# Degradation of Myosin by Enzymes of the Digestive System: Comparison between Native and Oxidatively Cross-Linked Protein

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We compared proteolysis of $H_2O_2$/hemin-cross-linked myosin and the native protein. Pepsin, trypsin, and chymotrypsin, representative enzymes of the mammalian digestive tract, and the plant enzyme papain were chosen as proteases. Under conditions which caused prominent degradation of the native myosin, only minimal degradation of the cross-linked protein was observed when individual enzymes were applied. Successive proteolysis by pepsin at gastric pH (1.85) followed by trypsin and chymotrypsin at duodenal pH (8.0) also showed retarded proteolysis of cross-linked myosin. At low pH both myosin forms precipitated, but by elevation of the pH only cross-linked myosin was partially resolubilized and the solubility was augmented following its minimal proteolytic degradation. It was concluded that although in general oxidatively modified proteins are susceptible to proteolysis, oxidatively cross-linked myosin is resistant. The data suggest that decreased digestibility of cross-linked myosin may lead to reduced quality of oxidized muscle foods.

**Keywords:** *Myosin; heme; peroxidation; cross-linking; proteolysis*

## INTRODUCTION

Myosin, the major muscle protein, is responsible together with actin for the specific physiological function of contractility, namely, conversion of chemical to mechanical energy. Hence it is highly probable that muscle malfunction involves myosin damage. A vast body of information indicates that hypoxia, namely, inadequate oxygen supply, is a main cause of functional failure of muscles, particularly of heart muscle which is crucial for life (Hess and Manson, 1984; Solaro, 1992). In muscles hypoxia may result from high oxygen consumption during exhaustive exercise (Witt et al., 1992) or due to lack of oxygen supply, namely, ischemia (McCord, 1985). Lack of oxygen was shown to be followed by formation of active cellular oxygen species including $H_2O_2$ resulting from mitochondrial, peroxisomal, and cytosolic enzymatic reactivity (Boveris, 1972; Chance et al., 1979). Thus, under such conditions, all cellular components are exposed to peroxidative damage. Lipids are easily oxidized to hydroperoxides which in turn are highly active as triggers of further oxidation of other cellular components like proteins (Tappel, 1952; Tappel, 1962; Govindarajan et al., 1977; Slater, 1984; Kanner and Harel, 1985; Dee et al., 1991). Oxidative damage to proteins is crucial due to their key roles in cellular structure and function. The distinct protein and oxidizer involved in oxidation determine the form of the oxidative protein products which may be either fragments or covalent cross-linked aggregates (Aft and Mueller, 1984, 1985; Wolf et al., 1986; Davies and Goldberg, 1987; Kelvin et al., 1987).

Muscles are efficiently supplied with oxygen by two globin hemoproteins: vascular hemoglobin and cellular myoglobin. Although these proteins are assigned to transport oxygen, many reports demonstrated their involvement in oxidation of various cell components as peroxidases. Hemoglobin was shown to be an active catalyzer of oxidative cross-linking of proteins like spectrin, the main red cell membrane cytoskeletal protein (Snyder et al., 1981; Shaklai et al., 1987), as well as of lipids (Paganga et al., 1992). Myoglobin was found to catalyze $H_2O_2$ oxidation of myosin. This peroxidative reactivity is heme-iron dependent. Globin-free hemin was found to associate with myosin and in a myosin-bound form triggers myosin oxidative cross-linking. The cross-linked products of both myoglobin and hemin peroxidized myosin were identified as non-S−S covalent aggregates of high molecular weight (>$10^6$ Da) (Bhoite-Solomon et al., 1990, 1992).

As muscles provide an important dietary protein source in the form of meat, the postmortem fate of muscle constituents is of great interest from a nutritional point of view. During maintenance and processing of muscle foods in absence of blood circulation and cellular metabolism, active oxygen species are formed (Portwitch and Aebi, 1960). Since, like in the living tissue, muscle foods contain high amounts of hemoglobin and myoglobin, all their components, including proteins, are prone to heme/$H_2O_2$-triggered oxidative damage. Because myosin comprises an appreciable portion of muscle protein, myosin enzymatic hydrolysis represents muscle quality as food.

The present study compared the ability of native and oxidatively cross-linked myosin to undergo proteolysis. The proteolytic enzymes chosen were papain, a plant protease used widely as a meat tenderizer, and the main enzymes responsible for protein degradation in the digestive tract: pepsin, trypsin, and chymotrypsin.

## MATERIALS AND METHODS

**Materials.** The following were purchased from Sigma: papain, EC 3.4.22.2, cat. no. P-4762; pepsin, EC 3.4.23.1, cat. no. P-7012; α-chymotrypsin type II, EC 3.4.21.1, cat. no. C-4129; trypsin type IX, EC 3.4.21.4, cat. no. T-0134; hemin, cat. no. H-2250; brilliant blue (Coomassie) R-250, cat. no. B-0149. Chemicals for SDS−PAGE were purchased from Bio-Rad. Molecular weight markers were purchased from Pharmacia, cat. no. 17-0-446-01. All other chemicals were of analytical grade.

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S0021-8561(95)00413-4 CCC: $12.00 © 1996 American Chemical Society

**Methods.** Myosin was prepared from New Zealand white male rabbit muscles of the hind legs and back (Spudich and Watt , 1971; Tonomura et al., 1966). Cross-linked myosin was prepared by myoglobin/$H_2O_2$ or hemin/$H_2O_2$ as described before (Bhoite-Solomon et al., 1992). Myosin maintenance was in a soluble form at high ionic strength of 0.6 M NaCl at 4 °C. To allow any further required pH change, no buffer was added. Myosin concentration was measured spectrophotometrically using 280 and 330 nm absorption (Spudich and Watt, 1971; Tonomura et al., 1966). Light scatter was eliminated by subtraction of the UV-extrapolated base line. Protein concentrations were measured by the Lowry method (Lowry et al., 1951). *P/D* is a term used throughout the study for protein content in supernatants (including protein degradation products). *P/D* was measured using OD 280 (corrected for light scatter). As supernatant *P/D* was expressed as percent of total protein in reaction mixture, no extinction coefficient was used. Protein patterns of reaction mixtures were assessed by SDS−PAGE (Laemmli, 1970) using slab gels of either homogenous 10% acrylamide and 0.27% *N,N*′-methylenebisacrylamide or biphasic 6% (0.16% *N,N*′-methylenebisacrylamide) and 12% acrylamide (0.32% *N,N*′-methylenebisacrylamide) in the upper and lower parts, respectively. Gels were run at a constant current of 40 mA/gel and stained with 0.2% Coomassie blue R-250.

*Apparatus.* Electrophoresis was carried out using Hoeffer minigels apparatus. Spectrophotometry was performed by a Varian 635 or Cary 219 instrument.

**Experimental Procedures.** *Myosin Proteolysis by Individual Enzymes.* Reaction mixtures contained 1−2 mg/mL of either myosin monomer (MM) or cross-linked myosin (CM) and were incubated with one of four enzymes: pepsin, trypsin, chymotrypsin, or papain. The temperature was 37 °C, and incubation duration was up to 10 h. The pH of the reaction mixtures were gastric pH of 1.85 for pepsin action, achieved by 0.033 M glycin buffer, or duodenal pH of 8.0, achieved by 0.1 M $NaHCO_3$, for the action of trypsin, chymotrypsin, and papain.

*Successive Proteolysis Experiments.* Reaction mixtures containing 1 mg/mL of either MM or CM were preincubated at 37 °C for 15 min in the presence of 0.033 M glycine, pH 1.85, buffer. Then, 400 U/mL pepsin was added in a minimal volume (less than 4% of total volume), and the reaction mixtures were further incubated at 37 °C for 30 min. This was followed by raising the solution pH to 8.0 by addition of 1.0 M $NaHCO_3$, pH 9.0, buffer (in 1/10 of the total reaction volume) containing 10 U/mL trypsin and 1.0 U/mL chymotrypsin. Reaction mixtures were further incubated at 37 °C for 10 min and terminated by transfer to 2 °C. Identical parallel reaction mixtures were used to determine proteolysis progress in each stage of the process. Samples were centrifuged at 20600*g* for 15 min in the cold. Aliquots of the supernatants were withdrawn, mixed with SDS and mercaptoethanol (final concentrations of 3% and 1.5%, respectively), and boiled for 3 min prior to SDS−PAGE analysis. The bulk of the supernatant served to measure protein and peptide content by 280 nm absorption.

## RESULTS

**Degradation of Monomeric and Cross-Linked Myosin by Individual Proteolytic Enzymes.** The proteolytic activity of the alkaline enzymes trypsin and chymotrypsin on isolated MM was studied under various protein/enzyme proportions and incubation times. All reactions were carried out at 37 °C and pH 8.0, resembling the duodenal pH conditions, using 0.1 M carbonate buffer. The same conditions were used to study papain activity. Proteolysis was evaluated by protein pattern on SDS−PAGE. For comparison of the activity of the individual enzymes on MM and CM, the conditions which caused substantial degradation of MM were chosen. Samples of MM and CM without enzymes, treated identically as the reaction mixtures, served as references (controls).

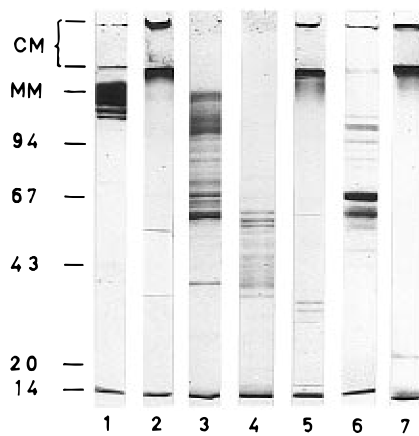


**Figure 1.** Protein patterns of trypsin- or chymotrypsin-treated MM and CM. Conditions: My, 1 mg/mL in lanes 1−5 and 1.5 mg/mL in lanes 6 and 7; buffer, 0.1 M carbonate (pH 8) + 0.6 M NaCl; temp of incubation, 37 °C; tryp treatment, lanes 3−5; chym treatment, lanes 6 and 7; amount of total protein loaded/lane, 20 μg in lanes 1−5 and 39 μg in lanes 6 and 7. Lane 1, MM; lane 2, CM; lane 3, MM + tryp (1.0 U/mL), 2 h; lane 4, MM + tryp (10 U/mL), 2 h; lane 5, CM + tryp (10 U/mL), 2 h; lane 6, MM + chym (0.1 U/mL), 40 min; lane 7, CM + chym (0.1 U/mL), 40 min.

*Trypsin Treatment.* MM and CM at identical concentrations were subjected to trypsin at pH 8.0 (see Methods) in a wide range of activities (0.1−100 U/mL per 1 mg/mL protein) and various incubation times (5 min−10 h). At time intervals samples were withdrawn, SDS-containing buffer was added immediately to terminate the reactions, and the samples were loaded on SDS−PAGE for protein analysis. Representative reactions are demonstrated in Figure 1. Comparison of protein patterns of trypsin treated and reference MM (lanes 3 and 1, respectively) showed that after 2 h of incubation with 1 U/mL trypsin, a small fraction of MM was left intact and peptides of heterogeneous sizes, 14−150 kDa, were formed. Increasing the enzyme reactivity by 1 order of magnitude to 10 U/mL resulted in a massive breakdown of the MM to small peptides, some of which were seen in the 35−67 kDa range while the rest appeared in the gel front (lane 4). It should be stated that very small peptides and amino acids are anticipated to leave the gel under the used SDS−PAGE conditions. The response of CM to trypsin action differed from that of MM: CM was resistant as demonstrated by the fact that even at 10 U/mL trypsin CM retained its original form and only a small fraction of peptides was produced (compare lanes 5 and 2).

*Chymotrypsin Treatment.* Application of chymotrypsin (0.01−1.0 U/mL/mg of protein) to MM and CM (for 5 min−10 h) showed the same trend as with trypsin: relative resistance of the CM protein. An example is demonstrated in Figure 1, lanes 6 and 7. Incubation of the myosins with 0.1 U/mL enzyme/mg of protein for 40 min resulted in a massive breakdown of the monomer as expressed by loss of the intact protein and appearance of smaller peptides mainly within the 43−67 kDa range (lane 6). Under identical conditions the cross-linked protein was left practically intact (lane 7).

*Papain Treatment.* Papain ranging between 0.0015 and 0.15 U/mL was applied to MM and CM for various incubation times (5 min−10 h). Under all conditions CM was more resistant than MM to papain hydrolysis. Comparison of protein patterns of papain-treated and reference MM (Figure 2, lanes 3 and 1, respectively) showed that after 2 h of incubation with 0.015 U/mL

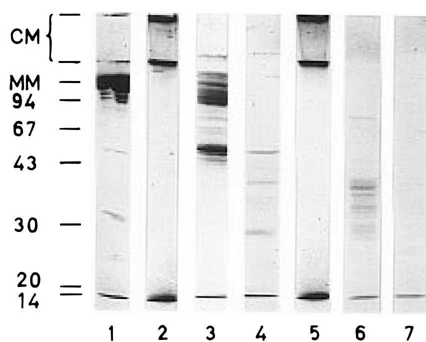


**Figure 2.** Protein patterns of papain- or pepsin-treated MM and CM. Myosin was cross-linked by myoglobin/$H_2O_2$ (according to Bhoite et al., 1992). Conditions: My, 1 mg/mL; buffers, lanes 1−5, 0.1 M carbonate (pH 8.0) + 0.6 M NaCl, and lanes 6 and 7, 0.6 M NaCl + 0.033 M glycine (pH 1.85); temp of incubation, 37 °C; amount of total protein loaded/lane, 20 $\mu$g. Lane 1, MM; lane 2, CM; lane 3, MM + papain (0.015 U/mL), 2 h; lane 4, MM + papain (0.15 U/mL), 2 h; lane 5, CM + papain (0.15 U/mL), 2 h; lane 6, MM + pepsin (10 U/mL), 1 h; lane 7, CM + pepsin (10−1000 U/mL), 2 h.

papain, a small fraction of MM was left intact and peptides of heterogeneous sizes, 43−150 kDa, were formed. A 1 order of magnitude increase in enzyme activity resulted in practically complete degradation of MM (compare lanes 4 and 1, Figure 2). Under the same conditions papain did not cause any degradation of the CM (compare lane 5 and 2, Figure 2).

*Pepsin Treatment.* Pepsin operates in vivo in the stomach where the pH can range from 1 to 5 (Brooks, 1974). We first measured the activity of pepsin on native myosin (MM) at this pH range (data not shown) and selected pH 1.85, which allows optimal pepsin activity on myosin, throughout this study. At this pH, both monomeric and cross-linked myosin forms were precipitated, but while MM precipitated as a compact solid block, the CM formed flakelike clouds, which precipitated slowly. The proteins were preincubated at pH 1.85 for 15−30 min prior to application of the enzyme. Various pepsin activities (0.01−2000 U/mL) were employed for different durations at 37 °C. All reaction mixtures were centrifuged at the end of incubation, and the supernatants were subjected to SDS−PAGE analysis. In reaction mixtures containing CM, barely any protein bands could be observed when 10−1000 U/mL pepsin was employed for 2 h. An example is demonstrated in lane 7 of Figure 2. In reaction mixtures containing MM, some degradation products were observed when 10 U/mL pepsin was employed for 1 h (lane 6, Figure 2), but the total protein in the bands comprised only a small fraction of the loaded amount.

The extent of myosin proteolytic degradation could be assessed by measuring the 280 nm absorption of the supernatants which contain small size peptides soluble even at the acidic pH. Supernatant *P/D* (defined in Methods) content of each sample was expressed as percent of myosin amount introduced initially to the reaction mixture. *P/D* measurements of all reaction mixtures of this study are presented in Figure 3. Protein traces were found in supernatants of both MM and CM controls incubated without pepsin (Figure 3, bar pair 1, MM−gray bar, CM−netted bar). The percent of soluble peptides produced after 30 min incubation of 400 U/mL pepsin with MM or CM is demonstrated in Figure 3, bars 2. As can be seen, in the CM supernatant, no additional protein content above control level was found (compare netted bars 2 and 1). In contrast, the MM supernatant contained an

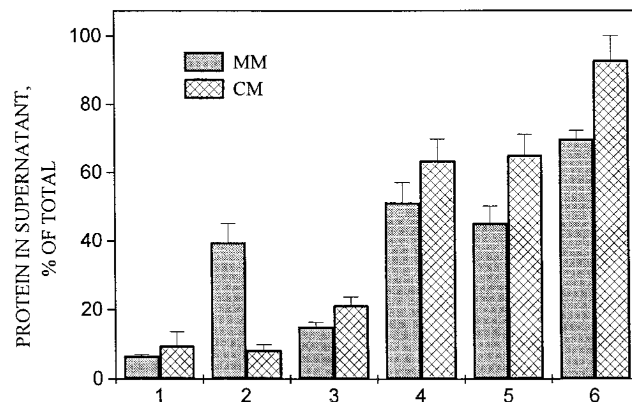


**Figure 3.** Fraction of protein in supernatant following various treatments. Concentration of reactants: myosins, 1 mg/mL; pepsin, 400 U/mL; trypsin, 10 U/mL; chymotrypsin, 1.0 U/mL; pH of reaction mixtures, 1.85 (bar pairs 1 and 3) or 8.0 (bar pairs 2, 4, 5, and 6); temp, 37 °C. Shown are mean ± SE values of three experiments: gray bars, My monomer; netted bars, cross-linked My. Bar pair 1, following incubation at pH 1.85; bar pair 2, pepsin treatment following preincubation at pH 1.85; bar pair 3, no enzymes added, preincubation at pH 1.85 and then elevation of pH to 8.0; bar pair 4, as in 2 and then elevation of pH to 8.0; bar pair 5, as in 3 followed by tryp/chym treatment; bar pair 6, as in 4 followed by tryp/chym treatment.

additional 33% more protein above control level (compare gray bars 2 and 1). SDS−PAGE of the supernatants served to demonstrate medium size peptide, as very small peptides are likely to leave the gel during electrophoresis. When these supernatants were run on SDS−PAGE, neither showed any protein bands (Figure 4, lanes 3, left and right). In the case of CM this is explained by the fact that no protein material was released into the supernatant following pepsin treatment (compare netted bars 2 and 1 in Figure 3). In the case of MM the fact that no protein bands appeared in the gel despite expression of a large fraction of the protein in the supernatant (compare gray bars 2 and 1 in Figure 3) indicated that this material was composed of small size peptides only.

**Degradation of Monomeric and Cross-Linked Myosin by Successive Enzymatic Proteolysis.** To simulate the protein fate of MM and CM in the digestive tract, we applied the main digestive enzymes in their respective order at their in vivo pH's. Following preincubation and pepsin treatment, the pH of the reaction mixtures was raised to 8.0 and a mixture of the alkaline enzymes trypsin and chymotrypsin (tryp/chym) was added. CM and MM supernatants at each step in the process were analyzed by both SDS−PAGE and 280 nm absorption. Representative results are displayed in Figures 3 and 4. In the absence of proteases, mere alkalinization of low-pH-precipitated protein samples led to increased supernatant *P/D* of both MM and CM above control levels (compare bar pairs 3 and 1, Figure 3). SDS−PAGE analysis of these supernatants showed small size peptides only in the MM sample (Figure 4, left, lane 2) but entirely cross-linked protein in the CM sample (Figure 4, right, lane 2). These findings indicate that unlike MM, CM, although aggregated, is resoluble. This CM feature is amplified upon alkalinization following pepsin treatment. Comparison of the netted bars 4 and 2 in Figure 3 indicated that by elevating the pH, the amount of soluble CM material was increased by ~55% above nonalkalinized levels while that of soluble MM increased only by ~10% (compare gray bars 4 and 2). The size of the soluble material was assessed by

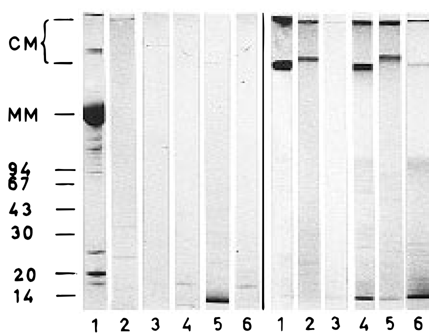


**Figure 4.** Protein patterns of supernatants following various treatments. Conditions: My, 1 mg/mL; buffers, (lane 1) 0.6 M NaCl, (lane 3) 0.6 M NaCl + 0.033 M glycine (pH 1.85), (lanes 2 and 4−6) 0.6 M NaCl + 0.033 M glycine + 0.1 M carbonate; final pH, 8; temp of incubation, 37 °C; amount of total protein loaded/lane, 20 $\mu$g; M, myosin monomer heavy chains; CM, cross-linked myosin heavy chains; enzyme activity, pepsin, 400 U/mL, trypsin, 10 U/mL, chymotrypsin, 1 U/mL. The left side of the figure refers to MM and the right side to CM, treated identically. Lane 1, untreated protein; lane 2, proteins incubated at pH 1.85 for 15 min and then pH elevated to 8.0 (with no enzymes added); lane 3, pepsin-treated proteins; lane 4, as in 3 followed by pH elevation to 8.0; Lane 5, as in 2 followed by combined tryp/chymo treatment; lane 6, action of all enzymes as described in the text.

SDS−PAGE. In the case of MM, only very small peptides were detected (Figure 4, left, lane 4), whereas in the case of the CM sample all the soluble protein was still cross-linked (Figure 4, right, lane 4).

Further incubation of the myosins with tryp/chym increased the fraction of soluble protein in both CM and MM samples (compare bar pairs 6 and 4 , Figure 3), reaching an average of 95% and 70%, respectively. Practically no protein bands were detected by SDS−PAGE in the MM supernatant (Figure 4, left, lane 6) indicating that all the soluble material consisted of degraded products of very small peptides or even amino acids which left the gel. In contrast, the supernatant of the CM form still contained some cross-linked material in addition to heterogeneous size peptides of 14−30 kDa (Figure 4, right, lane 6). The residual precipitates of both MM and CM were treated with 3% SDS, and the soluble material was loaded on SDS−PAGE. MM samples contained fragments of 50−70 kDa, whereas CM samples contained cross-linked protein only (data not shown).

## DISCUSSION

Oxidatively damaged proteins are generally considered to be prone to enhanced proteolysis (Davies, 1985; Fagan et al., 1986; Davies and Goldberg, 1987; Davies et al., 1987; Goldberg and Boches, 1982; Rivett, 1985; Grune et al., 1995). The $H_2O_2$/hemin oxidatively damaged myosin (CM), investigated in the current study, showed the reverse. In soluble form, CM was remarkably less susceptible to enzymatic hydrolysis than the monomeric native myosin (MM) when three proteases, namely, papain, trypsin, and chymotrypsin, were individually employed; under reaction conditions in which MM supernatant contained degraded material, that of CM contained mostly intact cross-linked material (Figures 1 and 2). Inaccessibility of proteases toward a substantial number of peptide bonds located within the core of the CM conglomerate may explain its resistance to proteolysis as compared to the native protein. Reduced accessibility of enzymes to polymeric protein structures is a general phenomenon. For example, decomposition of cellulose to glucose by cellulase relates to the level of cellulose crystallinity (Selbey, 1962).

When analyzing pepsin activity on the two myosins, one has to take into consideration not only the structural differences between MM and CM but also the fact that by subjecting these two proteins to low pH, pepsin is reacting on the protein precipitates. Following subjection of both precipitated myosins to pepsin, no protein bands could be detected on SDS−PAGE (Figure 4, lanes 3). In such a case one cannot distinguish between massive proteolysis yielding small peptides/amino acids escaping the gel and no proteolysis or a partial one which leaves large size peptides in a precipitated form. Spectrophotometric analysis of the supernatants revealed substantial *P/D* content in MM compared to a negligible *P/D* content in the CM (Figure 3, bar pair 2). This proved that no breakdown of the CM nor its breakdown to large size peptides occurred. Protein pattern of the precipitated CM fraction that could be solubilized by SDS indicated that it contained mostly intact cross-linked material (data not shown). Thus, no large size fragments were left in the precipitate. These results were interpreted as reflecting resistance of CM to pepsin proteolysis compared to MM.

To simulate the sequence of events in the digestive tract following pepsin activity at gastric pH, the protein had to reach duodenal conditions of pH 8.0 prior to tryp/chym action. In general, subjecting proteins to extreme pH conditions results in neutralization of their charged superficial groups. Concomitant irreversible unfolding (denaturation) and increased hydrophobicity leads to protein precipitation. Refolding of protein allowing resolubilization by pH elevation is generally impossible, as in the case of MM where only small size proteolytic products could be detected in all its supernatants. Unlike MM, existence of rigid intermolecular covalent bonds in the cross-linked protein (CM) prevented its complete unfolding under low pH. Consequently, in this case, neutralized superficial side chain groups *could be* recharged upon elevation of the pH leading to resolubilization of intact CM. Since the aggregate's surface group comprised a small portion of the total rechargeable side groups, only part of the CM could be solubilized by pH elevation alone (netted bar 3, Figure 3). As a matter of fact, minor pepsin proteolysis sufficed to expose an additional number of side chains, thus recharging additional cryptic groups. Thereby the solubilized intact CM amount increased considerably (compare netted bars of pairs 4 and 3, Figure 3). The same was true for direct tryp/chym treatment following pH elevation of the low-pH-precipitated CM (compare netted bars of pairs 5 and 3, Figure 3).

Successive protease treatment by enzymes attacking different peptide bonds eventually resulted in some extent of cross-linked degradation (Figure 4, right, lane 6). Seemingly, pepsin proteolytic attack "scrapes off" some of the conglomerate external cross-linked material, exposing inner cryptic sites for further proteolytic attack. Because of CM resolubility upon pH elevation, its ultimate supernatant contained both nondegraded cross-linked and degraded peptide material. Therefore CM supernatant content reached 95%, while MM supernatant (containing hydrolyzed material solely) reached only 70% of the total introduced protein (Figure 3, bar pair 6). The relative resistance of CM to proteolysis is stressed by the fact that despite its complete solubility and conditions which usually render the molecule accessible to proteases action, the cross-linked protein

underwent partial proteolysis only. It is noteworthy that the various systems showing degradation of oxidatively damaged proteins involved mostly non-cross-linked protein material, whereas the heme/$H_2O_2$-peroxidized myosin is a cross-linked protein. Few studies demonstrated that oxidative covalent cross-linking actually prevents proteolysis (Davies, 1986; Dizdaroglu, 1984). Thus, retarded hydrolysis of cross-linked proteins is apparently a general phenomenon.

CM was shown to be formed by cross-linking of myosin heavy chains via non-S−S covalent bonds (Bhoite-Solomon et al., 1992). Similar cross-linking of myosin heavy chains was reported in muscle food (Kim et al., 1984). As aforementioned, heme or the hemoproteins hemoglobin and myoglobin which exist in muscle foods serve as triggers for CM formation in the presence of $H_2O_2$. Hemoproteins themselves can serve as an $H_2O_2$ source by their autoxidation to met ($Fe^{III}$) forms in the presence of oxygen (Wallace et al., 1982). Indeed, generation of $H_2O_2$ by this nonenzymatic reaction was demonstrated in muscle tissues (Harel and Kanner, 1985). It was shown that cross-linking of myosin heavy chain molecules is associated with meat browning and with decreased protein extractability (Kim et al., 1984). Formation of methemoglobin/metmyoglobin and globin-free hemin is the main cause of brown color development in muscle food (Giddings, 1977; Igene et al., 1979; Faustman and Cassens, 1990). Therefore, it is likely that the in vitro heme/$H_2O_2$-cross-linked myosin investigated in the current study is representative of cross-linked myosin existing in muscle food.

In vitro digestibility tests are used for evaluating the bioavailability of amino acids (Hsu et al., 1977; Shetty and Kinsella, 1982; Rothenbuhler and Kinsella, 1985). Thus, the present investigation of myosin proteolysis is informative for amino acid availability, known to be the most important factor affected by digestibility (Kakade, 1974). As myosin comprises a major fraction of muscle protein, in vitro proteolysis of myosin can be used to evaluate muscle food digestibility. Thus, the findings of the present study, demonstrating reduced hydrolysis of CM by the digestive tract enzymes, indicate that browning of muscle foods reduces their nutritional value due to decreased digestibility and extractability.

## ABBREVIATIONS USED

CM, cross-linked myosin; chym, chymotrypsin; MM, monomeric myosin; My, myosin; MW, molecular weight; PAGE, polyacrylamide gel electrophoresis; *P/D*, protein content including degradation products; SDS, sodium dodecyl sulfate; tryp, trypsin; U/mL, units per milliliter.

## ACKNOWLEDGMENT

We thank Mr. A. Pinchasov for excellent photography and Mrs. R. Gutman for bibliographical assistance.

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Received for review July 5, 1995. Revised manuscript received March 4, 1996. Accepted April 25, 1996.[⊗] This study was supported by Grant 000044-6327-1-94 from the Israeli Science and Art Ministry.

JF950413X

[⊗] Abstract published in *Advance ACS Abstracts,* June 1, 1996.