Chemical, Physical, and Functional Properties of Cod Proteins Modified by a Nonenzymic Free-Radical-Generating System

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Chelated ferric iron, hydrogen peroxide, and ascorbate were used to generate hydroxyl radical in washed, minced cod muscle. Protein carbonyl content increased between 2 and 24 h of storage at 5 °C on treatment with the free-radical-generating system (FRGS); it increased less if that storage period included a freeze/thaw process. There was an equal loss of sulfhydryl groups on treatment with the FRGS for 2 or 24 h with or without a freeze/thaw treatment. Electrophoretic results suggested disulfide formation. Treatment with the FRGS and a freeze/thaw process decreased protein solubility; a combination of the two was synergistic. Washed, minced cod muscle had improved gelation and emulsification characteristics when treated with the FRGS if the muscle tissue was not frozen but had poorer qualities if it was frozen. A model is proposed to explain the improvement of gelation and emulsification properties on exposure to the FRGS as well as its loss when exposed to a freeze/thaw process.

Keywords: *Proteins; free radical effects on; free radical effects on proteins; oxidation of proteins; cod proteins*

INTRODUCTION

Although emphasis on oxidative deterioration in meat and fish has traditionally concentrated on lipid components, it is becoming increasingly clear that proteins may be important targets in muscle foods (Decker et al., 1993; Srinivasan and Hultin, 1995) as well as in vivo (Davies and Goldberg, 1987). This is not surprising in view of the fact that many of the pro-oxidants in muscle are found in the aqueous phase. If a highly reactive radical initiates the oxidative reaction, it could be expected that the damage would be done close to the point where the radical was produced (Dean et al., 1991); highly reactive radicals would not be expected to diffuse very far before they contacted molecules with which they would interact. Since the contractile proteins of muscle tissue are bathed in the aqueous phase of the cell, they should be susceptible to attack by radicals produced in the aqueous phase.

In a previous paper (Srinivasan and Hultin, 1995), we used an enzymic system of xanthine oxidase and hypoxanthine to produce hydroxyl radical in washed, minced cod muscle. It was shown that this led to oxidation of the protein with little change in the solubility. However, the oxidized protein lost its solubility much more rapidly than unoxidized protein when subjected to further stress treatments such as freezing and thawing or a low pH. There also appeared to be some cross-linking via disulfide bonds.

The principal purpose of this paper was to extend the observations on modification of fish muscle proteins by hydroxyl radicals to functional properties such as gel-forming ability and emulsification characteristics, while keeping the protein modification similar to what would be expected in stored fish muscle. Further, we wished to do this using a free-radical-generating system that would not have the possibility of modifying the results by the introduction of extraneous enzymes. Davies and Goldberg (1987) showed that there were proteases in commercial xanthine oxidase preparations. Thus, a nonenzymic free-radical-generating system was used in these studies.

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MATERIALS AND METHODS

Materials. Bled and gutted Atlantic cod (*Gadus morhua*) were obtained from local day boats in Gloucester, MA, and transported to the laboratory on ice. The sodium salt of methanesulfinic acid was obtained from Fairfield Chemical Co. (Blythewood, SC). Coomassie Brilliant Blue R-250 and 2,4-dinitrophenylhydrazine were purchased from Sigma Chemical Co. (St. Louis, MO). Fast blue BB salt was from Aldrich Chemical Co. (Milwaukee, WI). Coomassie protein assay reagent was obtained from Pierce Chemical Co. (Rockford, IL). Electrophoretic grade chemicals were obtained from Bio-Rad Laboratories (Richmond, VA). All other chemicals used were of analytical grade.

Preparation of Samples. Washed, minced cod muscle was prepared as described previously (Srinivasan and Hultin, 1995).

Stock solutions of the components of the free-radical-generating system were prepared immediately prior to use. Adenosine 5′-triphosphate (ATP) was used after thawing a 50 mM solution stored at −20 °C. Ferric iron as $FeCl_3 \cdot 7H_2O$ and ascorbic acid were prepared by dissolution in deionized distilled water. Hydrogen peroxide (H_2O_2) was diluted from a 30% solution. The components of the free-radical-generating system were mixed in the order of 0.04 M phosphate buffer (pH 6.8), 200 μM ATP, 100 μM ferric iron, 2 mM H_2O_2, and 100 μM ascorbic acid (concentrations represent final values). When the ascorbate concentration was increased to 500 μM and 1 mM, the concentration of H_2O_2 was increased to 10 and 20 mM, respectively, while all the other components of the free-radical-generating system remained the same. As soon as ascorbate was mixed with the other components of the free-radical-generating system, the solution was added to the washed mince and mixed thoroughly. Control mince contained buffer and deionized distilled water corresponding to the same

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volume as that in the free-radical-generating system. The final volume of the free-radical-generating system was 20 mL with 1 mL each of the ferric iron, ATP, hydrogen peroxide, and ascorbate solutions described above and 16 mL of phosphate buffer for 89 g of washed mince (90% moisture).

Samples with and without the free-radical-generating system were stored as follows. One set was stored for 2 h at 5 °C. A second set was stored for 24 h at 5 °C. A third set was stored for 2 h at 5 °C, 20 h at −10 °C, and 2 h at room temperature (20−25 °C) to thaw. This sample is referred to as the "frozen thawed" sample and was used to test the additional stress of freezing and thawing on protein stability in addition to that of free radicals. All samples (20 g/replication) except for those for determining gel-forming ability were kept in Petri dishes for storage in a still freezer (−10 °C) as detailed by Srinivasan and Hultin (1995).

Determination of Hydroxyl Radical. Hydroxyl radical was determined as methane sulfinic acid (MSA) formed from 1.0 M dimethyl sulfoxide (DMSO). The procedure of Smith et al. (1990) as modified by Srinivasan and Hultin (1995) was used.

Carbonyl Content of Proteins. Protein carbonyls were assayed as hydrazone derivatives by reacting with 2,4-dinitrophenylhydrazine (DNPH). The procedure of Levine et al. (1990) as modified by Srinivasan and Hultin (1995) was used.

Sulfhydryl Content of Proteins. Total sulfhydryls were determined by reacting with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as described by Simplicio et al. (1991). A 1.0 g sample of washed mince was homogenized in 50 mL of cold (5−7 °C) deionized distilled water with a Teflon pestle in a glass homogenization tube (6−10 passes). An aliquot of the homogenate was dissolved in a urea−SDS solution containing 8 M urea, 2 M thiourea, 3% SDS, and 0.05 M Tris (pH 8.0) at a rate of 1:1 homogenate:urea−SDS solution and immediately assayed. To 1 mL of the sample in urea−SDS solution were added 2.0 mL of 0.1 M phosphate buffer (pH 7.4), and 0.5 mL of 10 mM DTNB (in 0.1 M phosphate buffer, pH 7.4). A time scan was run at 412 nm for 8 min. The reference cuvette contained all of the components except DTNB. A blank was also run for 8 min to account for change in the absorbance of DTNB in the absence of proteins and the value subtracted. The concentration of sulfhydryls was estimated using a molar extinction coefficient of 11 400 M^{-1} cm^{-1}.

Free Amino Groups in Proteins. Free amino groups in proteins were determined by reacting with 2,4,6-trinitrobenzenesulfonic acid (TNBS) as described by Snyder and Sobocinski (1975). Glycine was used to construct a standard curve. Washed mince proteins were solubilized in 6 M guanidine hydrochloride and assayed.

Solubility of Proteins. Solubility of proteins was determined in 1.0 M LiCl and 0.02 M Li2CO3 (pH 7.2) as described by Kelleher and Hultin (1991).

Electrophoresis. A discontinuous SDS system of Laemmli (1970) with a 2.5% stacking gel and a precast 3−27% gradient separating gel (Integrated Separation Systems, Natick, MA) was used. Sample buffer with and without 75 mM dithiothreitol contained 8 M urea, 2 M thiourea, 3% SDS, 0.05 M tris (pH 8.0), and 0.05% bromophenol blue (Fritz and Greaser, 1991). Preparation of samples and gel running conditions were described in detail by Srinivasan and Hultin (1995).

Gel-Forming Ability. The gel-forming ability of washed mince was assessed by a torsion test as described by Wu et al. (1985). After being centrifuged at 100000*g* for 30 min to a moisture content of 76−78%, washed mince (500 g/sample) with or without treatments (free-radical-generating system and freeze/thaw cycle) was mixed with sorbitol (8%) and sodium tripolyphosphate (0.2%) and stored at −40 °C overnight. Thus, the sample exposed to the freeze/thaw cycle was frozen twice, the first time at −10 °C without cryoprotectants and then with cryoprotectants at −40 °C, while the control sample was frozen only once in the presence of cryoprotectants at −40 °C. The frozen samples were thawed at 5−7 °C for 2 h, cut into small pieces, and chopped for 30 s in a stainless steel bowl previously chilled to −20 °C. Sodium chloride with or without cold water (5−7 °C) was sprinkled on the chopped sample to give final values of 2% salt and 78−80% moisture. The chopping was continued until the temperature reached 5−7 °C (∼3 min).

The chopped samples were filled immediately into stainless steel tubes (19 mm i.d.) previously sprayed with a lecithin-containing Pam vegetable oil spray (American Home Products Inc., New York, NY). The filled tubes were sealed on both ends and incubated in a water bath at 25 °C for 3 h and cooked at 90 °C for 15 min. The cooked gels were immediately cooled on ice for 30 min and stored at 5−7 °C for 48 h. After equilibration at room temperature for 2 h, dumbbell-shaped specimens were prepared as detailed by Montejano et al. (1983). Torque and angular displacement were recorded while twisting samples to the point of failure in a Brookfield digital viscometer (Brookfield Engineering Laboratories, Stoughton, MA), and shear stress and true shear strain were calculated as described by Wu et al. (1985).

Expressible Moisture. The centrifugal procedure of Hseih and Regenstein (1984) for estimating water loss in cooked gel was used. Cooked gels of washed mince were prepared as described in the method for determining gel-forming ability.

Emulsifying Ability. The emulsifying ability of the washed mince was determined by the method of Pearce and Kinsella (1978). An aqueous suspension of washed mince (90 g) containing 5 mg of protein/mL was mixed with 27.23 g (equivalent to 30 mL) of 100% pure peanut oil in an Oster Mini-Blend jar of 1 cup capacity (Sunbeam-Oster Household Products, Milwaukee, WI). The protein and oil mixture was blended in an Osterizer (Sunbeam-Oster Household Products, Milwaukee, WI) at "liquefying speed" for 1 min. All operations were carried out at room temperature. This is a technique that measures the ability of the protein to emulsify a vegetable (peanut) oil as measured by turbidity (absorbance at 500 nm) after blending and dilution in SDS. It is expressed as the emulsifying activity index (EAI), calculated as $2T/jc$ (m^2/g), where T (turbidity) is a function of absorbance, j is the oil fraction volume, and c is the concentration of protein in the aqueous phase (g/cm^3). The stability of the emulsion formed was monitored by determining the EAI of the emulsion with time at room temperature.

Color Values. Color measurements were made with a Hunterlab Labscan II (Hunter Associates Laboratory, Inc., Reston, VA) with a 16 mm sample orifice, 10°, and D65 illuminant. The instrument was standardized with a white tile ($L = 91.03$, $a = -1.00$, $b = 1.57$; 2°; whiteness index = 75.05). Cooked gels of washed mince were prepared as described in the method for determining gel-forming ability. Tristimulus color values of "*L*", "*a*", and "*b*" of cooked gels (diameter 19 mm and height 28.7 mm) were measured by placing them on a disposable polystyrene culture dish (diameter 35 mm, thickness 10 mm).

Statistical Methods. All experiments were performed in duplicate, and in each experiment, at least two replicates were done unless otherwise specified. Washed mince was prepared from the same fish for each experiment. Analysis of variance was performed utilizing a General Linear Models procedure of the SAS system for personal computers (SAS, 1988). Mean separation was done with linear single degree of freedom comparisons.

RESULTS

Free-Radical-Generating System. Hydroxyl ($HO^{\bullet}$) radical generation was measured by the conversion of dimethyl sulfoxide (DMSO) to methanesulfinic acid (MSA) in the presence of ferric iron, ATP, hydrogen peroxide, and ascorbate. MSA production was measured with increasing ascorbate, hydrogen peroxide, and ATP concentrations (Figure 1). When ATP was added as a chelater, a small increase in MSA formation occurred irrespective of the ATP concentration over the range from 0.05 to 1 mM. Little MSA was formed in the absence of added hydrogen peroxide; the system was saturated at about 1 mM. On the other hand, increase in MSA production continued in a linear fashion as the ascorbate concentration was increased to 1 mM.

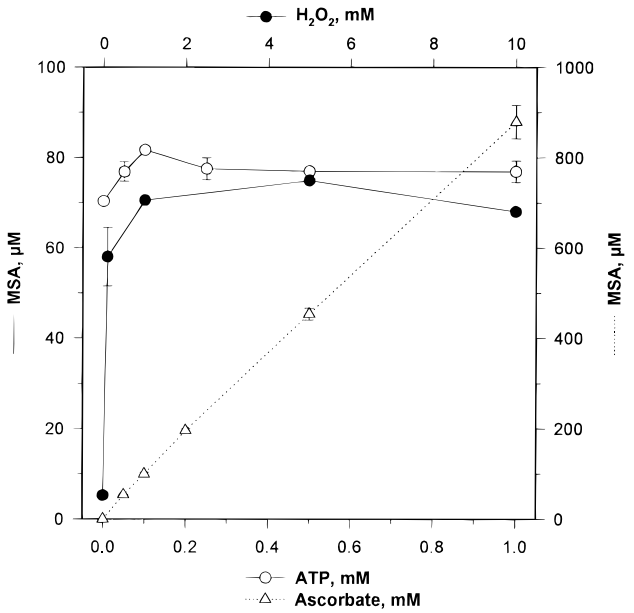

Figure 1. Production of MSA by a nonenzymic free-radical-generating system as a function of reactant concentrations. The reaction mixture contained 1.0 M DMSO, 100 μM ferric iron, 0.04 M phosphate buffer (pH 6.8), and varying concentrations of other reactants in a 2.0 mL final volume at 23 °C. When ATP concentration was varied, 100 μM ascorbate and 10 mM H_2O_2 were used. When H_2O_2 was varied, 100 μM ascorbate and 100 μM ATP were used. The concentration of ATP was 200 μM when ascorbate was varied from 0 to 1 mM. For ascorbate concentrations up to 200 μM, 2 mM H_2O_2 was used, and for 500 μM and 1 mM ascorbate, concentrations of H_2O_2 were 10 and 20 mM, respectively.

Table 1. Hydroxyl Radical Generation Measured as MSA Formed from DMSO by a Nonenzymic Free-Radical-Generating System in Phosphate Buffer at 5 °C

	μM MSA formed		
time at 5 °C	100 μM ascorbate[a]	500 μM ascorbate[a]	1 mM ascorbate[a]
30 min	29 ± 3^a	297 ± 24^a	537 ± 24^a
2 h	58 ± 2^b	352 ± 5^b	651 ± 15^b
24 h	80 ± 1^c	361 ± 6^b	580 ± 14^c

[a] The reaction mixture contained 1.0 M DMSO, 200 μM ATP, 100 μM ferric iron, and 0.04 M phosphate buffer (pH 6.8) with varying concentration of H_2O_2 in a 2.0 mL final volume. For 100 μM, 500 μM, and 1 mM ascorbate, the concentrations of H_2O_2 were 2, 10, and 20 mM, respectively. Means in the same column with the same superscript are not significantly different ($p > 0.05$).

For the generation of HO• in most of the experiments reported, the concentrations were 100 μM ferric iron, 200 mM ATP, 2 mM hydrogen peroxide, and 100 mM ascorbate in 0.04 M phosphate buffer (pH 6.8). The 100 mM ascorbate concentration was chosen because that is approximately the concentration found in fish muscle (Decker and Hultin, 1990; Phillippy and Hultin, 1993). This system generated 100 mM MSA at room temperature in 30 min and 29 mM MSA over the same time period at 5 °C (Table 1). Production of MSA continued past 2 h of incubation. Production of a given quantity of hydroxyl radical in buffer solution does not necessarily mean that a comparable amount of hydroxyl radical would be produced in the muscle sample.

Chemical Modifications. Treatment of the washed minced cod muscle with the free-radical-generating system caused a significant increase of protein carbonyl content in all samples, i.e., those stored for 2 and 24 h at 5 °C and those that were processed through the freeze/thaw cycle (Table 2). There was about a 5-fold increase in the carbonyls produced over 24 h at 5 °C compared to those that were produced in 2 h. The frozen/thawed sample, which was also stored for 24 h in total but which included a freeze/thaw process and about 20 h of storage at −10 °C, showed an increase in carbonyl groups approximately one-half that of the sample stored for 24 h at 5 °C.

Table 2. Changes in Carbonyl Content of Washed Mince Proteins Exposed to a Nonenzymic FRGS and a Freeze/Thaw Cycle

sample	incubation	carbonyl content (nmol/mg of protein)	increase in carbonyls[a] (nmol/mg of protein)
washed mince	2 h/5 °C	1.09 ± 0.12	0
	24 h/5 °C	1.28 ± 0.16	0.19
	2 h/5 °C and frozen/thawed	1.01 ± 0.04	−0.07
washed mince + FRGS	2 h/5 °C	1.39 ± 0.16	0.30
	24 h/5 °C	2.62 ± 0.22	1.53
	2 h/5 °C and frozen/thawed	1.84 ± 0.12	0.75

Significance of Contrasts[b]

	2 h	24 h	frozen/thawed
no FRGS vs FRGS	*	***	***

	no FRGS	FRGS
2 h vs 24 h	NS	***
2 h vs frozen/thawed	NS	***
24 h vs frozen/thawed	NS	***

[a] Increase in carbonyls with respect to washed mince incubated for 2 h at 5 °C as "0". FRGS: A free-radical-generating system consisting of 100 μM ascorbate, 200 μM ATP, 100 μM ferric iron, and 2 mM H_2O_2 in 0.04 M phosphate buffer (pH 6.8) was used. [b] NS, *, ***: nonsignificant or significant at $p \leq 0.05$ and 0.001, respectively.

The effect of the free-radical-generating system on changes in the sulfhydryl content of the washed minced proteins followed a different pattern than protein carbonyl production (Table 3). Although there were highly significant decreases for the samples whether processed for 2 h or 24 h or frozen/thawed ($p \leq 0.001$), there was no difference in the loss of sulfhydryl groups among the three samples; the loss was roughly 30% of the total. It is presumed that the total decrease in sulfhydryl content was over after 2 h of incubation at 5 °C.

The only significant change ($p \leq 0.05$) in amino groups under the three processing conditions in the presence and absence of the free-radical-generating system was an increase observed after 2 h of incubation at 5 °C in the sample treated with the free-radical-generating system (Table 4). This increase disappeared after exposure of the washed cod mince to the free-radical-generating system for 24 h.

Solubility Changes. Holding washed minced cod muscle for 2 h at 5 °C had no significant effect on protein solubility in the presence or absence of the free-radical-generating system (Table 5). However, after 24 h of incubation at 5 °C, there was a significant decrease in solubility brought on by the presence of the free-radical-generating system ($p \leq 0.001$). The process of freezing, storage at −10 °C, and thawing in the absence of the free-radical-generating system also caused a highly significant decrease in protein solubility (Table 5). However, this decrease of about 24% was only half of the loss of solubility that was caused by the same conditions in the presence of the free-radical-generating system (about 48%). The 48% decrease in solubility

Table 3. Changes in Sulfhydryl Content of Washed Mince Proteins Exposed to a Nonenzymic FRGS and a Freeze/Thaw Cycle

sample	incubation	sulfhydryl content (nmol/mg of protein)	decrease in sulfhydryls[a] (nmol/mg of protein)
washed mince	2 h/5 °C	51.9 ± 3.0	0
	24 h/5 °C	51.2 ± 0.9	0.7
	2 h/5 °C and frozen/thawed	53.4 ± 3.4	−1.5
washed mince + FRGS	2 h/5 °C	37.0 ± 4.0	14.9
	24 h/5 °C	34.5 ± 4.4	17.4
	2 h/5 °C and frozen/thawed	38.3 ± 0.7	13.6

Significance of Contrasts[b]

	2 h	24 h	frozen/thawed
no FRGS vs FRGS	***	***	***

	no FRGS	FRGS
2 h vs 24 h	NS	NS
2 h vs frozen/thawed	NS	NS
24 h vs frozen/thawed	NS	NS

[a] Decrease in sulfhydryl groups with respect to washed mince incubated for 2 h at 5 °C as "0". FRGS: A free-radical-generating system as described in Table 2 was used. [b] NS, ***: nonsignificant and significant at $p \leq 0.001$, respectively.

Table 4. Changes in Free Amino Groups of Washed Mince Proteins Exposed to a Nonenzymic FRGS and a Freeze/Thaw Cycle

sample	incubation	nmol of glycine equiv/mg of protein	decrease[a] (nmol of glycine equiv/mg of protein)
washed mince	2 h/5 °C	482 ± 22	0
	24 h/5 °C	457 ± 36	25
	2 h/5 °C and frozen/thawed	451 ± 14	31
washed mince + FRGS	2 h/5 °C	518 ± 28	−36
	24 h/5 °C	465 ± 18	17
	2 h/5 °C and frozen/thawed	461 ± 8	21

Significance of Contrasts[b]

	2 h	24 h	frozen/thawed
no FRGS vs FRGS	*	NS	NS

	no FRGS	FRGS
2 h vs 24 h	NS	***
2 h vs frozen/thawed	NS	***
24 h vs frozen/thawed	NS	NS

[a] Decrease in free amino groups with respect to washed mince incubated for 2 h at 5 °C as "0". FRGS: A free-radical-generating system as described in Table 2 was used. [b] NS, *, ***: nonsignificant or significant at $p \leq 0.05$ and 0.001, respectively.

observed in the sample that was treated with the free-radical-generating system and frozen and thawed was greater than the sum of the decreases in solubility observed on freezing and thawing without the free radical system and storage for 24 h at 5 °C in the presence of the free radical system. Thus the effects of freezing and thawing and the free-radical-generating system were synergistic, causing a greater loss of solubility than the sum of either of the processes separately.

Changes in Electrophoretic Patterns. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) patterns of the polypeptides of the washed minced cod muscle stored for 2 h at 5 °C in the presence

Table 5. Changes in Solubility of Washed Mince Proteins Exposed to a Nonenzymic FRGS and a Freeze/Thaw Cycle

sample	incubation	% decrease in solubility[a]
washed mince	2 h/5 °C	0
	24 h/5 °C	1.7 ± 0.4
	2 h/5 °C and frozen/thawed	23.7 ± 4.7
washed mince + FRGS	2 h/5 °C	2.1 ± 1.3
	24 h/5 °C	14.0 ± 9.9
	2 h/5 °C and frozen/thawed	47.8 ± 8.2

Significance of Contrasts[b]

	2 h	24 h	frozen/thawed
no FRGS vs FRGS	NS	***	***

	no FRGS	FRGS
2 h vs 24 h	NS	***
2 h vs frozen/thawed	***	***
24 h vs frozen/thawed	***	***

[a] The percent decrease in solubility was with respect to washed mince incubated for 2 h at 5 °C as "0%". Solubility of proteins of the washed mince incubated for 2 h at 5 °C was 90 ± 2%. FRGS: A free-radical-generating system as described in Table 2 was used. [b] NS, ***: nonsignificant or significant at $p \leq 0.001$, respectively.

and absence of the free-radical-generating system and a disulfide splitting component (dithiothreitol (DTT)) were obtained (Figure 2). Electrophoretic patterns were obtained for treatments at 100 μM, 500 μM, and 1 mM ascorbate with hydrogen peroxide concentrations of 2, 10, and 20 mM, respectively. In the presence of the disulfide splitting reagent DTT, there were no observable effects of the free-radical-generating system with the possible exception of a small loss in the uppermost band (possibly titin) at the highest ascorbate concentration (1 mM) (lanes 2−5, Figure 2). When DTT was not included in the electrophoretic medium, there was an increase in some very high molecular weight fractions (arrow). Presumably this represents polymerization through disulfide bonds. Observable changes in these high molecular weight polypeptides were detected in the sample treated the same as those used for studying chemical changes, solubility properties, and functional properties, i.e., 100 μM ascorbate and 2 mM hydrogen peroxide (lane 8, Figure 2). The changes at these concentrations of ascorbate and hydrogen peroxide were not as severe as those at the higher concentrations of the pro-oxidants (lanes 8−10, Figure 2). The intensity of a polypeptide band with a molecular mass between 66 and 97 kDa decreased with increasing concentrations of pro-oxidants.

A similar electrophoretic evaluation was made of the polypeptides after treatment with the free-radical-generating system for 24 h at 5 °C (Figure 3). Although little change could be observed in the sample that was treated with the 100 μM ascorbate and 2 mM hydrogen peroxide when the electrophoresis was conducted in the presence of DTT, some bands did change at the two higher concentrations of the pro-oxidant system (lanes 3−5, Figure 3). A faint band of very high molecular weight material appeared at the top of the gel. There was also a faint band that appeared slightly above the titin band (tentatively identified); the titin band decreased. Another faint band appeared between the titin and nebulin bands, and there was an increasing concentration of a band slightly below nebulin (these four bands are indicated by arrowheads). There also appeared to be some diminution in the myosin heavy chain

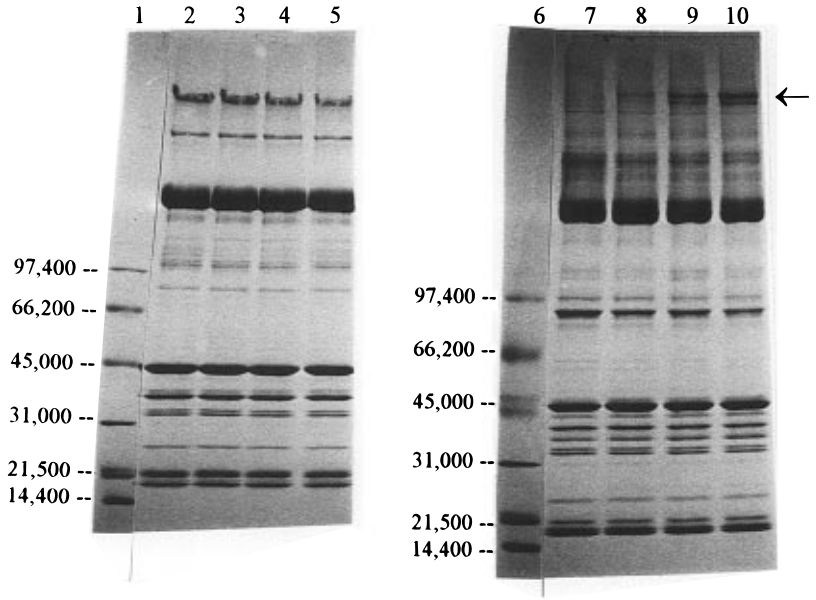

Figure 2. Electrophoresis pattern of washed mince proteins exposed to a nonenzymic free-radical-generating system for 2 h at 5 °C. Each lane contained 30 μg of protein. Electrophoresis of samples was done in the presence (lanes 2−5) and absence (lanes 7−10) of dithiothreitol: lanes 1 and 6, molecular weight marker proteins; lanes 2 and 7, washed mince; lanes 3 and 8, washed mince + 100 μM ascorbate; lanes 4 and 9, washed mince + 500 μM ascorbate; lanes 5 and 10, washed mince + 1 mM ascorbate. The other components of the free-radical-generating system were 100 μM ferric iron, 200 μM ATP, 0.04 M phosphate buffer (pH 6.8), and varying amounts of H_2O_2. For 100 μM, 500 μM, and 1 mM ascorbate, the concentrations of H_2O_2 were 2, 10, and 20 mM, respectively. The arrow indicates high molecular weight peptides (>200 kDa).

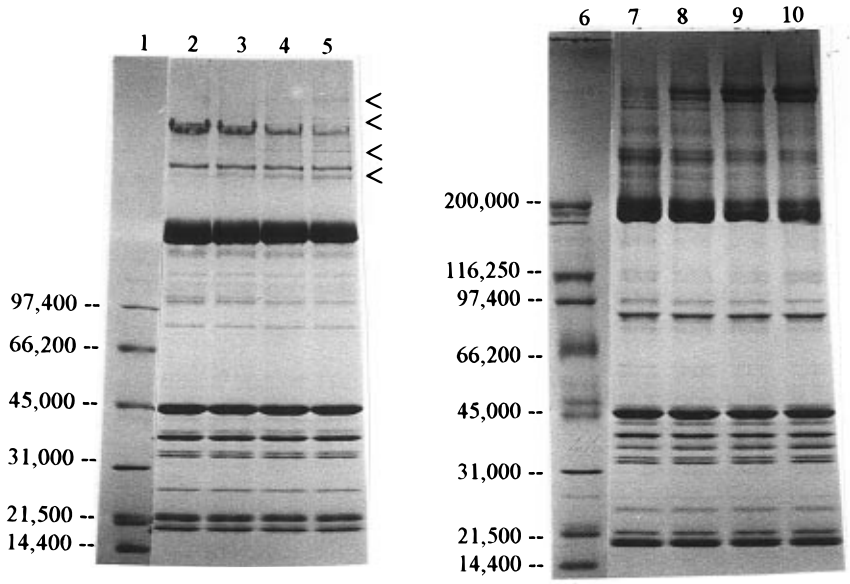

Figure 3. Electrophoresis pattern of washed mince proteins exposed to a nonenzymic free-radical-generating system for 24 h at 5 °C. Conditions were as described in Figure 2. The arrowheads indicate either the appearance of new peptide bands or an increase in the intensity of peptide bands (all bands >200 kDa).

with increasing concentration of pro-oxidants. The formation of a high molecular mass band above titin in samples treated with FRGS and solubilized with dithiothreitol could be due to nondisulfide covalent linkages. When the electrophoresis was carried out in the absence of DTT, the decrease in myosin heavy chain was clearly shown with increasing ascorbate concentration (lanes 8−10, Figure 3). In addition, there was a decrease in the bands directly above the myosin heavy chains and an accumulation of two very high molecular weight components. Samples treated with increasing concentrations of pro-oxidants and frozen and thawed gave electrophoretic patterns with and without DTT that were similar to those treated with the free-radical-generating system but without the freeze/thaw treatment (Figure 4 compared to Figure 3). New bands appeared between the 31.5 and 45 kDa bands and at approximately 21.5 kDa in all frozen/thawed samples (Figure 4). The new bands were probably due to greater band separation in the gel based on the relative intensities of bands (comparing Figures 3 and 4) and also as the dye front in Figure 4 migrated to the end of the gel.

Functional Properties. *Gelation.* A comparison was made of the gelation properties of the washed cod muscle samples that were treated or not with the free-radical-generating system. The samples tested included those that were held for 24 h at 5 °C and those that went through the freeze/thaw procedure, i.e., also stored for 24 h but with freezing, storage at −10 °C, and thawing. For these samples, the free-radical-generating system contained 100 μM ascorbate and 2 mM hydrogen peroxide. The samples were incubated in the

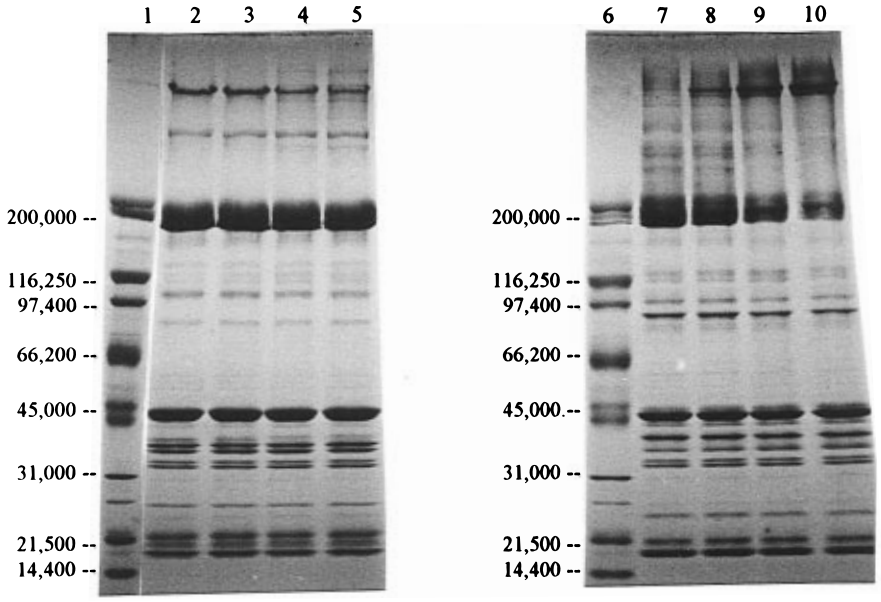

Figure 4. Electrophoresis pattern of washed mince proteins exposed to a nonenzymic free-radical-generating system for 2 h at 5 °C and frozen/thawed. Conditions were as described in Figure 2.

Table 6. Some Properties of Cooked Gel from Washed Mince Exposed to a Nonenzymic FRGS and a Freeze/Thaw Cycle

	properties of cooked gel			
sample	moisture (%)	stress (kPa)	true strain	EM[a]
24 h/5 °C				
washed mince	77.1 ± 0.1	27.4 ± 2.6	1.71 ± 0.11	24.0 ± 0.2
washed mince + FRGS	76.9 ± 0.1	46.1 ± 2.0	2.06 ± 0.13	25.2 ± 1.0
2 h/5 °C and frozen/thawed				
washed mince	76.9 ± 0.1	30.4 ± 2.8	1.17 ± 0.03	28.7 ± 1.0
washed mince + FRGS	77.3 ± 0.1	32.3 ± 2.8	1.10 ± 0.04	30.0 ± 1.0

Significance of Contrasts[b]

		stress	true strain	EM
no FRGS vs FRGS	24 h	***	***	NS
	frozed/thawed	NS	NS	NS
24 h vs frozen/thawed	no FRGS	NS	***	***
	FRGS	***	***	***

[a] EM: Expressible moisture of cooked gel was determined as grams of water lost per 100 g of water in the gel. FRGS: A free-radical-generating system as described in Table 2 was used. [b] NS, ***: nonsignificant or significant at $p \leq 0.001$, respectively.

absence of any cryoprotectants even when the samples were to be frozen and thawed. This was done to sharpen the distinction between the samples that had been treated with the free-radical-generating system and those that had not. Cryoprotectants were added to all the minces before the gels were produced. In the samples that were not frozen and thawed, there was a 68% increase in the stress value in the sample that was treated with the free-radical-generating system compared to the sample not treated (Table 6). Moisture contents of the samples were similar and were probably not large enough to account for the differences observed (Hamann and MacDonald, 1992). In the case of the samples that were frozen and thawed, the free-radical-generating system had no effect on the value of stress.

True strain of the washed mince stored for 24 h at 5 °C was also significantly increased when the sample was exposed to the free-radical-generating system (Table 6). The freeze/thaw treatment, with or without the free-radical-generating system, decreased true strain values. In these experiments, cryoprotectants were not added to the minces that were frozen and thawed. No differences were observed in expressible moisture by addition of the free radical system to the samples stored at 5 °C for 24 h or for the samples put through the freeze/thaw process. However, the process of freezing and thawing did cause an increase in expressible moisture of the samples with or without treatment by the free-radical-generating system.

Color, Hunter "*L*", "*a*", and "*b*" values, and the whiteness index of the gels made from the three washed cod mince samples with or without exposure to the free-radical-generating system using 100 μM ascorbate and 2 mM hydrogen peroxide are shown in Table 7. There was a decrease in the whiteness index and an increase in the "*b*" values (indicating more yellowness) in all three samples that were treated with the free-radical-generating system. The changes in these values were especially marked after 24 h. High "*b*" values are typical of surimis made from fatty fish. It may be that these high "*b*" values are a reflection of the oxidations occurring in the fatty fish after exposure to free radicals (Srinivasan and Hultin, 1995).

Emulsifying Activity. The ability of the cod muscle proteins to form and maintain an emulsion was evaluated (Figure 5). Comparisons were made between samples stored for 24 h either at 5 °C or through a freeze/thaw cycle after 2 h at 5 °C with or without incubation with a free-radical-generating system. The system contained 100 μM ascorbate and 2 mM hydrogen peroxide. Conditions for the study of the emulsification were chosen that gave a relatively high emulsifying activity index but moderate stability for the sample stored for 24 h at 5 °C without the free-radical-generating system. The conditions were chosen to make it possible to see either an enhancement or a decline in emulsifying capacity and stability of the proteins with other treatments. The sample that was stored for 24 h at 5 °C in the presence of the free-radical-generating system showed no decline in emulsifying activity index (EAI) over a period of 90 min. Over the same period of time, the same sample not exposed to the free-radical-generating system lost about one-half of its EAI value.

Table 7. Color Values of Cooked Gels from Washed Mince Exposed to a Nonenzymic FRGS and a Freeze/Thaw Cycle[a]

sample	incubation	*L*	*a*	*b*	whiteness index
washed mince	2 h/5 °C	77 ± 1	−3.1 ± 0.1	0.9 ± 0.1	57 ± 1
	24 h/5 °C	79 ± 1	−3.0 ± 0.1	2.1 ± 0.1	54 ± 1
	2 h/5 °C and frozen/thawed	80 ± 1	−3.2 ± 0.1	2.5 ± 0.1	53 ± 1
washed mince + FRGS	2 h/5 °C	79 ± 1	−2.8 ± 0.1	2.4 ± 0.2	52 ± 1
	24 h/5 °C	78 ± 1	−2.3 ± 0.1	7.1 ± 0.3	28 ± 1
	2 h/5 °C and frozen/thawed	78 ± 1	−2.3 ± 0.1	6.9 ± 0.1	30 ± 1

Significance of Contrasts[b]

		2 h	24 h	frozen/thawed
no FRGS vs FRGS	*L*	NS	NS	NS
	a	***	***	***
	b	***	***	***
	WI	***	***	***

No FRGS

	L	*a*	*b*	WI
2 h vs 24 h	NS	NS	***	**
2 h vs frozen/thawed	*	NS	***	***
24 h vs frozen/thawed	NS	**	**	NS

FRGS

	L	*a*	*b*	WI
2 h vs 24 h	NS	***	***	***
2 h vs frozen/thawed	NS	***	***	***
24 h vs frozen/thawed	NS	NS	*	NS

[a] Data represents mean ± SD of one experiment done in six replicates. FRGS: A free-radical-generating system as described in Table 2 was used. [b] WI: whiteness index. NS, *, **, ***: nonsignificant or significant at $p \leq 0.05$, 0.01, and 0.001, respectively.

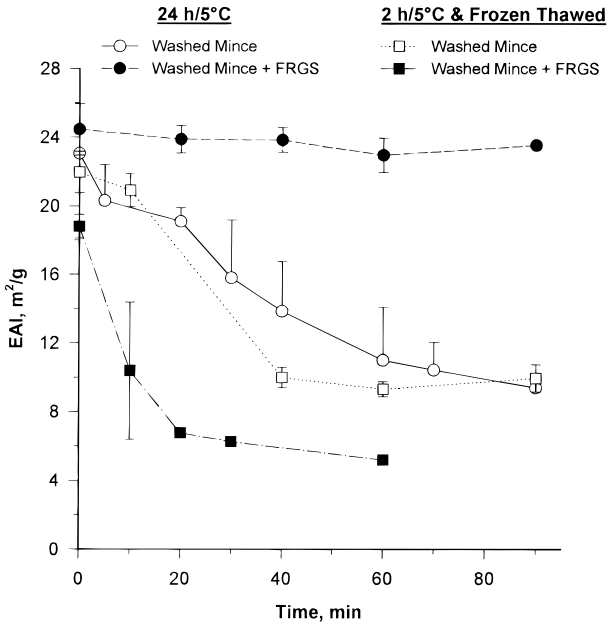

Figure 5. Emulsifying activity index and emulsion stability of washed mince exposed to a nonenzymic free-radical-generating system and a freeze/thaw cycle. FRGS: A free-radical-generating system as described in Table 2 was used.

The improvement in EAI of the sample exposed to the free-radical-generating system when the sample was not frozen was contrary to what was observed in the sample that underwent the freeze/thaw process. In this case, the sample that was treated with the free-radical-generating system showed a lower initial EAI and a more rapid decrease with time of storage compared to the sample that was not treated with the free-radical-generating system. Thus, in the unfrozen sample, oxidizing the protein by generating HO• improved the emulsification properties of the protein, while in the sample that underwent the freeze/thaw process, the oxidation of the proteins lowered the emulsifying ability.

DISCUSSION

Treatment of washed minced cod muscle with a mixture of inorganic phosphate, ATP, ferric iron, hydrogen peroxide, and ascorbate led to chemical, physical, and functional changes in the proteins of the muscle via the hydroxyl radical (HO•). The HO• was detected by measuring methanesulfinic acid (MSA), a product of the oxidation of dimethyl sulfoxide (DMSO). Changes were observed in protein carbonyl, sulfhydryl, and free amino groups, solubility, peptide distribution, gelation, and emulsifying activity. In an earlier study (Srinivasan and Hultin, 1995), some of these properties were evaluated after treating washed cod mince with a system that produced HO• enzymically (as measured by MSA). This enzymic system contained phosphate, EDTA, ferric iron, hypoxanthine, and xanthine oxidase. The measured amount of MSA formed from the enzymic system in buffer was 132 μM, and this production was attained by 2 h. The nonenzymic system used in the present study produced a total of 80 μM MSA when 100 μM ascorbate and 2 mM hydrogen peroxide were used. The production of HO• was greater at 24 h than at 2 h. In both studies, the production of carbonyl groups followed the pattern of HO• production. That is, in the present study where HO• was produced over a 24 h period, the carbonyl content of the proteins also increased over that period. In the earlier study using the enzymic free-radical-generating system, where HO• production was over after 2 h, the production of protein carbonyls also terminated after 2 h. Thus it seems likely that the production of protein carbonyls was in direct response to the production of the hydroxyl radical by the respective systems.

It had been assumed that the amount of MSA formed from DMSO by HO• was stoichiometric and cumulative

(Steiner and Babbs, 1990). However, in a recent paper, Scaduto (1995) demonstrated that the methanesulfinic acid formed from DMSO can be converted to methanesulfonic acid by the hydroxyl radical. Thus this measurement would underestimate the actual amount of HO• formed. The rate of HO• production could play a role in whether the radical reacts with a molecule of DMSO or MSA. If this is the case, formation of MSA may not accurately measure the amount of hydroxyl radical produced, and the percentage of the MSA that is oxidized to the methane sulfonic acid may be dependent on reaction conditions. The rate of HO• production in the muscle tissue may determine in part the groups on the proteins with which it interacts. The production of protein carbonyls is only one of the many possible protein reactions (Stadtman, 1993). A change in the relative reaction rates with different protein groups would alter the spectrum of products obtained when HO• is produced at different rates. Production of protein carbonyls probably represents a relatively small fraction of the total reaction in the muscle brought about by the HO•. The total change in carbonyl content was 1 order of magnitude less than that of the disappearance of sulfhydryl groups.

It is interesting that the amount of protein carbonyls produced by the nonenzymic free-radical-generating system in this study was about twice as great as the amount of carbonyls that were produced in our previous study (Srinivasan and Hultin, 1995) even though the amount of HO• produced in 24 h was less than 60% of that formed in the enzymic system. Autoxidation products of ascorbic acid, such as threose, may form adducts with lysine, and these adducts may react with 2,4-dinitrophenylhydrazine (Dunn et al., 1990). Thus, the adducts formed from the autoxidation products of ascorbic acid may also contribute to the pool of protein carbonyls. Further evidence that the nonenzymic free-radical-generating system did not produce the same effects as did the enzymic system is shown by the results on protein solubility. With the nonenzymic system, there was a significant difference between the loss of protein solubility in the sample that was stored for 24 h at 5 °C in the absence of the free-radical-generating system compared to the sample that was stored under the same conditions but in the presence of the free-radical-generating system. In the case of the enzymic system (Srinivasan and Hultin, 1995), there was no difference between these samples in their loss of protein solubility. The values obtained in the previous study (Srinivasan and Hultin, 1995) were similar to what was observed in this study for the sample that was stored for 24 h at 5 °C in the absence of the free-radical-generating system. In other words, when the nonenzymic free-radical-generating system was used, it produced a loss of protein solubility whereas the enzymic system did not. This further substantiates the greater deleterious effect that the nonenzymic free-radical-generating system had on the proteins compared to the enzymic system.

The pattern of the loss of sulfhydryl groups was very different from the increase in the carbonyls. All of the loss in sulfhydryls had taken place within the first 2 h of reaction with the free-radical-generating system. In addition, there was no significant change ($p > 0.05$) in sulfhydryl group content on freezing (Table 3). Carbonyl content was five times greater at 24 h than it was at 2 h, and the freeze/thaw process decreased the concentration of the carbonyl group by 50%. The loss of the sulfhydryl groups occurred within the first 2 h, although in the model buffer system, the nonenzymic free-radical-generating system produced HO• throughout the 24 h period. It is possible that only a certain fraction of the sulfhydryl groups of the proteins of cod mince are exposed and subject to oxidation by the system.

The loss of the sulfhydryl groups may have been due to the formation of disulfide bonds either within polypeptides or between polypeptides. If the latter were the case, it would be expected that the electrophoretic pattern of the polypeptide bands would be different between samples that were not treated with the free-radical-generating system compared to samples that were. That this was in fact the case is shown in Figure 2 (lanes 7 and 8) and Figure 3 (lanes 7 and 8). In both cases, there was an increase in a high molecular weight band in the samples that had been treated with the free-radical-generating system for either 2 or 24 h when the electrophoresis was carried out in the absence of an agent that would reduce disulfide bonds. The high molecular weight band that was formed in the sample stored for 24 h at 5 °C stained considerably darker than did that from the sample stored for 2 h at 5 °C. This would indicate that more cross-linking had taken place over the longer time period. The total loss of sulfhydryl groups in the two samples were not statistically different ($p > 0.05$), however. These results could be reconciled by the possibility that initially intramolecular polypeptide disulfide bonds formed which later rearranged by interaction with sulfhydryl groups on other polypeptides. The interpeptide bonds would cause cross-linking while the initial intrapeptide bonds would not.

For samples held for either 2 or 24 h at 5 °C, there was no indication of any changes in the band patterns of polypeptides with molecular masses less than 200 kDa. Thus the formation of interpeptide disulfide bonds occurred primarily in the high molecular weight fractions. It seems possible that a considerable part of this cross-linking occurred through myosin heavy chains. The changes in the polypeptide bands observed at a concentration of 100 μM ascorbate and 2 mM hydrogen peroxide were not large enough to identify myosin as participating in formation of high molecular weight fractions. When higher concentrations of ascorbate and hydrogen peroxide were used, the decrease in the myosin heavy chain could be clearly seen along with the formation of a very high molecular weight fraction at the top of the gel (Figure 3). This loss in myosin heavy chain was greater in the sample held for 24 h than at 2 h (lanes 9 and 10 in Figure 3 vs lanes 9 and 10 in Figure 2), which suggests the possibility that the intermolecular rearrangement of disulfide bonds might have occurred within the myosin thick filament. The close arrangement of myosin molecules in the thick filament might encourage such an exchange. All of the sulfhydryl groups that were to be lost had been lost at 2 h.

The difficulty of looking for small changes from relatively large initial values hampered the usefulness of the data on change in free amino groups caused by the free-radical-generating system. However, the one difference that was observed was that there was an initial increase in free amino groups, i.e., on 2 h exposure at 5 °C to the free-radical-generating system, which was followed by a decrease (Table 4). We suggest the possibility that the initial increase comes from fragmentation of the peptide chains to produce free amino groups. The later decrease observed after storage

for 24 h either at 5 °C or in the frozen/thawed regimen could be from deamination reactions. Davies (1987) has shown that hydroxyl radical in the presence of superoxide and oxygen causes protein fragmentation but little aggregation. Hydroxyl radical alone produces covalently bound proteins and causes extensive changes in the electrical charge (chemical changes of side groups) but with few fragmented products. It is conceivable that in the early stages of our reaction there is sufficient oxygen mixed into the mince to facilitate fragmentation and exposure of polypeptide amino groups. With time, the oxygen becomes used up and then the hydroxyl radical preferentially modifies side chains.

A decrease in the protein solubility of the cod mince was observed on freeze/thawing the control sample or after 24 h of treatment at 5 °C with the free-radical-generating system. When these two processes were combined, i.e., freeze/thawing and treatment with the free-radical-generating system, the percent decrease in solubility was greater than the sum of the decreases observed in the individual steps. Cross-linking of proteins could make them more susceptible to aggregation and precipitation and thus lower their solubility. The evidence obtained does not seem to support this as a major cause of the loss of solubility. First of all, it is clear that a decrease in solubility can take place without any cross-linking. This is evidenced by the substantial loss of solubility that is observed in the frozen/thawed sample which was not treated with the free-radical-generating system. No loss of sulfhydryl groups was seen under these conditions. There was a substantial decrease in solubility of the sample treated with the free-radical-generating system for 24 h compared to the sample that was treated for only 2 h, 14.0% vs 2.1% (Table 5). There was no significant difference, however, in the loss of sulfhydryl groups under these conditions. As previously mentioned, there could be some rearrangement of the disulfide groups due to interaction with free sulfhydryl groups. This could modify the proteins without changing the sulfhydryl content.

Carbonyl content also increased in the presence of the free-radical-generating system. It is possible that formation of carbonyl groups could lead to cross-linking. For example, this is a common way in which collagen is cross-linked. Lysyl residues are oxidized to aldehydes. The aldehydes can then interact and condense with free amino groups or there could be an Aldol condensation reaction between two carbonyl groups. In theory, the process of freeze/thawing could favor these interactions (Ang and Hultin, 1989). Treatment with the free-radical-generating system of 100 μM ascorbate and 2 mM hydrogen peroxide did not have any observable effects on the distribution of peptide bands on SDS-PAGE, suggesting that cross-linking through aldehydic groups probably did not occur to any extent under these conditions. The lower content of carbonyl groups in the frozen/thawed sample might simply have been a reflection of less oxidation due to the fact that the muscle tissue was stored at a lower temperature and in the frozen state.

Whatever contribution cross-linking might make to the loss of protein solubility when exposed to the free-radical-generating system, a comparison of the loss of solubility in the sample that was treated with the free-radical-generating system and stored for 24 h at 5 °C (14.0%) with the sample that was not treated with the free-radical-generating system but was frozen and thawed (23.7%) indicates that cross-linking was not the only factor responsible for the loss in protein solubility. We suggest that an additional factor in the loss of solubility of protein is caused by modification of side chains. Modification of protein side chains could destabilize the protein by modifying the stabilizing and destabilizing forces of the native conformation (Hultin, 1986). Any amino acid side chain may be oxidized by hydroxyl radicals dependent on such factors as the location of the transistion metal on the protein (Stadtman and Oliver, 1991). Amino acids particularly susceptible to oxidation include histidine, proline, arginine, threonine, tyrosine (Stadtman, 1993), methionine (Vogt, 1995), and tryptophan (Uchida et al., 1989) in addition to lysine and cysteine. If the oxidation of the side chains were severe enough, the changes could be sufficient to cause denaturation. It is also possible that modifications could be such that under conditions in which the reactions were carried out the thermodynamic forces which stabilize the protein had not been sufficiently changed to cause an unfolding of the molecule. However, a further stress such as heating or a freeze/thaw process could change the balance of forces such that the denatured state was favored. This is illustrated in Figure 6, which shows the response of a protein to denaturation as a function of any destabilizing factor. The graph uses a two-step denaturation curve to illustrate the process. The denaturing stress increases from left to right. A shift in the sigmoidal curve illustrates a decrease (to the left) or an increase (to the right) in the stability of the protein. Modification of the side chains of the protein by $HO^{\bullet}$ oxidation would shift the curve describing denaturation to the left, i.e., the protein would denature under less severe conditions. Under conditions where any stress becomes severe ("C" on the abscissa), it would not make any difference if the protein were in the native or modified form, since the force is sufficient to denature them both. On the other hand, if the destabilizing forces are relatively low ("A" on the abscissa), they may not be sufficient to destabilize even the modified protein. This could correspond to what was observed for the oxidation of the protein without the freeze/thaw treatment. If the stress is such that there is a difference in stability of the native and modified protein ("B" on the abscissa), one would expect the modified protein to be more susceptible to denaturation than the native protein. This increased stress could have been induced in these experiments by the freeze/thaw process which was in addition to the stress caused by the chemical change of the side groups

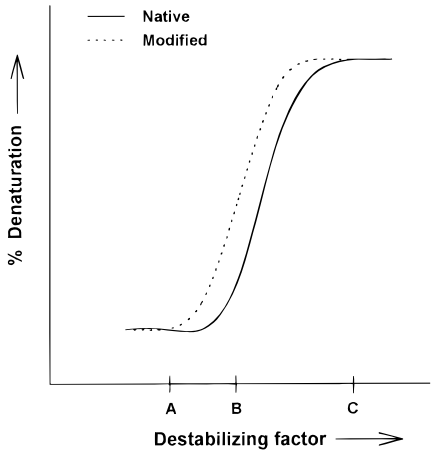

Figure 6. Theoretical two-step denaturation curves for a native protein and a protein with modified side chains.

brought on by the free radicals. A similar hypothesis has been used to explain the denaturing effect of formaldehyde in fish susceptible to TMAO break down (Ang and Hultin, 1989).

Formation of disulfide cross bridges between peptides and destabilization of the proteins by side group modification may explain the gelation and emulsifying properties that were observed. In evaluating the effect of the free-radical-generating system on both of these properties, samples that were stored for 24 h at 5 °C were compared with samples that were stored for the same length of time but went through a freeze/thaw process. In evaluating each of the samples, some of the proteins were treated with the free-radical-generating system and some of the proteins were not.

In relation to gel formation, a 68% increase in stress and a 20% increase in true strain were observed in the sample that was stored for 24 h at 5 °C and treated with the free-radical-generating system. Cross-linking of peptides via disulfide and possibly other types of linkages could have been responsible for the improvement. These cross-linkages aid in developing the three-dimensional network of protein molecules. This network is stabilized by noncovalent as well as covalent interactions, including disulfide bonds (Niwa, 1992). Disulfide bonds could improve gel properties. Nishimura et al. (1990) has shown that incubation of surimi with ascorbate brought about polymerization of actomyosin and an increase in gel strength of Alaska pollock surimi. They hypothesized that ascorbate improved the quality of the heat-induced gels by accelerating the formation of disulfide linkages during heating. These authors observed an approximately 30% decrease in sulfhydryl groups when Alaska pollock surimi was incubated with 0.2% ascorbate for 1 h at 40 °C and slightly greater than 40% decrease when the incubation was at 90 °C for the same period. A 0.2% ascorbate solution is approximately 14 mM (based on an estimated 80% moisture in the fish). The value these workers obtained at 40 °C was similar to what we observed under our three conditions, i.e., 5 °C for either 2 or 24 h and the freeze/thaw process. Decker et al. (1993) reported that oxidation of turkey white muscle myofibrillar proteins by iron or copper and ascorbate caused a lowering of the gel strength compared to controls. These workers used lower iron concentrations (25 μM) than we did, but much higher ascorbate concentrations. Although their incubation period was only 6 h, it was done at 23 °C. In their iron-catalyzed system, carbonyl production was considerably higher than we observed in our system, while copper was only slightly so. They found a major loss of both myosin and actin. It seems likely that the total oxidative effect in their experiments was considerably larger than what we achieved in the samples used for determination of functional properties. This might account for the lowering of gel quality which they observed compared to the improvement of gel quality which was observed in our experiments. At higher concentrations of ascorbate (500 or 1 mM), we also observed considerable loss of myosin. The samples we chose for evaluating functional properties were treated with 100 mM ascorbate. This was done because it is similar to what is found in fish muscle and it produced changes in protein carbonyl groups that were similar to what occurred in mackerel muscle during refrigerated storage (Srinivasan and Hultin, 1995).

When our oxidized cod muscle samples were subjected to a freeze/thaw treatment before addition of cryoprotectants, there was a precipitous drop in the value of true strain, 32% in the case of the samples that were not exposed to the free-radical-generating system and 47% in the samples that were exposed. True strain has been considered a measure of protein quality (Hamann and Lanier, 1987). These samples were not protected with the cryoprotectants usually used in surimi production. Not only was the percentage decrease in true strain of the frozen/thawed sample treated with the free-radical-generating system greater than that of the sample that was not exposed to the $HO^{\bullet}$ but frozen/thawed, but the final absolute value of true strain was significantly lower in the former, though not by a large amount. The improved stress value of the sample that was exposed to the free-radical-generating system but not frozen/thawed was essentially lost when the sample was put through the freeze/thaw cycle.

The emulsifying properties of the cod mince proteins were improved by treatment with the free-radical-generating system. The sample treated for 24 h with the free-radical-generating system produced only a slightly higher initial emulsifying activity index (EAI) than the sample not treated, but this EAI was stable for at least 90 min. The EAI of the sample not exposed to the free-radical-generating system declined throughout that period, losing somewhat more than 50% of its EAI value. Cross-linking of the proteins via oxidation of sulfhydryl groups to disulfides could have produced polymers whose greater size allowed them to form a more stable protein film on the surface of the oil droplets. When the samples with addition of the free-radical-generating system were subjected to a freeze/thaw treatment, there was a decline in the initial EAI value of the sample. This initial value decreased rapidly with time. The frozen/thawed sample that was not oxidized, on the other hand, was not greatly different from the sample that was neither frozen/thawed nor exposed to the free-radical-generating system. Modification of side groups by oxidation may have led to less stable proteins that denatured on freezing. The denatured proteins were not able to form a stable emulsion.

A mild oxidation can improve some functional properties of washed cod mince. Future work should be directed to determine if this improvement can be maintained during frozen storage by the use of cryoprotectants. Work by Nishimura et al. (1990, 1992a,b) would indicate that, to a certain extent at least, it can. It would seem likely that this will be related to the amount of disulfide formation versus other modifications of side groups which are readily susceptible to oxidation by $HO^{\bullet}$. One has to be concerned, however, about potential changes in other attributes of the product due to exposure to an oxidizing system. Lipid oxidation may produce undesirable flavors and odors. The increase we observed in "*b*" value indicates that discoloration could also be a problem as has been previously observed (Nishimura et al., 1992a).

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