurs before an eventual change in protein solubility. Another explanation could be that some high molecular weight proteins not visible on the gel are degraded and the degradation products lie within this area. Alternatively, the increase in protein carbonylation could derive from a combination of carbonylation and fragmentation of large proteins usually not resolved in 2D-GE applied here. For the low-salt-soluble proteins the immunoblot in just defrosted rainbow trout shows about nine carbonylated proteins, practically all with sizes between 37 and 97 kDa and with p*I* values >5. After incubation at room temperature for 48 h, there also is an increase in the number of carbonylated proteins. It is notable that all carbonylated proteins in the low-salt-soluble fraction have sizes of 37−97 kDa and lie within the p*I* range of 5−7. However, it is likely that higher levels of protein oxidation might be induced by other factors, as, for instance, prolonged frozen storage at high temperatures.

The results show that only a certain number of proteins present in either the low-salt-soluble or the high-salt-soluble fraction of the muscle are oxidized. Furthermore, the increase in the number of oxidized proteins during decay is rather distinct with respect to the proteins prone to oxidation. A rough estimate of the ratio of carbonylated proteins to total number of proteins shows that about 1% (16/1500) and 0.5% (9/2000) of the high-salt- and low-salt-soluble proteins, respectively, are carbonylated in defrosted fish. After 48 h at room temperature, this number increases to ∼1% (15/2000) for the low-salt-soluble proteins. For the high-salt-soluble proteins it is difficult to make a similar estimate due to the large increase in carbonylation intensity combined with the relatively bad resolution of the high

molecular weight proteins; however, there is no doubt that there is a strong increase in carbonylated proteins after 48 h at room temperature.

Comparing the patterns of protein carbonylation emerging from the SDS-PAGE (**Figure 1B**) and 2D-GE immunoblotting (**Figures 2C** and **3C**) demonstrates the strength of 2D-GE, and it is a good illustration of the complexity of proteins hidden in a single SDS-PAGE protein band. The advantage of 2D-GE is separation of a high load of proteins in two dimensions. Furthermore, the precipitation step in the DNPH−HCl method applied for proteins separated on 2D-GE enables the up-concentration of low-salt-soluble proteins. The applied amount is 500 μg of low-salt-soluble protein and 350 μg of high-salt-soluble protein. For the SDS-PAGE experiment 8 μg of low-salt-soluble protein and 18 μg of high-salt-soluble protein are applied. This relative increase of low-salt-soluble protein applied to 2D-GE as compared to SDS-PAGE enables the detection of carbonylated proteins in the low-salt-soluble fraction. The protein applied to 2D gels corresponds to 180 and 120 mg of muscle for low-salt- and high-salt-soluble proteins, respectively, whereas for SDS-PAGE the amount of muscle corresponds to 0.333 mg for whole muscle homogenate and low-salt- and high-salt-soluble proteins. Comparing SDS-PAGE with 2D-GE reveals a much higher amount of proteins with a size of 200 kDa or above present in SDS-PAGE; furthermore, these proteins are relative heavily carbonylated. A likely explanation for this discrepancy is found by a difference in acrylamide concentration, which is 10% in SDS-PAGE compared to 14% in 2D-GE, thereby giving a better resolution of high molecular weight proteins in SDS-PAGE; furthermore, high molecular weight proteins are difficult to analyze by 2D-GE (*35*). Also, migration of high molecular weight proteins is often a problem during isoelectric focusing gels, and it is simpler for proteins to enter SDS-PAGE. Furthermore, 2D-GE is the most complex technique, with more equilibration steps during which proteins may be lost, due to diminished solubility. It is, however, likely that a more comprehensive pattern of protein carbonylation can be achieved by changing both p*I* gradient and acrylamide concentration in 2D-GE.

Protein oxidation in meat has been studied in a variety of different model systems.With respect to myofibrillar beef proteins it has been shown that the carbonyl content after 10 days of maturation is equivalent to the carbonyl level reached after 1 h of induced protein oxidation with different free-radical-generating systems (*36*). In chicken (*33*) myosin oxidized with a nonenzymatic free-radical-generating system has shown oxidation to induce fragmentation and polymerization of myosin. With respect to fish meat the majority of protein oxidation studies have been conducted on fish mince (*10*−*13*, *15*), showing that the level of protein oxidation in bulk protein preparations increases during incubation with different free-radical-generating systems. Protein oxidation in whole mackerel fillets has also been followed (*12*), revealing carbonyl levels to increase during 8 days of storage on ice and the extent of carbonyl groups formed to be greatest in the tail portion of the fish. However, how an increase in protein oxidation affects single proteins during spontaneous deterioration has not been studied previously. Application of the 2D immunoblot approach, as described here, has shown the oxidation pattern of the individual proteins to change rather specifically. Proteomics has been used to investigate marine species and seafood products for the past 15 years (*37*), and 2D immunoblot measurements of protein oxidation might add new knowledge to the state-of-the-art evaluation of freshness and quality in fish and seafood products.

In conclusion, 2D immunoblotting shows a distinct number of rainbow trout muscle proteins to be carbonylated. Also, different proteins have different oxidation levels, indicating that oxidative modifications are protein specific. The fractionation step reveals relatively more high-salt-soluble than low-salt-soluble proteins to be carbonylated. In the low-salt-soluble fraction, the oxidized proteins all have sizes between 37 and 97 kDa, and the level of oxidation is more or less evenly distributed. After 48 h at room temperature, there is a relative increase in carbonylation within the groups of proteins that are already carbonylated in freshly defrosted fish. In the high-salt-soluble fraction carbonylation primarily can be ascribed to six proteins in the just thawed fish. However, after incubation for 48 h at room temperature, one major increase in carbonylation in this fraction seems to originate from low-salt-soluble proteins that concurrently have lost solubility and become carbonylated or simultaneously fragmentation and carbonylation of large proteins. Another decay-related increase in protein carbonylation comes from myosin heavy chain with concurrent fragmentation. The results presented here demonstrate the strength of 2D immunoblotting, enabling a detailed picture of the actual number and level of oxidized/carbonylated proteins to emerge. This elucidates the pattern of protein oxidation in fish muscle and reveals certain proteins to be more oxidized than others. Furthermore, it is demonstrated that fish muscle deterioration led to an increase of carbonylation level for distinct proteins. Future work on protein oxidation in fish would preferentially lead to the identification of these proteins with mass spectroscopy. This might clarify if oxidized proteins have a specific cellular or biochemical function and if there is a link between protein and lipid oxidation. The results achieved here provide an important basis for future studies elucidating how protein oxidation in fish is affected by, for example, different storage conditions or different fish feed combinations.

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