JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2004,** *52,* 3967−3974 **3967**

Identification of Specific Oxidatively Modified Proteins in Chicken Muscles Using a Combined Immunologic and Proteomic Approach

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Muscle proteins are generally believed to be key players in free radical processes that eventually lead to oxidative deterioration or modifications of meat proteins resulting in alterations in functionality, for example, gel-forming ability, emulsification properties, and water-binding capacity. This study addresses protein oxidation in chicken muscles using a combined immunologic and proteomic approach and identifies specific proteins that contain carbonyls and/or 3-nitrotyrosine (3-NT). Whereas α-enolase was the predominant carbonyl-reactive species among the water-soluble muscle proteins, several other proteins (actin, heat shock protein 70, and creatine kinase) contained carbonyls and/or 3-nitrotyrosine. Finally, this approach was used to demonstrate differential susceptibility of water-soluble muscle proteins toward oxidation in chickens fed a low-antioxidant diet compared with chickens fed a diet supplemented with antioxidant-rich fruits/vegetables.

KEYWORDS: Protein oxidation; carbonyls; 3-nitrotyrosine; oxidative stress; meat

INTRODUCTION

Aerobic metabolism inevitably oxidizes important biomolecules such as lipids, carbohydrates, proteins, and nucleic acids, and critical antioxidative defense mechanisms normally counteract these oxidative modifications (*1*). In vivo oxidative stress is assumed to shift this balance, resulting in irreparable oxidation of vital molecules, which then must be degraded and replaced to avoid cellular damage (*2*). Several products of excessive oxidative modifications have been described and used as biomarkers for oxidative stress (*3*), although it is apparent that redox-based protein modification is a ubiquitous and, when reversible, normal mechanism for regulation of the functional properties of a wide variety of proteins (*4*).

Proteins may be instrumental in early oxidative events because of their propensity to bind transition metal ions, such as ferrous and cuprous ions, and may themselves be oxidized to more or less stable products (*5*). Oxidation of proteins has been described in a number of pathological conditions (*6*, *7*), but identification and characterization of oxidative modifications in specific proteins have only recently progressed substantially through the use of combined immunologic and proteomic methods (*8*−*12*).

Applying a combined immunologic and proteomic approach should provide useful information about possible relationships between protein oxidation and meat quality. Until now, most studies of protein oxidation and the implications for quality of muscle-based foods have relied on the determination of protein solubility (*13*−*15*), formation of total protein carbonyls (*16*−*18*), or degree of polymerization (*19*−*21*) as related to specific meat quality traits. However, most of the previously reported relationships between degree of protein oxidation and meat quality have not included characterization of the specific proteins involved. Consequently, a specific understanding of which proteins are oxidized during the post-mortem process, storage, and processing could identify alterations of importance for physical characteristics related to the quality of muscle-based foods and could subsequently be useful in the quality control of such foods.

Immunologic techniques have previously been described for the detection of carbonyls (C═O), which arise from oxidation of various amino acid residues (*22*, *23*), and for specific oxidation of tyrosine to form 3-NT (*24*, *25*). The latter may occur through reaction with peroxynitrite arising from NO and superoxide (*26*) or, even more relevant to muscle-based foods, through peroxidase/hemeprotein-catalyzed nitration of tyrosine via a H_2O_2-dependent oxidation of nitrite and tyrosine (*27*−*31*), with maximum efficiency at meat pH (5.3−5.9) (*32*, *33*).

We have previously addressed oxidative stability in muscles from chickens fed a low-antioxidant diet and the effect of supplementation with fruits and vegetables. The total content of carbonyls in water-soluble muscle proteins appeared to be unaffected, whereas a significant decrease in carbonyl content of insoluble proteins following the dietary supplementation was observed (*34*). We reasoned that oxidation of specific proteins might be differentially affected by dietary conditions, and we therefore combined immunologic and proteomic methods to test

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10.1021/jf035503d CCC: $27.50
Published on Web 05/15/2004

the feed-induced differences in the antioxidative capacity of chicken muscles.

MATERIALS AND METHODS

Chemicals. 2,4-Dinitrophenylhydrazine (DNPH) was obtained from Lancaster Synthesis, Morecombe, U.K. Antibody against 3-NT was obtained from Oxis, Portland, OR. Secondary antisera conjugated to HRP were from Dako, Glostrup, Denmark. Anti-2,4-dinitrophenylalanine antiserum and all other regents (analytical grade) were obtained from Sigma, St. Louis, MO.

Animals. One-day-old female chickens (Ross 208) were obtained from a commercial hatchery and raised at our local facility under standard conditions with free access to water and feed. Chickens were housed in pens (20 chickens per 1.44 m^2) and fed a standard diet as previously described (*34*). These standard chickens were used for development of assays and conditions (**Figures 1**−**3**). For the experiment shown in **Figure 4**, chickens were—from day 21—fed either sweet corn (*Zea mays* L. var. *Saccharata*) or a mixture of apple (Cox's Orange) and broccoli (*Brassica oleracea* L. var. *italica*) (1:1 w/w) constituting ∼10% based on energy or continued on standard diet low in antioxidants (LAD) as described (*34*) until slaughter, at 42 days. Chickens ate the supplements before they were given free access to the LAD diet. Samples of breast muscle (M. pectoralis major) or thigh muscle (M. iliotibialis) were rapidly excised and frozen in liquid nitrogen within a few minutes post-mortem. Samples were stored at −80 °C until homogenization.

Tissue Homogenization. Tissue samples (100 mg) were homogenized in 0.5 mL of standard homogenization buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 255 mM sucrose) using an Ultraturrax blender (13000 rpm for 5 s on ice). The homogenate was centrifuged (21000*g* for 30 min at 4 °C) and the supernatant stored at −80 °C until use. Protein content was determined after appropriate dilution in water using the bicinchoninic acid method as recommended by the manufacturer (Sigma) and using bovine serum albumin as standard.

For the solubility experiment, distilled water, one-dimensional (1D) sample buffer (0.1 M Tris-HCl, pH 8, 6 M urea, 10% SDS), or two-dimensional (2D) sample buffer (7 M urea, 2 M thiourea, 2% CHAPS, 1% dithiotreitol) was used for homogenization in addition to the standard homogenization buffer.

Protein Derivatization and Immunoblot Detection of Oxidized Proteins. Supernatants from tissue homogenates prepared as described above were diluted in SDS sample buffer [100 mM Tris, pH 6.5, 10% (w/v) SDS, 30% (v/v) glycerol, 100 mM dithiotreitol] to a protein content of ∼1 mg/mL and heated at 95 °C for 5 min. Proteins (∼50 μg) were separated on 10% acrylamide/SDS gels and transferred to PVDF membranes by semidry electroblotting. Membranes for detection of 3-NT-containing proteins were blocked and incubated with primary and secondary antibodies as described below. Detection of protein carbonyls was as described (*23*). Briefly, membranes were rinsed in 2 N HCl and incubated with 2,4-dinitrophenylhydrazine (DNPH; 2 mg/mL dissolved in 2 N HCl) for 5 min at room temperature on a platform rocker. Unreacted DNPH was removed by washing six times in methanol and once in TBS (10 mM Tris, pH 8.0, 100 mM NaCl).

Membranes were blocked in TBS containing 2% (v/v) Tween-20 and 3% (w/v) nonfat dry milk powder and incubated with primary and secondary antibodies in TBS with 0.1% Tween-20 and 1% milk powder.

For the detection of DNP-derivatized proteins, anti-DNP antiserum was used at 1:10000, followed by secondary HRP-conjugated antiserum also at 1:10000. For detection of 3-NT-containing proteins, the antibody was diluted 1:1000 and the secondary HRP-conjugated antiserum 1:10000. Incubations with primary antibodies were performed overnight at 4 °C, whereafter the membranes were washed thrice in TBS. Incubations with secondary antisera were at room temperature for 1 h followed by three washes with TBS. Membranes were subsequently processed for enzymatic chemiluminescence (ECL). Quantity-one software (Bio-Rad, Hercules, CA) was used for quantification of band intensities.

2D Electrophoresis. Samples were extracted, separated, and analyzed as previously described (*35*). Briefly, 100 mg of muscle tissue samples was homogenized in 7 M urea, 2 M thiourea, 2% CHAPS, and 1% DTT in a hand-held glass homogenizer. 2D separation was performed with immobilized pH 4−7 gradients, whereas SDS-PAGE for the second dimension separations was 10% Laemmli gels. Analytic or preparative gels were loaded with aliquots of 50 or 500 μg of protein, respectively. Analytic gels were either silver-stained (*36*) or electroblotted to PVDF membranes for further immunologic detection as described above. Preparative 2D gels were stained by imidazole-zinc precipitation as described (*37*).

In-Gel Digestion of Proteins. Samples for matrix-assisted laser desorption ionization time of flight (MALDI-TOF) analyses were excised from preparative 2D gels or from 1D 10% SDS-PAGE. In-gel digestion of the individual protein spots was performed as described (*38*). Briefly, the excised gel pieces were washed in 50 mM NH_4CO_3, pH 7.8, followed by washes in 50 and 100% acetonitrile and dried by vacuum centrifugation. Fifteen microliters of trypsin (sequencing grade, Roche, Mannheim, Germany), dissolved in 50 mM NH_4CO_3, pH 7.8 (12 ng/μL), was added to the dry gel pieces and left to reswell on ice for 1 h, whereafter the supernatant was removed, 30 μL of 50 mM NH_4CO_3, pH 7.8, was added, and the digestion was incubated overnight at 37 °C. Prior to mass spectrometry analyses, the samples were desalted and concentrated as described (*39*).

Peptide-Mass Mapping by MALDI-TOF MS. A Bruker REFLEX IV model MALDI-TOF mass spectrometer (Bruker-Franzen Analytik GmbH, Bremen, Germany) was used in positive-ion reflector mode for mass analysis of tryptic peptide mixtures (peptide-mass mapping). The ion acceleration voltage was 20 kV. Calibration was performed using the autolytic trypsin peaks at *m*/*z* 842.11 and 2211.1. The average deviation in mass accuracy was 50 ppm. Protein identifications were performed using the database search program Mascot, searching the Swiss-Prot database. The peptide-mass maps and protein identifications were evaluated as described (*38*).

Data Analysis. Statistical analysis was carried out using the Statistical Analysis System, version 8.00 (SAS Institute, Cary, NC). The General Linear Models (GLM) procedure was used for analysis of variance. Least-squares means were considered to be significantly different if $P < 0.05$.

RESULTS

Detection of Protein Carbonyls. Soluble proteins (**Figure 1**) were extracted from chicken breast muscle (M. pectoralis major) or thigh muscle (M. iliotibialis) with homogenization buffer and separated by reducing SDS-PAGE. After derivatization of carbonyl groups with DNPH and blotting with anti-DNP antiserum, the soluble muscle protein fraction contained only a single major carbonyl-reactive species of MW ∼45 kDa (**Figure 1**). This band was at least 10-fold more intense than less abundant DNPH-reactive proteins as estimated from the 10 and 120 s exposures. The 120 s exposure revealed minor DNPH-reactive proteins of MW ∼75, ∼65, ∼45, ∼40, and ∼35 kDa. These blots demonstrate that carbonyl reactivities are similar for the two types of muscles, although clear differences in specific bands could be observed. Interestingly, two bands (MW ∼75 and ∼45 kDa) were much more carbonylated in thigh muscle than in breast. In contrast, breast muscle displayed some minor bands (MW ∼65 and ∼35 kDa) that were not detected in thigh muscle. None of these bands could be unambiguously identified from the Coomassie-stained gels.

Blots that were either not derivatized with DNPH or reduced with sodium borohydride prior to derivatization and subsequently processed parallel to DNPH-derivatized blots gave no detectable signal (data not shown).

Identification of Oxidized Proteins by 2D Electrophoresis and MALDI-TOF. After the analyses shown in **Figure 1** using DNPH derivatization and immunoblotting techniques, we also analyzed for 3-NT using specific antibodies against this oxidation product (**Figures 2** and **3**).

Figure 2 shows a silver-stained gel of proteins from chicken breast muscle that could be resolved by 2D electrophoresis and

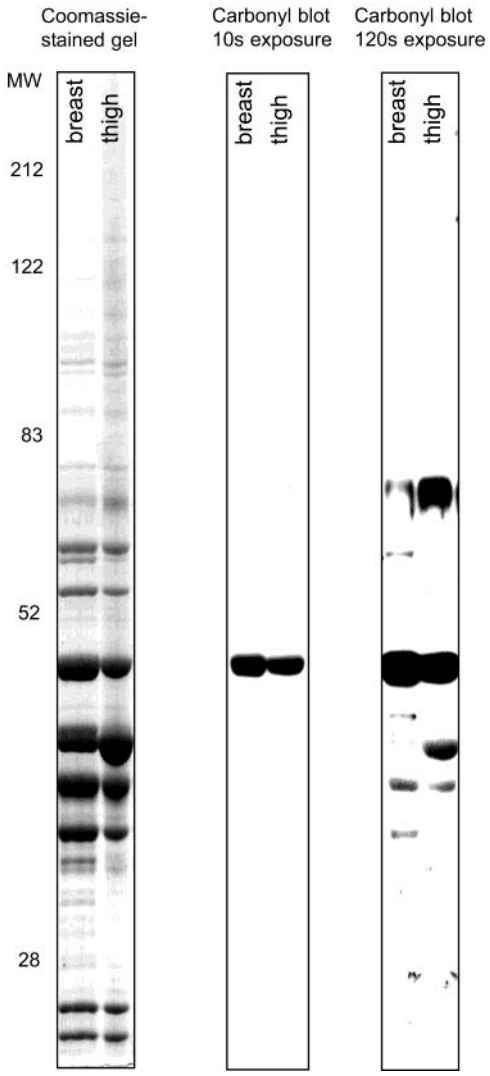

Figure 1. Soluble muscle proteins from chicken breast (M. pectoralis major) or thigh (M. iliotibialis) muscles. Coomassie-stained gel (left) and carbonyl blot after 10 s of exposure (middle) or after 120 s of exposure (right) are shown. Molecular weights (MW) of standards are indicated.

the corresponding carbonyl and 3-NT blots. The major carbonyl-reactive protein was detected with relative migration (MW ~45 kDa) in the second dimension consistent with the 1D analysis in **Figure 1** and was identified by MALDI-TOF MS as α-enolase. This protein occurred as multiple spots of the same apparent molecular weight, but with different isoelectric points in the silver-stained gels. The major isoform had an apparent p*I* ~6.3, and there was at least one isoform with higher p*I* and four more acidic forms as seen in the silver-stained gel. The two carbonyl-reactive enolase species had p*I* ~6.3 and ~6.2, and both enolase isoforms as well as an additional, more acidic isoform were detected by anti-3-NT antibody.

A protein of MW ~100 kDa and p*I* ~5.3 (actually a doublet) also gave strong carbonyl and 3-NT reactivity. Both spots were identified as α-actinin, a structural protein of myofibrils. This protein was not apparent in **Figure 1**, most likely because of its low solubility in the extraction buffer used for that experiment (this issue is further addressed below).

Actin, a major component of myofibrils and characteristically identifiable as indicated in **Figure 2**, showed weak carbonyl reactivity but somewhat stronger 3-NT reactivity. It is apparent from the 2D gels that the different actin isoforms had differential reactivities for carbonyls and 3-NT, as shown by the different shapes of the actin spot in **Figure 2**.

A protein that gave a weak carbonyl but a stronger 3-NT signal was identified as albumin, and a slightly larger protein, which gave a very faint carbonyl but strong 3-NT reactivity, was identified as a member of the heat shock protein 70 family of molecular chaperones (hsp70). Finally, creatine kinase, which is an abundant protein in muscle, was identified as shown in **Figure 2**, and found to contain significant 3-NT reactivity but no carbonyls. Additional carbonyl or 3-NT reactive protein spots in **Figure 2** were present in insufficient amounts to be unequivocally identified by the current analysis. **Table 1** summarizes the proteins identified by 2D electrophoresis and MALDI-TOF.

Differential Solubility. The different protein solubilization procedures used for the one- and two-dimensional analyses apparently gave somewhat different results, and this prompted us to compare various solubilization schemes. **Figure 3** shows a side-by-side comparison of chicken thigh muscle homogenized in water, homogenization buffer, 2D sample buffer, or 1D sample buffer. Proteins were visualized with Coomassie staining of gels either before or after transfer to the membrane for immunodetection with DNPH derivatization and anti-DNP antiserum or 3-NT antibody as indicated in **Figure 3**.

The presence of urea in the homogenization buffer (2D and 1D sample buffers) resulted in solubilization of high molecular weight proteins, predominantly myosin, but also large amounts of water-insoluble actin became soluble as seen by the major bands on Coomassie-stained gels. Most proteins below ~90 kDa were efficiently transferred to the membrane. Actin was fairly soluble in water and standard homogenization buffer and had few carbonyls (faint band below the strong enolase band in **Figure 3**), but the relatively larger amount of actin solubilized in both 2D and 1D sample buffers also gave a larger carbonyl band. Interestingly, the carbonyl-reactive actin solubilized in 2D and 1D sample buffers apparently had no 3-NT reactivity, although water-soluble actin contained some 3-NT in line with the results of 2D analysis. Myosin was expectedly insoluble in water and standard homogenization buffer, but readily solubilized in both 2D and 1D sample buffers; however, it was poorly transferred to the membrane. Several protein bands below myosin contained both carbonyls and 3-NT, and some of these proteins were solubilized only in 2D sample buffer, maybe due to the presence of dithiotreitol in this buffer. Multiple bands with 3-NT content in the range of 80−100 kDa were all largely insoluble in water and standard homogenization buffer, and some were soluble only in 2D sample buffer. These bands showed clear differences regarding content of carbonyls and 3-NT. Actinin, which has a molecular weight of ~105 kDa, likely represented one of these bands in line with the results of the 2D blots.

A protein band (MW ~75 kDa) appeared to be specific for carbonyls and soluble in all four buffers but not a major band by Coomassie staining. A protein ~65 kDa was strongly and specifically anti-3NT reactive and was soluble in all buffers. The major carbonyl-reactive band consistent with enolase also reacted with 3-NT antibody and was fully soluble in water. Finally, a specifically and strongly 3-NT reactive protein, in fact, the most intense band on short exposures (data not shown), with MW ~27 kDa was soluble in all buffers.

Although proteins of molecular weight >~100 kDa contained relatively large amounts of both carbonyl and 3-NT, these proteins were inefficiently transferred to the membrane, and comparisons to proteins that are quantitatively transferred or

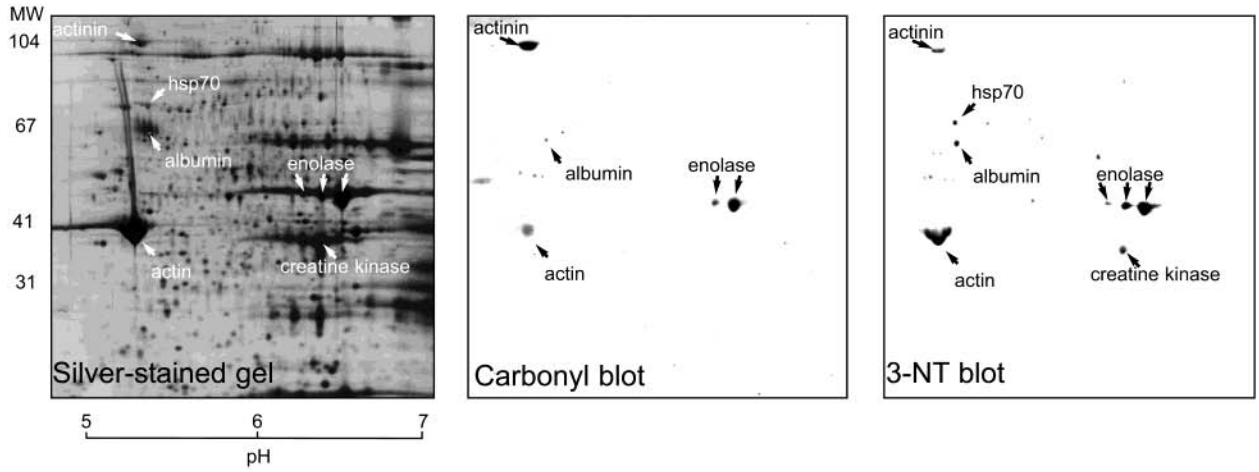

Figure 2. 2D electrophoresis and immunodetection. Representative silver-stained gel, carbonyl blot, and 3-NT blot are shown. At least four replicates were performed for each analysis. The pH gradient used for isoelectric focusing and molecular weights (MW) of standards are indicated for the silver-stained gel.

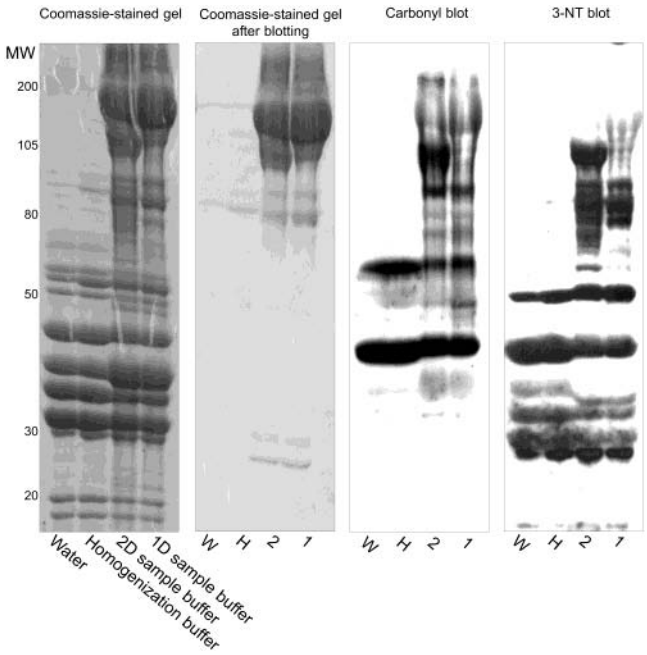

Figure 3. Solubility of chicken thigh muscle proteins. Each display is a 1D SDS electrophoresis of thigh muscle homogenized in water (W), standard homogenization buffer (H), 2D sample buffer (2), or 1D sample buffer (1). Coomassie-stained gels before or after transfer to membrane, as well as immunodetection of carbonyls or 3-NT by ECL after transfer to membranes, are shown. Molecular weights (MW) of protein standards are indicated to the left. Displays shown are representative of three replicates from one experiment.

between different gels are therefore difficult. Other analytical methods, for example, partial hydrolysis to reduce molecular weight, are required for quantitative determination of oxidative modification in these high molecular weight proteins.

Quantitation of Protein Oxidation following Dietary Supplementation. Soluble proteins were prepared from breast muscles of 32 chickens from four different feeding regiments (eight chickens per group): low-antioxidant diet (lad), lad supplemented with a mixture of apple/broccoli or corn, and finally a conventionally fed group. Proteins were analyzed for content of both carbonyl and 3-NT by Western blotting and band intensity quantified by densitometry, which is shown in **Figure 4**. Only one unidentified protein (MW ~55) was

Table 1. Proteins Identified by 2D Electrophoresis and MALDI-TOF

identified proteins[a]	Mascot score[b]	matching peptides[c]	sequence coverage[d]	SWISS-PROT[e]	calcd MW[f]	estd MW[g]	calcd pI[h]	estd pI[i]
α-actinin	354	46	55	P20111	104779	104	5.26	5.3
HSP70	161	18	63	P08106	69750	70	5.53	5.4
albumin	240	29	42	P19121	65667	67	5.35	5.4
α-enolase	97	14	42	P51913	47486	47	6.16	6.2–6.3
actin	70	13	35	P02568	42476	42	5.39	5.3
creatine kinase	107	16	46	P00565	43529	40	6.50	6.2

[a] Proteins identified by peptide mass fingerprinting. [b] Mascot score is $-10 \times \text{Log}(P)$, where P is the probability that the observed match is a random event. Protein scores >72 are significant ($p < 0.05$). [c] Number of matched peptides in the database search. [d] Coverage of the matched peptides in relation to the full-length sequence. [e] Primary accession number in the SWISS–PROT database. [f] Calculated molecular mass (Da) of full-length chicken protein. [g] Molecular mass (kDa) of the identified spot as estimated from **Figure 2**. [h] Calculated isolectric point of the full-length chicken protein. [i] Isoelectric point of protein spot as estimated from **Figure 2**.

significantly more oxidized, as analyzed for content of carbonyls, in muscles from chickens fed the low-antioxidant diet (lad) compared to muscles from chickens fed lad + broccoli/apple ($p = 0.0048$), lad + corn ($p = 0.003$), and conventional diet ($p = 0.0175$), respectively. A protein with MW ~45 kDa, likely enolase, was significantly more oxidized, as analyzed for content of 3-NT, in muscles from chickens fed lad + corn compared to muscles from chickens fed lad ($p = 0.0139$) or conventional diet (0.0081), respectively. Moreover, another unidentified muscle protein (MW ~27 in **Figure 4**) contained significantly more 3-NT in muscles from chickens fed conventional diet compared to chickens fed lad ($p = 0.0453$), whereas there was no significant difference from the two groups supplemented with vegetables.

DISCUSSION

As a first step in trying to understand the occurrence and influence of protein oxidation on oxidative stability of muscle and muscle-derived foods, we have identified proteins in chicken breast and thigh muscle that contained either carbonyls or 3-NT, or both.

Enolase appeared to be extensively oxidized in both muscle types and contained both carbonyls and 3-NT. It is a major glycolytic enzyme, catalyzing the dehydration of 2-phospho-D-glycerate into phosphoenolpyruvate, and it is relatively

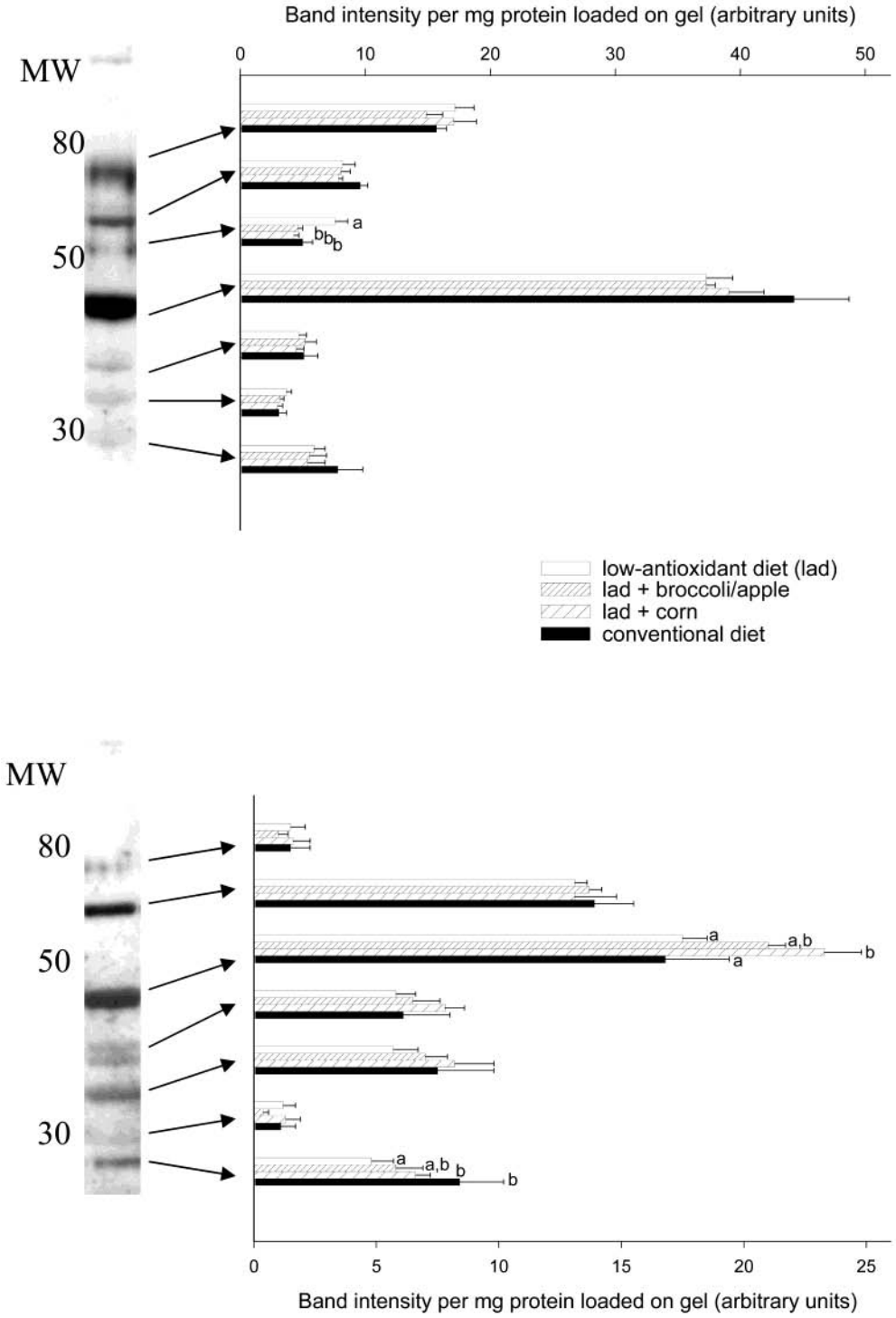

Figure 4. Quantitation of carbonyls (top) and 3-NT (bottom) in soluble breast muscle proteins. A representative lane for detection of carbonyls or 3-NT is shown, and molecular weights (MW) of standards are indicated. The bars represent means and SEM from eight chickens per dietary group. Different letters indicate that band intensities are significantly different.

abundant in muscle as evident from **Figures 1** and **2**. Formation of carbonyl groups in enolase has previously been observed in bacteria (*40*), where it is the major carbonyl-reactive protein following challenge with hydrogen peroxide. Enolase carbonyls have also been described in yeast (*41*) and under certain pathological conditions (Alzheimer's disease) in the human brain (*9*). Enolase appears to be protected against oxidative modifications by the glutathione redox buffer as it is extensively glutathionylated even under basal conditions in T lymphocytes, probably because it is exquisitely sensitive to oxidation (*42*). The enolase isoform that is predominantly expressed in thigh muscle and extensively carbonylated was shown by MALDI-TOF to be the α isoform. The intense oxidation of enolase in chicken muscle is interesting, as it might be a good indicator of the antioxidative status of the muscle, even though further investigations are needed to confirm this. Oxidation of enolase, however, is presumably without influence on the functionality of muscle for further processing, where the critical components are the structural muscle proteins.

The polymerization and dissociation of actin is known to be affected by nitration (*43*), and excessive 3-NT formation in actin is associated with sickle-cell disease (*44*). Carbonylation of actin has also been described to destabilize polymerization (*45*), and increased occurrence of carbonyl-containing actin has been observed in both Alzheimer's disease brains and ischemic hearts. It might be hypothesized that carbonylation and/or nitration of actin have implications for both texture and water-holding capacity of fresh meat and, therefore, that such changes may affect the functionality of the muscle, as actin is a critical component in many of the protein networks created during further processing. Consequently, more investigations are indeed needed to elaborate this issue.

Albumin is present in the systemic circulation, and residual blood in the muscle samples must be the source of the oxidized albumin detected in the blots. The higher amount of albumin in the preparation of soluble thigh muscle proteins compared to breast is in line with more vascularization of the former. Oxidized albumin is also present in the soluble protein fraction from liver (data not shown), where it is synthesized and secreted for circulation.

Creatine kinase is also relatively abundant in muscle. Creatine kinase in the water-soluble muscle fraction was identified in **Figure 2** as 3NT-reactive. Creatine kinase has previously been identified as oxidation-sensitive (*46*) and shown to be specifically carbonylated in Alzheimer's disease (*47*, *48*) and during atrial fibrillation (*49*), the latter also with increased 3-NT.

The presence of carbonyl groups in heat shock proteins (hsp), and in particular the hsp70 family, has been described previously in Alzheimer's disease (*9*). As with enolase, the degree of oxidation of both creatine kinase and hsp's might be good indicators for evaluation of the oxidative status of the muscle in relation to storage life.

Peroxynitrite ($NO^{\bullet} + O_2^{\bullet -}$) has for some time been speculated to be a potential pro-oxidant in muscle foods (*50*−*52*), and 3-NT is known to be a marker of oxidative stress; however, our demonstration of nitrosylation of specific muscle proteins is the first evidence for this taking place in muscle foods. Whether peroxynitrite is formed through the reaction between NO and superoxide (*26*) or through peroxidase/hemeprotein-catalyzed nitration of tyrosine via a H_2O_2-dependent oxidation of nitrite and tyrosine (*27*−*31*) cannot be explored by the present data; however, this observation encourages further studies of the potential influence of the formation of both peroxynitrite and 3-NT on the quality development in fresh and nitrite-cured meat products.

We have previously shown that the gross carbonyl content in water-soluble and -insoluble proteins appeared to be differentially affected after chickens had been fed antioxidant-rich diets (*34*). Thus, water-soluble protein carbonyls did not show any overall effect of dietary supplementation, whereas water-insoluble proteins, likely dominated by myosin and actin, contained significantly fewer cabonyls after supplementation of a low-antioxidant diet with either apple/broccoli or corn. Chickens fed a conventional diet, that is, containing various natural and synthetic antioxidants, also had a lower content of carbonyls in water-insoluble breast muscle proteins. Subsequently, we speculated that similar gross carbonyl contents might conceal important differences in individual proteins and that oxidative modifications of particular water-soluble proteins, conveniently measurable using immunological reagents, could be markers for oxidative stability of meat derived from different feeding strategies. To test this, and at the same time support the potential applicability of the combined immunologic and proteomic approach demonstrated above in a food-related area, we have analyzed specific water-soluble breast muscle proteins from the above study for degree of carbonylation and nitrosylation. Thus, the analyses for carbonyl and 3-NT in the soluble breast muscle proteins depicted in **Figure 4** indicate that oxidative differences in individual proteins indeed occur depending on the given diets. However, we want to caution that these analyses may not be perfectly quantitative, as unknown differences in solubility of individual proteins could influence the efficiency of detection.

The present study shows that the introduction of a combined immunologic and proteomic approach will provide a further and more fundamental understanding of protein oxidation and its implications for the quality of muscle-based foods and should be of great advantage in future studies.

ABBREVIATIONS USED

ECL, enzymatic chemiluminescence; DNPH, 2,4-dinitrophenylhydrazine; DNP, 2,4-dinitrophenylalanine; 3-NT, 3-nitrotyrosine; TBS, Tris-buffered saline; 1D, one-dimensional; 2D, two-dimensional.

ACKNOWLEDGMENT

We appreciate the skillful technical assistance of Rita Albrechtsen and Hanne Søndergård-Møller. We thank Dr. René Lametsch for identification of some proteins by MALDI-TOF. Moreover, we thank Dr. Anders Hans Karlsson for assistance with the data analysis.

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Received for review December 23, 2003. Revised manuscript received March 19, 2004. Accepted April 14, 2004.

JF035503D