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# Effect of Microbial Rennets on Meat Protein Fractions

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Proteases of *Mucor miehei* and *Endothia parasitica*, used commercially as microbial rennets, were incubated with isolated myofibrillar, sarcoplasmic, and insoluble connective tissue fractions of meat to evaluate their potential for meat tenderization. SDS–polyacrylamide gel electrophoresis of incubation mixtures demonstrated that *E. parasitica* proteases cleaved all myofibrillar and sarcoplasmic proteins within 24 h, as did a commercial papain preparation. In addition, only papain demonstrated effective proteolysis of the insoluble connective tissue. A stable 130 000–140 000 Da degradation product of myosin resulted from action of *M. miehei* enzymes. These results suggest that *M. miehei* proteases may prove useful in meat tenderization when limited proteolysis of myosin is desired.

## INTRODUCTION

Tenderness in meat can be directly related to the structure of components of its myofibrillar and connective tissue fractions (Bailey, 1972). Approaches to increasing the perceived tenderness have utilized either enzymatic or mechanical means to alter these structures. The majority of the enzymatic efforts have utilized proteases of animal or plant origin with economic considerations favoring the use of plant proteases such as bromelain, papain, and ficin. Varying positive results have been attained by antemortem injection or spraying, dipping, or multiple needle injection of meat pieces with solutions of the plant enzymes (Flynn, 1975). Papain, often containing chymopapain, is presently being used for the tenderization of beef (Brocklehurst et al., 1981).

The major effect of papain on meats has been shown to be the proteolysis of the myofibrillar protein fraction (Kang and Rice, 1970) especially of actin and myosin (Rattrie and Regenstein, 1977). Additional reports (Miyada and Tappel, 1956; Kang and Warner, 1974) also suggest proteolysis of connective tissue components by papain; however, it has not been clearly demonstrated that this action is on the insoluble connective tissue. Nevertheless, increased tenderness is observed following papain treatment. An undesirable "mushy" mouth feel results, however, if proteolysis is too extensive.

Comminution of selected meat cuts and restructuring of the pieces into formed products is another method of improving textural qualities. This effect is exerted primarily on the connective tissue. For selected products a combination of mechanical and enzymatic approaches may produce the best results. A significant reduction in binding of comminuted meat pieces occurred with the addition of papain, however (Schnell et al., 1973). This decrease in binding was probably due to degradative action of the papain on extracted myosin, the primary component responsible for binding of meat pieces (Macfarlane et al., 1977; Ford et al., 1978; Turner et al., 1979; Siegel and Schmidt, 1979; Schmidt and Trout, 1982).

In order to identify proteases having more desirable specificities than papain toward muscle proteins, i.e. less activity on myofibrillar proteins and more activity on connective tissue components, selected microbial proteases were evaluated for activity on isolated meat fractions. Protease preparations of *Mucor miehei* and *Endothia parasitica*, commercially available and widely used by the cheese industry as microbial rennets, were selected since they demonstrate differing specific peptide bond cleavage (Whitaker, 1970; Ottesen and Rickert, 1970).

## MATERIALS AND METHODS

**Enzymes.** Rennet preparations from *M. miehei* were obtained from Chr. Hansen's Laboratory, Inc. (*Mucor miehei*$_1$), and Pfizer Chemical Division (*Mucor miehei*$_2$) both of Milwaukee, WI. A rennet preparation from *E. parasitica* was also supplied by Pfizer Chemical Division. A commercial preparation of papain, donated by Swift Independent Packing Co., Oak Brook Terrace, IL, was used for comparison of enzyme effects. Chromatographically purified collagenase from *Clostridium histolyticum* was purchased from Sigma Chemical Co., St. Louis, MO.

Proteolytic activities were determined by a modification of the method of Arnon and Shapira (1967) using a purified $\alpha_s$-casein complex substrate (gift of Dr. H. M. Farrell, Jr.). An aliquot (825 μL) of a prewarmed 1% casein solution in 0.05 M sodium acetate, pH 5.5, was added to 175 μL of buffer containing varying amounts of enzyme (5–350 μg) in the presence or absence of papain activating agents, 2 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM cysteine. After 10-min incubation at 37 °C, 1.5 mL of 5% trichloroacetic acid (TCA) was added, and the mixture was

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kept at 25 °C for 1 h before it was filtered through Whatman No. 1 paper. The absorbance at 280 nm of the resulting filtrates was measured against a filtered casein blank incubated similarly but without enzyme. Activities were calculated as change in absorbance at 280 nm per milligram of enzyme per 10 min at 37 °C.

**Preparation of Meat Protein Fractions.** Insoluble endomysial and perimysial connective tissue was isolated from a retail sample of beef chuck by the dry-blending method of McClain (1969) after the removal of gross layers of fat and adhering epimysial connective tissue. Strands of white connective tissue were removed from the pulverized muscle with forceps, washed for 3 h with 0.15 M NaCl at 5 °C, then rinsed with and exhaustively dialyzed against deionized water, and lyophilized. This material was characterized by amino acid analysis on a Beckman Model 119CL analyzer after hydrolysis in 6 N HCl at 110 °C for 20 h in vacuo. Before incubation with enzymes the lyophilized connective tissue was ground in a Wiley mill to pass a 20-mesh screen.

Sarcoplasmic proteins, those muscle proteins soluble in neutral solutions of low ionic strength (Szent-Györgyi, 1960), were extracted from 250 g of the fragmented muscle fraction remaining from the previous procedure by homogenization at 5 °C with 1 L of 0.15 M NaCl, 0.05 M Tris-HCl at pH 7.4, containing 20 mM EDTA, 1 mM *N*-ethylmaleimide (NEM), and 1 mM (phenylmethyl)-sulfonyl fluoride (PMSF). Tissue homogenates were centrifuged (12000*g*) at 4 °C for 1 h, and the supernatants were filtered through glass wool and pooled. The pellet was extracted twice more with the low-salt buffer, and the extracts were centrifuged and filtered. Supernatants were pooled with those of the initial extract.

The sedimented material resulting from sarcoplasmic protein extraction was homogenized three times at 5 °C with 1.0 M NaCl, 0.05 M Tris-HCl, pH 7.4, containing EDTA, NEM, and PMSF as before to extract myofibrillar proteins which require neutral-salt solutions of high ionic strength ($\Gamma/2 > 0.5$) for initial solubilization (Szent-Györgyi, 1960). Sarcoplasmic and myofibrillar protein preparations were stored at −80 °C. Before use they were thawed slowly to 5 °C, concentrated in dialysis tubing under negative pressure, and dialyzed exhaustively against deionized water.

**Incubation of Meat Proteins with Enzymes.** The protein concentrations of enzyme solutions and sarcoplasmic and myofibrillar slurries were determined by the method of Lowry et al. (1951) using a bovine serum albumin standard.

Concentrated myofibrillar and sarcoplasmic protein preparations were diluted to 10.5 mg $mL^{-1}$ in 0.05 M sodium acetate at pH 5.5, the pH of meat. Aliquots (240 $\mu$L) of these solutions were incubated for 15 min to 24 h at 25 °C with 10 $\mu$L of enzyme solution or buffer without enzyme. At the end of incubation an equal volume of electrophoresis sample buffer (0.13 M Tris-HCl at pH 6.8, 5% sodium dodecylsulfate (SDS), 1.4 M $\beta$-mercaptoethanol, 25% glycerol) was added, and the sample was heated at 100 °C for 2 min to inactivate the proteases and to prepare the samples for electrophoresis.

Samples of ground, lyophilized, insoluble connective tissue (1.25 mg) were incubated at 25 °C in a total volume of 0.25 mL of 0.05 M sodium acetate at pH 5.5 containing enzyme for 4 h. Proteolysis was stopped by the addition of an equal volume of sample buffer and heating at 100 °C for 2 min. Digests were centrifuged at approximately 13000*g* for 3 min in an Eppendorf microfuge, and the soluble supernatant components were analyzed by SDS–polyacrylamide gel electrophoresis.

**Electrophoresis.** Electrophoresis reagents were products of BioRad Laboratories, Richmond, CA.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed by the discontinuous system of Laemmli (1970) as modified by Basch et al. (1985), using a 10% acrylamide separating gel slab with a 4% acrylamide stacking gel. With this modification thiourea is the cross-linking agent and polymerization is catalyzed with $H_2O_2$. Molecular weight standards used and their corresponding approximate molecular weights were as follows: myosin, 200 000; $\beta$-galactosidase, 116 000; phosphorylase b, 94 000; bovine serum albumin, 68 000; actin, 42 000; troponins including troponin T, 37 000; tropomyosin, 35 000; carbonic anhydrase, 29 000.

Gels were fixed in a solution of 10% TCA, 10% methanol, and 7% acetic acid and stained by the addition of 1% Coomassie blue R-250 in acetic acid–methanol–$H_2O$ (10:50:40) to a final 0.03% concentration. After 1.5 h of staining at 25 °C, the gels were destained in a solution of 10% methanol and 7% acetic acid.

Stained gels were scanned at approximately 560 nm on a Gelman ACD-18 densitometer, and integrated peak areas were used to evaluate degradation of specific proteins and the appearance of degradation products.

## RESULTS AND DISCUSSION

Analyses of the isolated meat fractions by SDS–PAGE indicated that they contained the proteins expected. The molecular weights of major components of the myofibrillar preparation were consistent with those of literature values (Harrington, 1979) for myosin heavy chain, actin, troponin T, and tropomyosin. In addition, their gel positions coincided with those of similarly run standards for those proteins (data not shown). Gels of the sarcoplasmic preparation showed a protein pattern unlike that of the myofibrillar preparation, in that it did not contain any of the major myofibrillar proteins but did contain numerous bands of protein with molecular weights between 26 000 and 186 000 Da. On the basis of its amino acid composition, the isolated insoluble connective tissue preparation was essentially collagen with only minor contamination.

All of the microbial enzyme preparations appeared to be highly purified when examined by SDS–PAGE (data not shown). Both preparations of enzyme(s) from *M. miehei* exhibited a major band of protein of approximately 43 000 Da and a minor band at 23 000 Da. The enzyme preparation from *E. parasitica* contained a sharp major band of protein of approximately 36 000 Da. The papain preparation showed a major band of protein with a molecular weight of 23 000 Da and another broad, heavy band smeared between 27 000 and 35 000 Da, which demonstrated the presence of proteins other than papain or chymopapain ($M_r$ 23 000 and 27 000, respectively).

Because of the apparent high purity of the microbial enzyme preparations, specific activities against a common substrate were measured without further purification. Therefore, proteolytic activity was measured against $\alpha_s$-casein complex as described in the presence or absence of papain activating agents. Similar levels of activity were found for both *Mucor* and *Endothia* proteases, which were significantly higher ($p < 0.01$ by the least significant difference test) than that of unactivated papain (Table I). The activating agents had neither an inhibiting nor an activating effect on the fungal proteases.

Results of the $\alpha_s$-casein assay demonstrated that the proteolytic activity of 1 $\mu$g of papain was equivalent to that of 10 $\mu$g of fungal protease. Because of this difference in specific activities, incubations of these enzymes with meat

**Table I. Specific Activity of Microbial Proteases and Papain on $\alpha_s$-Casein Complex**

| enzyme preparation | activity[a] without act. agents[b] | activity[a] with act. agents |
|---|---|---|
| papain | 0.6 | 20.6 |
| *Mucor miehei*$_1$ | 1.8 | 1.7 |
| *Mucor miehei*$_2$ | 2.0 | 2.1 |
| *Endothia parasitica* | 1.8 | 1.6 |

[a] Increase in $A_{280}$ units per milligram of protein per 10 min of incubation at pH 5.5 and 37 °C. [b] 2 mM EDTA, 5 mM cysteine.

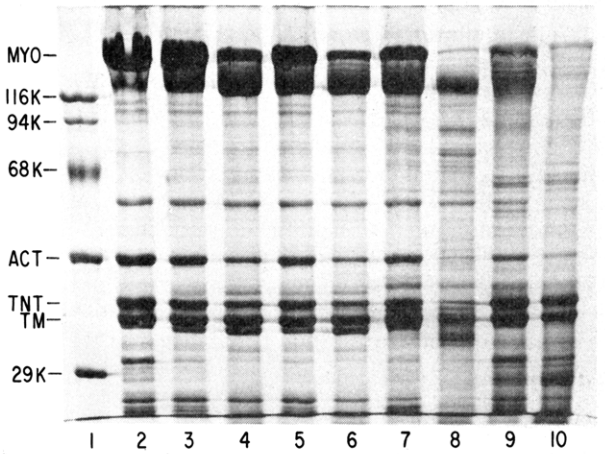


**Figure 1.** SDS–PAGE of myofibrillar proteins incubated with enzymes. (1) Molecular weight standards; myofibrillar proteins (2) at 0 time and (3–10) incubated with enzyme preparations of *Mucor meihei*$_1$ (3) for 15 min and (4) for 4 h, *Mucor miehei*$_2$ (5) for 15 min and (6) for 4 h, *Endothia parasitica* (7) for 15 min and (8) for 4 h, and papain (9) for 15 min and (10) for 4 h. Enzyme to substrate ratios are 1:50 by weight for microbial enzymes and 1:500 for papain. Key: MYO = myosin; ACT = actin; TNT = troponin T; TM = tropomyosin.

proteins were performed using equivalent amounts of $\alpha_s$-casein digestion units. Interestingly, when papain was incubated with myofibrillar proteins as described in the presence or absence of activating agents and the resulting gel patterns were compared, the proteins in both mixtures were similarly degraded over the entire incubation period (data not shown). This result suggests that components of the muscle preparation were able to completely activate the papain.

Preferential cleavage of actin and myosin by all of the enzymes was observed after incubation for 15 min (Figure 1). The two *Mucor* preparations demonstrated similar patterns of proteolysis. Analysis of their gel scans showed approximately 28% of the myosin was degraded in 15 min, creating a major band of proteins of approximately 130 000–140 000 Da that remained resistant to further proteolysis throughout 24 h of incubation. A pattern of gradual degradation of myosin continued over time, with approximately 70% of the myosin degraded by the *Mucor* enzymes in 4 h. Actin was similarly degraded 26% in 15 min and 63% in 4 h. The *Endothia* preparation appeared more active than the *Mucor* preparations, cleaving 67% and 87% of myosin in 15 min and 4 h, respectively, and 42% and 86% of actin at the same times. Initial major cleavage products of myosin were proteins of 130 000–140 000 Da as with the *Mucor* enzymes; by contrast, however, these proteins continued to be degraded by the *Endothia* enzyme and were totally degraded in 24 h.

The other major myofibrillar components, troponin T and tropomyosin, appeared to be less affected than actin and myosin by the fungal proteases during early times of incubation. Increases in calculated areas at these gel positions indicate, however, that degradation products from higher molecular weight proteins are present in these bands as well, making conclusions about their susceptibility to proteolysis difficult. These protein bands persisted throughout the incubation with *Mucor* proteases, however. In contrast, the *Endothia* enzyme(s) degraded all the myofibrillar proteins in 24 h.

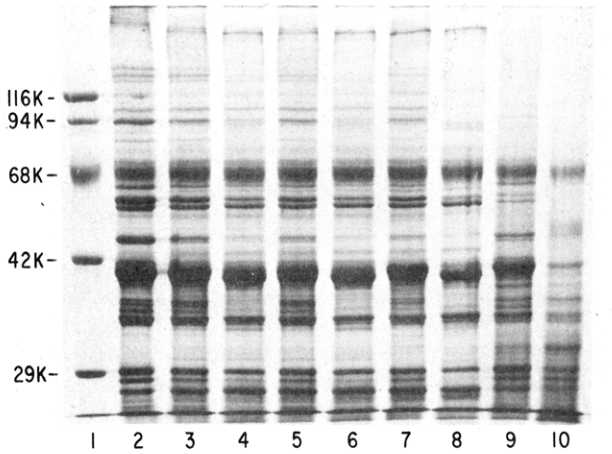


**Figure 2.** SDS–PAGE of sarcoplasmic proteins incubated with enzymes. Lane designations and enzyme to substrate ratios are identical with those in Figure 1.

The pattern of myofibrillar protein degradation by papain differed from those of the microbial enzymes. Papain degraded most (81%) of the myosin within 15 min and did not produce any major stable high molecular weight products. High molecular weight proteins were slowly degraded with time. Approximately 70% of actin was also degraded within the first 15 min of incubation. In contrast to the microbial enzymes, papain had little activity on tropopin T and tropomyosin over the 24-h period. Although cleavage products from larger proteins may have appeared in the region of these protein bands, the sharpness and consistent intensity of the bands suggest that they contained predominantly the original proteins at near initial concentrations.

If nearly complete degradation of myosin similar to that observed with papain is responsible for the "mushy" mouth feel of treated meats, then these data suggest that a limited cleavage of myosin by the proteases of *M. miehei* may permit tenderization of meat without total loss of meat structure.

Although the sarcoplasmic proteins are not known to perform a structural function either in the native muscle or in the binding of comminuted meat pieces, the enzymes were tested for their effect on this class of proteins. SDS–polyacrylamide gels of incubation mixtures showed that sarcoplasmic proteins were fairly stable to digestion by the *Mucor* proteases for 4 h, with the exception of a broad major protein band of 40 000 Da, a band of 46 000 Da, and a minor high molecular weight band >120 000Da (Figure 2). Increasing degradation of the 40 000-Da proteins and proteins >68 000 Da was evident with longer times of incubation. Protease(s) of the *Endothia* preparation degraded a greater number of sarcoplasmic proteins to smaller peptides than did the *Mucor* proteases after 4 h of incubation. These activities were in contrast to that of papain that degraded most of the proteins over the 4-h period.

Effects of these enzymes on insoluble connective tissue are important considerations in evaluation of their possible use in meat tenderization. Treatment of insoluble connective tissue with the sample buffer and heat in prepa-

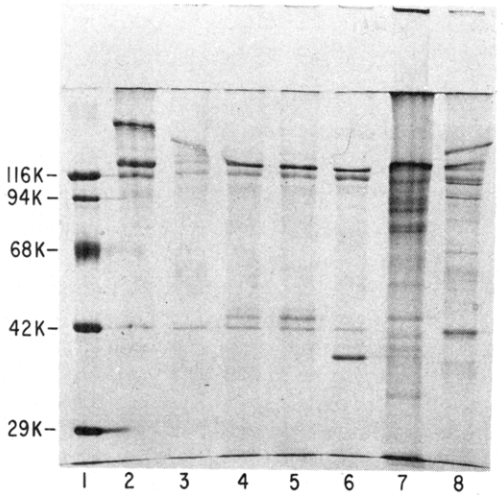


**Figure 3.** SDS–PAGE of proteins and peptides solubilized by enzymatic digestion of insoluble connective tissue. (1) Molecular weight standards; (2) soluble type I collagen standard; 4-h incubations with (3) no enzyme and preparations of (4) *Mucor meihei*$_1$, (5) *Mucor meihei*$_2$, (6) *Endothia parasitica*, (7) papain, and (8) collagenase.

ration for gel electrophoresis did release into the supernatant some monomeric collagen and a small amount of high molecular weight protein that could not enter the 10% gel (Figure 3). No additional collagen was released from the insoluble connective tissue by the action of the microbial enzymes in the 4-h incubation at 25 °C. Papain digestion, however, did solubilize additional protein as shown by a faint band of protein unable to enter the 4% gel, a protein band unable to enter the 10% gel, and additional bands of monomeric collagen and breakdown products. These bands can be attributed to collagen since they stained metachromatically (pink) with the Coomassie blue dye (Duhamel, 1983), unlike the standard proteins and microbial enzymes which stained blue.

It is interesting to note in Figure 3 that the activity of papain in solubilization of insoluble connective tissue was apparently greater than that of purified collagenase from *Clostridium histolyticum* tested in the same system at the same molar ratios of enzyme to substrate. Although collagenase is more specific than papain is for the collagen substrate, the difference seen may result from the pH of the meat system (5.5), which is almost 2 units lower than the optimal pH for collagenase activity. In addition, collagenase cleaves at various sites along the triple helical region of the collagen molecule, but papain cleaves at a site near the intermolecular cross-link, thereby permitting separation of molecules and even fiber sections from each other.

On the basis of the data presented, papain would clearly be the protease of choice for general, rapid tenderization of meat. However, in those applications in which proteolysis by papain leads to an unacceptable "mushy" texture, *M. meihei* proteases could be used. None of the proteases studied were capable of producing the selective connective tissue degradation necessary for the desired specific application.

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**Registry No.** *E. parasitica* protease, 37205-60-0; *M. miehei* protease, 9073-79-4; papain, 9001-73-4.

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